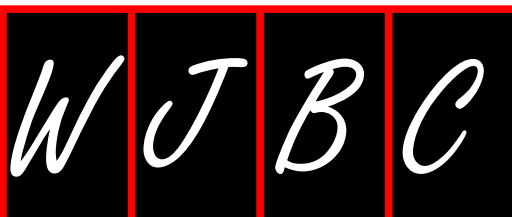


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Role of α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor regulation in stress-induced pain chronification

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Abstract

Persistent postsurgical pain is a serious issue in public health, which has received increased interest in recent years. Previous studies have reported that psychological factors promote the development of chronic postsurgical pain. However, it is unclear how chronification of postsurgical pain occurs. The α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor (AMPA) phosphorylation in the central nervous system plays a critical role in synaptic plasticity and contributes to central sensitization and chronic pain development. Here, we discuss the role of AMPA receptor regulation in stress-induced pain chronification after surgery.

Key words: α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor phosphorylation; Stress; Pain chronification

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Core tip: The α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor phosphorylation contributes to stress-induced pain chronification after surgery.

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PAIN CHRONIFICATION AFTER SURGERY

After surgery, patients experience either short-term or long-lasting pain. In some patients, pain post-surgery can persist even after surgical incision has recovered. Chronic postsurgical pain is primarily neuropathic in nature. As we

know, acute postoperative pain is an adaptive response to surgical damage, but chronic postsurgical pain is maladaptive since it is not protective. However, the central mechanisms underlying pain chronification after surgery remain to be illustrated^[1].

Pain chronification after surgery is a process involving multiple biological systems^[2]. Chronic postsurgical pain provides a special opportunity to understand pathogenic mechanisms for the transition from acute to chronic pain. Previous studies have indicated that psychological factors promote the development of chronic postsurgical pain^[3,4]. Psychological stress can disturb the physiological homeostasis of an organism^[5,6]. People who experience stress in their early life or even before birth may have chronic susceptibility to developing pain in their whole life^[7]. Thus, it is very possible that early stress could cause permanent changes in pain signaling in the central nervous system. In addition, surgery produces the release of inflammatory mediators, such as prostaglandins and cytokines^[8], which can sensitize primary sensory afferents. Stress might increase sensitivity to the hyperalgesic effects of proinflammatory cytokines^[9]. Moreover, stress-induced hyperalgesic priming, a neuroplastic change in primary afferent nociceptors, has been implicated in chronic generalized pain syndromes and other chronic pain conditions^[10-15]. Therefore, stress may be involved in the development of chronic pain after surgery.

STRESS ENHANCES AMPA RECEPTOR REGULATION AND SYNAPTIC PLASTICITY

Psychological stress produces physiological and behavioral changes that cause long-term adaptive responses^[5]. Two major reactions can occur in response to stress. One reaction after stress is rapid activation of the autonomic nervous system and subsequent release of the stress hormones epinephrine and norepinephrine^[16]. Norepinephrine can activate cAMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase II^[17,18], which can phosphorylate α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluA1 at the Ser845 and Ser831 sites, respectively^[19-21]. Targeted mutant mice with the mutations of these phosphorylation sites that block phosphorylation at Ser845 and Ser831 sites of GluA1 display impairment in synaptic plasticity and learning^[22]. Thus, stress through releasing the stress hormone norepinephrine can induce GluA1 phosphorylation at the Ser831 and Ser845 sites and then facilitate long-term potentiation induction^[23]. Phosphorylation at these sites is sufficient to reduce the threshold for GluA1 synaptic incorporation during long-term potentiation^[23]. Another reaction after stress is the stimulation of the hypothalamus-pituitary-adrenal axis and subsequent release of the stress hormone glucocorticoids (a type of corticosteroid hormone) from the adrenal glands^[16]. Glucocorticoids are able to bind to two types of re-

ceptors in the central nervous system: Mineralocorticoid receptors and glucocorticoid receptors. Mineralocorticoid receptors have a high affinity for corticosterone (the main glucocorticoid in rodents) and are bound when the hormone level is low. Glucocorticoid receptors have a lower affinity for corticosterone than do mineralocorticoid receptors, which are activated only when the hormone level is high enough^[16]. Both mineralocorticoid receptors and glucocorticoid receptors are expressed in the central nervous system^[16,24]. By activating the two receptor subtypes, corticosterone rapidly and persistently regulates AMPA receptor GluA2 trafficking, which plays an important role in synaptic transmission and plasticity^[5,25]. Therefore, the stress hormone corticosterone can effectively enhance the synaptic content of AMPA receptors and then produce synaptic potentiation^[5,25].

MECHANISMS UNDERLYING STRESS-INDUCED PAIN CHRONIFICATION AFTER SURGERY

After injury and injury-induced pain, a "supersystem" consisting of nervous system, endocrine system, and immune system may act together to regulate the functional activities in these systems^[26]. Thus, the development of chronic postsurgical pain could be caused by dysregulation of the supersystem. For instance, the nervous system and endocrine system can cooperate in the response to stress, which has been referred to as the neuroendocrine stress response^[26]. Recently, we have utilized these concepts to develop a new animal model to study acute-to-chronic pain transition after surgery. In this model, we found that social defeat stress enhances plantar incision-induced spinal AMPA receptor phosphorylation and thereby prolongs incisional pain and that stress hormones regulate AMPA receptor activities in the spinal cord during the pain prolongation^[27]. We also found that the social defeat stress not only increases GluA1 membrane expression, but also enhances GluA2 intracellular expression in the spinal dorsal horn neurons^[27]. Our study identifies stress as a risk factor for pain chronification after surgery. Our recent study indicates that intrathecal injection of a Ca^{2+} -permeable AMPA receptor blocker significantly inhibits the stress-induced postsurgical pain prolongation (unpublished data). Therefore, we hypothesize that by releasing two types of stress hormones (norepinephrine and corticosterone), stress regulates AMPA receptor activities (such as phosphorylation and trafficking), which leads to GluA1 membrane insertion and GluA2 internalization and causes a switch from Ca^{2+} -impermeable (GluA2-containing) to Ca^{2+} -permeable (GluA2-lacking) AMPA receptors. This switch will enhance Ca^{2+} influx and further activate Ca^{2+} -dependent protein kinases, thereby promoting AMPA receptor phosphorylation and other phosphorylation-triggered activities (Figure 1). This positive feedback loop may contribute to the molecular mechanisms that underlie stress-induced pain chronification after surgery.

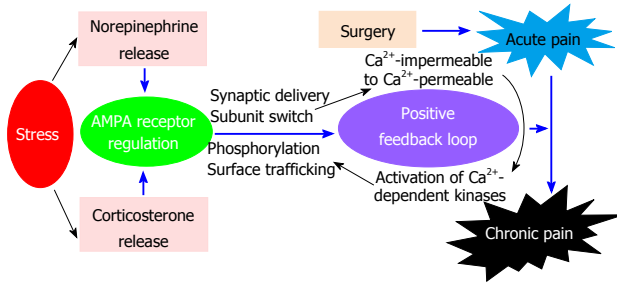


Figure 1 Mechanisms underlying stress-induced pain chronification after surgery. Note that α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor switch from Ca^{2+} -impermeable (GluA2-containing) to Ca^{2+} -permeable (GluA2-lacking) will enhance Ca^{2+} influx and further activate Ca^{2+} -dependent protein kinases, thereby promoting AMPA receptor phosphorylation and other phosphorylation-triggered activities.

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Modern trends in animal venom research - omics and nanomaterials

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Abstract

Animal venom research is a specialized investigation field, in which a number of different methods are used and this array is constantly expanding. Thus, recently emerged omics and nanotechnologies have already been successfully applied to venom research. Animal venoms have been studied for quite a long time. The traditional reductionist approach has been to isolate individual toxins and then study their structure and function. Unfortunately, the characterization of the venom as a whole system and its multiple effects on an entire organism were not possible until recent times. The development of new methods in mass spectrometry and sequencing have allowed such characterizations of venom, encompassing the identification of new toxins present in venoms at extremely low concentrations to changes in metabolism of prey organisms after envenomation. In particular, this type of comprehensive research has become possible due to the development of the various omics technologies: Proteomics, peptidomics, transcriptomics, genomics and metabolomics. As in other research fields, these omics technologies ushered in a revolution for venom studies, which is now entering the era of big data. Nanotechnology is a very new branch of technology and developing at an extremely rapid pace. It has found application in many spheres and has not bypassed the venom studies. Nanomaterials are quite promising in medicine, and most studies combining venoms and nanomaterials are dedicated to medical applications. Conjugates of nanoparticles with venom components have been proposed for use as drugs or diagnostics. For example, nanoparticles conjugated with chlorotoxin - a toxin in scorpion venom, which has been shown to bind specifically to glioma cells - are considered as potential glioma-targeted drugs, and conjugates of neurotoxins with fluorescent semiconductor nanoparticles or quantum dots may be used to detect endogenous targets expressed in live cells. The data on application of omics and nanotechnologies in venom research are systematized concisely in this paper.

Key words: Animal venom; Proteomics; Peptidomics; Transcriptomics; Nanotechnology; Genomics; Quantum dots; Nanoparticles; Metabolomics

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Core tip: A number of different methods are used in animal venom research, and this array is constantly expanding. The development of new methods in mass spectrometry and sequencing have allowed for the characterization of venom at different levels, ranging from identification of new toxins to profiling the changes in metabolism of an envenomed organism. The various omics technologies-proteomics, peptidomics, transcriptomics, genomics and metabolomics-have played key roles, as has nanotechnology. Nanomaterials are promising in medicine, and most studies combining venoms and nanomaterials are directed to medical applications, with conjugates of nanoparticles and venom components being proposed for use as drugs or diagnostics.

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INTRODUCTION

Animal venoms are complex mixtures of toxic substances, which are used to defend against predators and/or to hunt. They are extremely rich and complex natural sources of biologically active molecules, having a variety of molecular targets and functions. Venoms are aqueous solutions containing a large number of components, mostly protein and peptide in nature. Several hundred different substances, including toxins, may be found in venom from a single animal. Exposure of an envenomed organism to a toxin leads to significant dysfunction of the nervous, cardiovascular and muscular systems. The best known venomous animals are snakes, scorpions and spiders, and their toxins are used widely in research as molecular tools. Because toxin molecules often have a very strong potential for clinical structural optimization, some have been developed into new drugs (based on the optimized structures).

In attempts to understand the basis of venom biological activity, scientists have used a reductionist approach to venom composition and aimed to isolate the active compounds. Traditionally, this activity-driven approach was applied for purification of toxins. Having been initiated several decades ago, the present biochemical venom studies have advanced a long way from use of standard chemical fractionation methods, based in particular on differences in solubility, to modern high-performance liquid chromatography methods.

Most recently, venom research was revolutionized by the introduction of new mass spectrometry (MS)

methods and the development of "omics" technologies, including but not limited to genomics, proteomics and metabolomics. These English-language neologisms specify fields of study in biology that deal with very large-scale data collection and analysis, in particular characterization and quantification of pools of biological molecules^[1]. In molecular biology, the suffix -ome refers to a "totality of some sort" and is used to address the objects of omics studies, such as the genome, proteome or metabolome.

Three main categories within omics technologies are genomics, proteomics, and metabolomics/metabonomics. Genomics techniques are used to analyze the structure and function of genomes, while proteomic techniques deal with cellular and tissue-wide protein expression and metabolomics techniques are concerned with the identification and quantification of all the metabolites in a biological system. Within or in addition to these main techniques, some other omics techniques exist, such as transcriptomics, peptidomics, etc., Several omics techniques have already been applied to venom studies, resulting in more comprehensive characterization of venoms and their effects on organisms.

Another modern technology that carries substantial promise for having a high impact on venom studies is nanotechnology. Nanotechnology handles materials at the nanometer size level. Nanomaterials represent physical objects possessing size between 1 nm and 150 nm, in at least one dimension. This scale reduction results in significant changes of typical physical and chemical properties of materials, making them very attractive for novel and innovative applications in various fields, and they have garnered particular interest in medicine, pharmacy and medical diagnostics. Indeed, nanomaterials have been used successfully in magnetic and fluorescent bioimaging, as carriers for drugs, and even as medicines themselves (e.g., antimicrobial agents).

Among the different methodological directions being taken in venom studies, the development of new drugs based on venom components is the most prospective and rewarding. The combination of venom components with nanomaterials, and their application in treatment, diagnostics and disease prevention, will benefit the health and quality of human life. However, only the first steps considered in this Editorial have been made in this direction, thus far.

OMICS IN VENOM RESEARCH

In their quest to better understand the nature of venoms, researchers are constantly looking for new analytical methods of investigation. The recently emerged powerful analytical approaches united by the term "omics" have been successfully applied for venom studies. The relationship within "omics" technologies in venom studies is summarized in Figure 1.

Among all omics technologies, proteomics has been the most frequently used in venom research. The first publication^[2] on venom proteomics appeared at the

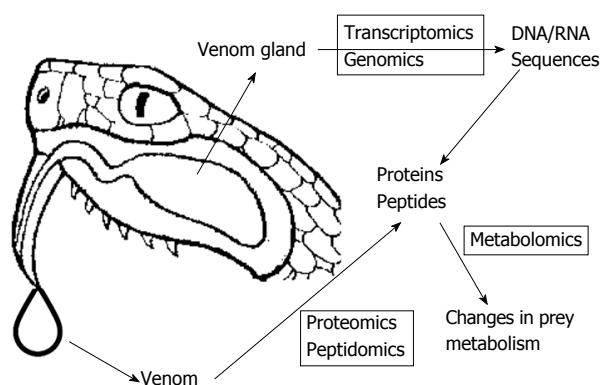


Figure 1 The relationship between “omics” technologies.

beginning of the millennium, and since then the number of works using this technology for venom studies has increased steadily. The special term “venomics” was introduced^[3], which refers to omics methods applied to venom studies. Considering this rapid growth, a key question emerges: What are the main advantages of proteomics over traditional methods? One of the major benefits is that proteomics gives a complete description of all the polypeptides present in a venom sample. This data may contribute to a deeper comprehension of the nature of a venom’s toxic effects. Quantitative proteomics is especially beneficial for this purpose, as it not only describes the polypeptide array but determines their amounts as well^[4], yielding an exhaustive catalog of the toxins present in the venom. Information about the toxins can be used in several ways; for example, some toxins are valuable molecular tools for studying physiological processes and may be applied for basic research. Venom proteomes also represent great potential for clinical diagnosis and development of new medicines for clinical use.

Proteomics contributes greatly to the development of antivenoms, which are currently the most efficient medications available for the treatment of bites or stings. The new term “antivenomics” was invented to specify an omics method used to identify venom proteins bearing epitope(s) that is(are) recognized by an antivenom^[5]. The basis of antivenomics is immunoaffinity chromatography, through which toxins bound to immobilized antivenom are identified by proteomics and in parallel the venom components that failed to raise antibodies in the antivenom, or which triggered the production of low-affinity antibodies and therefore remain unbound to antivenom, are identified. Antivenomics provides qualitative as well as quantitative data on both the toxins inducing antibodies with high affinity and those toxins exhibiting poor immunoreactivity.

It should be noted that proteomic analysis is capable of revealing many toxin variants belonging to known toxin families. In addition to this, several toxins of protein families not found in earlier venom studies have been identified and characterized by proteomics. For example, two proteins recently identified in a venom analysis of

the dog-faced water snake *Cerberus rynchops* showed sequence homology to ficolin, a mammalian protein with collagen-like and fibrinogen-like domains^[6]. These proteins were named as ryncolin 1 and ryncolin 2 (ryncops ficolin), and this new family of snake venom proteins was called veficolins (venom ficolins). The authors speculated that the ryncolins may induce platelet aggregation and/or initiate complement activation in the envenomed organism. Similarly, investigation of the venom proteome of the rear-fanged snake *Thamnodynastes strigatus* lead to the discovery of a new kind of matrix metalloproteinase (MMP) that is unrelated to the classical snake venom metalloproteinases found in all snake families^[7]. In that study, a protein related to lactadherin was newly identified and suggested to be a venom component^[7]. Finally, investigation of the venom of a cryptic Australian elapid snake *Drysdalia coronoides* resulted in the discovery of a new structural type of toxin from the three-finger toxin superfamily, and a new family phospholipase B was identified as well^[8]. These data highlight the great potential of proteomics in the discovery of new toxins and toxin families.

As was mentioned earlier in this Editorial, animal venoms are complex mixtures of different substances, but mostly proteins and peptides. In some venoms, the content of peptides is quite high; this is especially true for spider venoms^[9]. The proteomic approach is also used to study peptide components of the venom. This peptide-aimed type of study was given the name “peptidomics”. Thus far, peptidomics has been used mostly for peptide profiling of invertebrate venoms and its application has resulted in the discovery of several new toxins^[10,11].

Despite having been successfully used for many studies of venoms, proteomics continues to present several unresolved challenges. The usual first step in a proteomic venom study is the application of tandem MS/MS and database searching for the identification of protein and peptide toxins. In this so-called bottom-up approach, the comparison of experimentally determined sequences produced by MS/MS analysis with those obtained by *in silico* digestion of proteins present in a given database is performed. The problems encountered at this step, however, are the absence of tryptic cleavage or miscleavage at sites that differ from the classical trypsin sites. The absence of tryptic cleavage might be explained by post-translational modifications proximal to a tryptic cleavage site, which may result in the loss of trypsin activity. The activity of venom proteases might also result in the loss of some trypsin cleavage sites; such activity could also produce an artificial venom peptidome^[5].

Another challenge in proteomic studies is related to the absence of comprehensive databases containing venom protein sequences. To address this, several specialized toxin databases have been generated and released during the last decade. For example, the Animal Toxin Database supported by the Hunan Normal University^[12] contains more than 9000 toxins, while the

ArachnoServer, a manually-curated database containing information on the toxins derived from spider venom^[13] contains more than 1500 entries and the most recent Kalium database of potassium channel toxins from scorpion venom^[14] contains about 200 entries. Moreover, in the last UniProt release, the current number of entries for the query "snake venom" was around 29000 (UniProt release 2016_07). Nevertheless, this mass of information is still not sufficient and to describe the proteome of any particular venom completely and precisely, and as such transcriptomic and/or genomic data are necessary.

Genomics represents another versatile omics technology that is used widely in the life sciences. By definition "genomics is a discipline in genetics that applies recombinant DNA, DNA sequencing methods, and bioinformatics to sequence, assemble, and analyze the function and structure of genomes"^[15]. Yet, genomics has not been so widely applied as proteomics in venom studies. The genomes of poisonous animals that have been sequenced so far include the honeybee *Apis mellifera*^[16], the Chinese scorpion *Mesobuthus martensii*^[17], the Brazilian whiteknee tarantula *Acanthoscurria geniculata*^[18] and the King cobra *Ophiophagus hannah*^[19]. The rapid progress in sequencing methods has resulted in cost effective high-throughput sequencing (or next-generation sequencing) technologies that allow for the obtainment of millions of sequences at once. This makes the use of genomics more attractive for scientists involved in venom studies. And, according to a search of the up-to-date publicly available literature, among the snake species, the genomes of more than 10 are currently being studied^[20].

Venom proteins are produced by the venom gland, wherein translation of the information encoded by messenger (m)RNA takes place. This means that the data about venom protein composition can be obtained from an array of mRNA present in the venom gland. This approach is the basis of transcriptomic studies. Regarded sometimes as a part of genomics, transcriptomics can be defined as the study of the expression level of mRNAs in a given cell population, often by using high-throughput techniques. Transcriptomics has been embraced by venom researchers and its application has provided valuable information on the anticipated protein array in a given venom gland under a given biological condition. However, the presence of a given mRNA in the gland does not mean that it is guaranteed to be translated to a protein. Moreover, the transcriptomics approach does not give any information about the post-translational modification of a protein.

Nevertheless, compared to proteomics, which requires some initial data for protein identification, transcriptomics has a greater potential for discovery of new toxins. Thus, the transcriptomic approach has been applied to investigations of the composition of venom gland peptides from the *Hadogenes troglodytes* scorpion^[21]. A total of 121 novel peptides were discovered, including 20 new-type peptides cross-linked with 1, 2, 3, 4, 5 or 7 disulfide bridges, 11 novel K⁺-channel toxin-like peptides, 2 novel ryanodine receptors-

specific toxin-like peptides, a unique peptide containing the cysteine knots of spider toxins, 15 novel La1-like toxins, 3 novel TIL-domain containing peptides, 5 novel peptides with atypical cysteine patterns, 19 novel antimicrobial peptides, 6 novel cysteine-free peptides and 39 new-type cysteine-free peptides^[21]. These data demonstrate the great potential of transcriptomics for discovery of toxins.

The main disadvantage of the transcriptomic approach is the necessity to sacrifice the animal under investigation. However, it has been shown that mRNA encoding toxins can be recovered from the venom of several species^[22]. Subsequent studies have supported this finding and showed its applicability to RNA-based venom studies^[23,24].

Venoms have a great impact on the vital system in the prey organism that should result in substantial changes in the metabolism of the envenomed organism, which in principle may be revealed by metabolomics. The aims of metabolomics are the identification and quantification of all the metabolites in a biological system (cell, tissue, organ, organism) under given conditions. Metabonomics, in contrast, focus on the dynamic metabolic response of living systems to some biological stimuli. The task therein is to understand systemic change through time in complex multicellular systems. However, only a few publications in the literature to date report on the changes in prey metabolism after envenomation. One of these studies aimed to assess the response to a honeybee venom by analyzing serum levels of 34 free amino acids^[25]. To achieve this, serum samples were collected from 27 beekeepers within 3 h after they had been stung and after a minimum of 6 wk following the last sting. The two analyzed groups showed statistically significant differences in serum levels of L-glutamine, L-glutamic acid, L-methionine and 3-methyl-L-histidine. Moreover, L-glutamine and L-glutamic acid were found to be the most important metabolites for distinguishing the beekeepers who had been tested shortly after a bee sting from those who had been tested at least 6 wk later. The results obtained may contribute to better understanding of the human body's response to the honeybee sting^[25].

In other work, the toxic mechanism of scorpion *Hemiscorpius lepturus* venom was investigated by metabolome profiling of the victims using proton nuclear magnetic resonance (commonly known as ¹H NMR)^[26]. In parallel, the physiological effects of this venom on biochemical pathways and organs were investigated. The results obtained showed that the most affected pathways were pyrimidine, histidine and tyrosine metabolisms, and steroid hormone biosynthesis. The crude venom was found to affect mostly the pancreas and spleen. Mitochondria and nerve cells were attacked, resulting in acute seizures that resembled the early markers of myocardial injury and seizure disorders^[26].

The data discussed above indicate that omics technologies have contributed greatly to increasing our understanding of different aspects of venom's impact on a living organism. The application of these tech-

nologies in venom studies is constantly increasing, and the popularity of omics in venom study is evidenced by the fact that, to date, among the snake venoms alone over 150 species have already been examined by these techniques.

NANOMATERIALS IN VENOM RESEARCH

Most investigations of venom composition using analytical "omics" methods, as discussed in the previous section, are not goals in and of themselves, but are aimed at finding new compounds that can be of practical use. From this perspective, any enhancements that may improve the useful properties and applicability of these compounds will be of great value. One of these is the combination of venoms or toxins with nanomaterials.

Nanomaterials have numerous applications and are used in many areas, including research, technology and medicine. They can be used alone or in combination with other materials, such as venoms or toxins, as has been accomplished in magnetic and fluorescent bioimaging, as carriers for drugs, and as medicines.

The unusual properties of nanoparticles (NPs) can be exploited to improve the pharmacologic and therapeutic properties of drugs. While larger molecules may be eliminated from the body in a relatively short period of time, the cells can capture NPs very efficiently. To study the effectiveness of NP-based drug delivery to a biological target, hydrophilic NPs such as chitosan, nanogold, nano-silver and dendrimers, to name a few, were conjugated to potent animal toxins. Both crude venoms and isolated toxins have been used for this purpose.

Nanoparticles loaded with crude venoms

Cobra *Naja naja oxiana* venom was encapsulated in chitosan NPs, and such NPs were evaluated for their antigen delivery potential^[27]. The authors concluded by suggesting the possible application of chitosan NPs as an alternative to the currently used adjuvants.

In another study, poly(D,L-Lactide)-based NPs were combined with toxic fractions obtained from *Androctonus australis hector* and *Buthus occitanus tunetanus* scorpions' venoms in order to safely vaccinate animals to induce production of sera with neutralizing activity and achieve an immunoprotection outcome^[28]. The results obtained showed that polylactide NPs coated with toxic venom fraction preserve antigenicity and enhance immune response to weakly immunogenic toxins. This method can be easily extrapolated for horse therapeutic immune sera production, thereby reducing production costs. Moreover, PLA-NPs could be used for prophylactic purposes, as a biodegradable adjuvant to promote long-lasting immune protection against accidental envenomation of humans in hazardous areas^[28].

NPs loaded with anticancer agents have also shown great promise for the treatment of cancer. To study the effects of venom extracted from the cobra *Walterinnesia aegyptia* (WEV) on the proliferation and apoptosis of

human breast cancer cells, either the venom alone or in combination with silica NPs (WEV+NP) were investigated through monitoring caspase activity and free radical levels^[29]. Cells that had been isolated from female patients clinically diagnosed with breast cancer were used. It was found that both WEV and WEV+NP inhibited the growth of breast cancer cells in dose- and time-dependent manners, with the WEV+NP combination showing greater efficacy against the cancer cells. Thus, the combination of NPs with WEV significantly increased the antitumor effects of the venom.

To enhance the medicinal activity of bee venom (BV) acupuncture, it was loaded into biodegradable poly(D,L-Lactide-co-glycolide) NPs (BV-PLGA-NPs). The BV-PLGA-NPs were found to prolong the analgesic effect of BV in formalin-induced pain test in rats. PLGA-encapsulation of BV was also found to be effective in alleviating edema induced by allergens in BV^[30].

The above data indicate that NPs can significantly improve therapeutic potential of animal venoms. This improvement was more evident when individual toxins were used.

Nanoparticles conjugated with individual toxins

As was mentioned in the previous section, crude venoms are very complex mixtures of different compounds, some of which may show beneficial effects while others may show adverse effects. Thus, it is evident that the use of isolated compounds with beneficial effects may greatly enhance their potential.

Bombesin, the peptide isolated from the poisonous skin of the frog *Bombina bombina*, shows high affinity for the gastrin-releasing peptide (GRP)-receptors, which are widely represented in prostate cancer cells, breast cancer cells and small cell lung cancer *in vivo*. When bombesin was conjugated with gold NPs and radioisotope label was attached to these conjugates^[31], a high degree of specific binding to GRP-receptor and a high selectivity for the GRP-receptors of prostate tumor cells in mice was attained. The intraperitoneal drug delivery method was found to be effective for the bombesin-gold conjugate, avoiding absorption by the reticuloendothelial system of healthy organs and facilitating a concomitant increase in uptake of the drug by tumor cells.

The cytolytic peptide melittin, which is derived from BV, is considered a good candidate for cancer control. To overcome its main disadvantages - toxicity to non-targeted cells, non-specificity and unfavorable pharmacokinetic - the melittin nanoconjugate was developed, whereby the toxin has been inserted into the outer monolayer of perfluorocarbon NPs^[32]. This nano-carrier approach allows melittin to accumulate in tumors *in vivo* in mice and significantly reduce the tumor growth without any visible signs of envenomation. Moreover, such nanocarriers are capable of delivering melittin selectively to several tumor targets through the polydiffusion mechanism when the integrity of the cell membrane is maintained.

Nicotinic acetylcholine receptor of alpha7 type ($\alpha 7$ -

nAChR) is among several receptors that are regarded as tumor targets owing to expression specificity and significance for cancer. The venom isolate alpha-conotoxin ImI from the cone snail *Conus imperialis* possesses high affinity for $\alpha 7$ -nAChR and has been used to increase targeted drug delivery to $\alpha 7$ -nAChR-overexpressing tumors. In particular, the toxin was conjugated to the polyethylene glycol-grafted distearoylphosphatidylethanolamine micelles (ImI-PMs), forming spherical NPs of about 20 nm size with high drug encapsulation potential^[33]. To evaluate the targeting efficacy, the anti-tumor drug paclitaxel, which is frequently used in clinical for the treatment of breast cancer, was loaded in ImI-PMs. It was found that paclitaxel-loaded ImI-PMs exhibited greater cytotoxicity and induced more cell apoptosis *in vitro*, as well as displayed stronger antitumor efficacy in MCF-7 tumor-bearing mice. Thus, the alpha-conotoxin ImI-modified nanocarrier showed great potential for targeting $\alpha 7$ -nAChR-overexpressing tumors.

Snake venom toxin NKCT1 (GNP-NKCT1) tagged to gold NPs was studied as a potential anticancer agent using the human leukemic U937 and K562 cell lines as targets. Treatment of cells with GNP-NKCT1 resulted in the loss of mitochondrial membrane potential and a strong increase in reactive oxygen species (ROS). The conjugate induced apoptosis in leukemic cells, but upon suppression of apoptosis an alternative cell death pathway (in the form of autophagy) was observed^[34].

Fluorescent nanocrystals, otherwise known as quantum dots (QDs), have received great attention during the last year due to their outstanding properties. Recent advances in QD technology have made it possible to track the motion of individual molecules. Thus, the QDs have been used to monitor the receptors and ion channels in neuronal synapses, in particular in tracking movements of diffuse acetylcholine receptors on the surface of cultured myocytes^[35]. In cultured myocytes, acetylcholine receptors often form clusters in the absence of stimulation. These clusters can be easily visualized by alpha-bungarotoxin (a neurotoxin isolated from the venom of the *Bungarus multicinctus* krait) that is fluorescently labeled with organic fluorophores. However, this method was unable to detect the individual acetylcholine receptors that are diffusely distributed throughout the cell surface. To visualize such diffuse receptors, the myocytes were labeled with biotinylated alpha-bungarotoxin and then with streptavidin-conjugated QDs. In this manner, both clusters and individual acetylcholine receptors on the surface of the myocytes were finally able to be observed.

Chlorotoxin nanoconjugates - the best example of nanomaterials in venom studies

Chlorotoxin (CTX) found in the Israeli *Leiurus quinquestriatus* scorpion's venom binds preferentially to glioma cells, as compared with non-neoplastic cells or normal brain cells. This finding has allowed for the development of new methods for the treatment and diagnosis of several types of cancer with CTX-targeted NPs now being used fairly often for imaging and therapy

of gliomas^[36].

Using supermagnetic iron oxide as a nano-vector, a CTX conjugate with methotrexate was obtained^[37]. This conjugate demonstrated preferential accumulation in and high cytotoxicity against glioma cells *in vitro*. Moreover, the prolonged retention of this NP conjugate was observed in tumor cells *in vivo*.

In another study, CTX conjugated with an amine-functionalized polysilane and supermagnetic iron oxide NPs was developed^[38]. As a result, there was an increased uptake of the toxin conjugate into cancer cells and the tumor invasiveness was retarded compared to cells treated with the unconjugated CTX (98% vs 45% respectively). Moreover, the CTX-conjugates deactivated membrane-bound MMP2 and caused an increase in the internalization of lipid rafts. Because of its therapeutic action, this type of conjugate is considered a possible candidate for use both in non-invasive diagnosis and in treatment of various tumors.

A CTX-conjugate with near-infrared fluorophore and iron oxide particles coated with a biocompatible polyethylene glycol-modified chitosan was obtained^[39]. This conjugate was able to cross the blood-brain barrier, capable of mainly targeting brain tumor cells. The obtained compound showed no toxic properties and remained for a long time in the tumor cells.

Polyethyleneglycol-mediated synthesis was used to create highly stable iron oxide NPs. These NPs were conjugated to CTX and Cy5.5 fluorescent dye of near-infrared range. Near-infrared fluorescence imaging showed the specific accumulation of this conjugate in mice glioblastoma cells^[40].

CTX was also used to develop the upconversion nanoprobe that have proven useful for tumor targeting and visualization in living animals. Polyethylenimine-coated hexagonal-phase NaYF₄(4):Yb,Er/Ce NPs were prepared and conjugated with recombinant CTX^[41]. The resultant conjugates were visualized by laser scanning upconversion fluorescence microscopy. Animal studies provided high-contrast images, demonstrating highly specific tumor binding and direct tumor visualization with bright red fluorescence under 980 nm near-infrared irradiation. The high sensitivity and specificity of the CTX nanoconjugate represent substantial improvements that will benefit the diagnostic and therapeutic approaches for patients suffering from cancer.

Thus, using different conjugation methods, CTX can be tethered to iron oxide NPs, QDs, and upconversion NPs for targeted imaging of gliomas. In addition, CTX nanoconjugates can also be used as carriers to deliver anticancer drugs to gliomas.

The data about application of nanomaterials for the venom research are summarized in Table 1.

Potential adverse effects of nanomaterials

Although nanomaterials have numerous applications and possess great advantages over the traditional materials, they can have dangerous properties, which have not yet

Table 1 The application of nanomaterials in venom research

Nanomaterial	Toxin/Venom	Application
Polyethylenimine-coated hexagonal-phase NaYF(4):Yb,Er/Ce NPs	CTX from <i>Leiurus quinquestriatus</i> scorpion's venom	Glioma visualization by laser scanning upconversion fluorescence microscopy
Highly stable iron oxide NPs conjugated to CTX and Cy5.5 fluorescent dye	CTX	Near-infrared fluorescence imaging
Amine-functionalized polysilane and supermagnetic iron oxide NPs	CTX	Antitumor drug retarding tumor invasiveness and deactivating membrane-bound MMP2
Supermagnetic iron oxide conjugated with methotrexate and CTX	CTX	Antitumor drug with high cytotoxicity against glioma cells <i>in vitro</i>
Streptavidin-conjugated QDs	Biotinylated alpha-bungarotoxin from <i>Bungarus multicinctus</i> krait's venom	Visualization of nicotinic acetylcholine receptor in the myocytes
Gold NPs	Snake venom toxin NKCT1	Potential anticancer agent
Polyethylene glycol-grafted distearoylphosphatidylethanolamine NPs conjugated with ImI and loaded with paclitaxel	Alpha-conotoxin ImI from the cone snail <i>Conus imperialis</i>	Targeted antitumor drug
Perfluorocarbon NPs	The cytolytic peptide melittin, derived from bee venom	Antitumor drug with reduced acute toxicity
Gold NPs with radioisotope label	Bombesin from the frog <i>Bombina bombina</i>	Increase in uptake of the drug by tumor cells
Biodegradable poly(D,L-Lactide-co-glycolide) NPs	Bee venom	Prolonged analgesic effect of the venom
Silica NPs	<i>Walterinnesia aegyptia</i> cobra's venom	Significantly increased antitumor effects of the venom
Poly(D,L-Lactide)-based NPs	Toxic fractions obtained from <i>Androctonus australis hector</i> and <i>Buthus occitanus tunetanus</i> scorpions' venoms	Enhanced immune response to weakly immunogenic toxins
Chitosan NPs	Cobra <i>Naja naja oxiana</i> venom	Alternative to the currently used adjuvants

CTX: Chlorotoxin; NPs: Nanoparticles; QDs: Quantum dots; MMP: Matrix metalloproteinase.

been completely studied and can cause adverse effects in humans. NPs can enter the human body through several routes, including inhalation, ingestion, skin penetration or injection^[42,43]. After entering the body, NPs can interact with different components and localize in various organs, wherein they may remain intact or be subjected to modification or metabolism. NPs can cross cell boundaries, accumulating within the cells. Once in the cell, they may bind to DNA or proteins and interfere with normal cell functions or trigger an inflammatory response. The production of excess ROS^[44], including free radicals, which can cause oxidative stress, inflammation and other cellular damage, is one of the main known toxicity mechanisms of NPs. Similar to the toxicity of the NPs' parent bulk materials, the NPs themselves have toxicity that is determined by their chemical composition; however, size, surface chemistry, shape, and/or surface smoothness or roughness may enhance the toxicity profile of an NP, and all of these features can be altered substantially. While some negative NP effects have become understandable through detailed research, considerable efforts are still needed to study the physiological effects of acute and chronic exposure to NPs. Concerning the safety of NP conjugates with venoms or toxins, their application *in vivo* should be carried out with great care, keeping in mind the toxicity of starting materials.

CONCLUSION

Modern technologies, namely omics and nanotechnology, have made a great impact on the development of different branches of science and have contributed sub-

stantially to recent advancements in the field of venom research. Application of omics technologies have led to a better understanding of venom composition and of the roles played by different toxins in a given venom's effects. Preparation of conjugates of animal toxins with NPs opens a new path to creation of novel and innovative effective drugs, having better therapeutic potential and biocompatibility, as well as to the generation of more advanced systems to better deliver these drugs. However, NPs can generate ROS and induce apoptosis in the affected cells. These effects, on the one hand, may kill cancer cells, while, on the other hand, they may produce negative effects on normal cells. Thus, the possible adverse effects of NPs on human health should be taken into account when considering their application.

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Biochemical strategies for the detection and detoxification of toxic chemicals in the environment

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Abstract

Addressing the problems related to the widespread presence of an increasing number of chemicals released into the environment by human activities represents one of the most important challenges of this century. In the last few years, to replace the high cost, in terms

of time and money, of conventional technologies, the scientific community has directed considerable research towards the development both of new detection systems for the measurement of the contamination levels of chemicals in people's body fluids and tissue, as well as in the environment, and of new remediation strategies for the removal of such chemicals from the environment, as a means of the prevention of human diseases. New emerging biosensors for the analysis of environmental chemicals have been proposed, including VHH antibodies, that combine the antibody performance with the affinity for small molecules, genetically engineered microorganisms, aptamers and new highly stable enzymes. However, the advances in the field of chemicals monitoring are still far from producing a continuous real-time and on-line system for their detection. Better results have been obtained in the development of strategies which use organisms (microorganisms, plants and animals) or metabolic pathway-based approaches (single enzymes or more complex enzymatic solutions) for the fixation, degradation and detoxification of chemicals in the environment. Systems for enzymatic detoxification and degradation of toxic agents in wastewater from chemical and manufacturing industries, such as ligninolytic enzymes for the treatment of wastewater from the textile industry, have been proposed. Considering the high value of these research studies, in terms of the protection of human health and of the ecosystem, science must play a major role in guiding policy changes in this field.

Key words: Biosensors; Biomonitoring; Bioremediation; Toxic compounds; Chemicals pollution; Human health; Environmental pollutants

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Core tip: The increasing focus on the presence of hazardous chemicals in the environment is directing scientific research towards the development of new and eco-sustainable strategies for their control. Such

advances in technology are enabling scientists to improve the detection limits of these substances, in the environment, in food and the human body, as well as to develop new strategies for their removal from their surroundings. However, further research is required to achieve the goal of a continuous monitoring of the environment and of providing, in real time, information on its current state.

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INTRODUCTION

Addressing the issue of chemicals in the environment is not easy. The world's chemical production increased 400 fold in the last century, leading to the introduction of an increasing number of toxic substances into the environment, all directly related to technological progress and improvements in agricultural and industrial methods. The 2014 inventory of the Environmental Protection Agency includes approximately 85000 different chemicals available for use in just the United States^[1], and only a small subset of these chemicals has been sufficiently characterized in order to draw conclusions about their toxicity^[2,3]. While some chemicals may be harmless, others can be very toxic for human health. In particular, special attention has been focused on chemicals that persist in the environment and can accumulate in the human body, interfering with hormones (endocrine disruptors), causing cancer or damaging DNA.

In 2009, the "Fourth National Report on Human Exposure to Environmental Chemicals" of the Department of Health and Human Services (Centers for Disease Control and Prevention - National Center for Environmental Health - United States)^[4], aimed at bio-monitoring the United States population by measuring the chemicals in people's urine and blood, revealed for the first time the presence of 75 new toxic chemicals in the United States population. The update of this report in August 2014 (Updated Tables, July 2014)^[5] presents data relating to 35 additional chemicals.

The priority list of hazardous substances (<https://www.atsdr.cdc.gov/spl/>) includes more than 700 compounds, belonging to groups such as Metals and Metalloids, Disinfection By-Products, Environmental Phenols, Phthalates, Polycyclic Aromatic Hydrocarbons, Volatile Organic Compounds, Fungicides, Herbicides and Pesticides (Carbamates, Organochlorines and Organophosphates).

IMPACT ON HUMAN HEALTH OF LOW LEVEL EXPOSURE TO TOXIC CHEMICALS

The toxic effects of several chemicals on human health

and on the environment (animals and plants) are well known. For instance, phthalates, causing epigenetic alterations, play an important role in adverse offspring neurodevelopment and in complex diseases such as cancers and diabetes^[6,7].

Considerable legislation, in many countries of Europe, America and Asia, is aimed at regulating the relative amounts that can be released into the environment and the human exposure time in relation to the majority of these chemicals. Recommended limit values of exposure to chemicals in the environment in terms of time and quantity have been determined following toxicological studies best suited to the evaluation of health risks^[8-10]. These limits are not absolute, but subject to change depending on advances in scientific knowledge. Unfortunately, this approach has resulted in potentially dangerous practices, such as the massive introduction of chemicals insufficiently characterized from a toxicological point of view, as well as the use of complex mixtures in which all the individual components are under the threshold but their combination may be hazardous. However, the effects of a prolonged exposure to low concentrations of these chemicals, as well as the cumulative effects of mixtures of these substances, even in traces, still remain unclear. Recently, the failure to explain the vast majority of chronic diseases afflicting an increasing percentage of the population^[11] by means of genome-wide association studies, together with the reported transformation in developed countries of many infectious diseases into chronic diseases, has led to a fundamental reconsideration of the health impact of environmental exposure to chemicals^[12]. In a recent article, Bijlsma and Cohen highlight the growing awareness that several chemicals present at low concentrations in the environment (in air, water, soil, food, buildings and household products) contribute to the contraction of many of the chronic diseases typically seen in routine medical practice^[13].

TOBACCO SMOKING: A BAD HABIT CONTRIBUTING TO CHEMICAL POLLUTION

Although it may sound excessive, a bad habit, such as cigarette smoking, may introduce over 4500 chemicals to the environment, including metals^[14], *via* cigarette particulate matter and mainstream smoke^[15], contributing to an increase in the level of harmful compounds in more populated areas. In particular, tobacco product waste contains all the toxins, nicotine, and carcinogens found in tobacco products, and becomes a public pollution problem because chemicals can leach into aquatic environments and are toxic to aquatic micro-organisms and fish^[16,17].

In spite of the undeniable evidence of harm associated with cigarette smoking, which can affect male fertility^[18], induce oxidative stress and pro-inflammatory responses^[19,20], promote cancer and other pulmonary and cardiovascular diseases^[21], the habit remains unacceptably prevalent in both developed and developing countries.

In relation to tobacco smoking, in order to avoid the

introduction of harmful chemicals other than those that are really necessary to maintain the current technological status, we should simply reduce, or even better stop, the consumption of cigarettes.

In conclusion, we must agree with Vassallo *et al*^[22] in their opinion that cigarette smoking is a leading cause of preventable mortality in the world.

THE CASE OF PESTICIDES

Currently, the most dangerous chemical pollution is represented by pesticides. The fact that five of the nine new persistent organic pollutants (POPs)^[23-25], which bioaccumulate in humans and animals, are pesticides (<https://www.environment.gov.au/protection/chemicals-management/pops/new-pops>), highlights the importance that must be attached to the presence of these substances in the environment. Differently from other harmful chemicals, introduced into the environment by natural processes, or produced as by-products of industrial processes and other human activities, pesticides are designed to be toxic and are used as poisons to kill organisms. They are extensively applied over large areas in agriculture and in urban settings, becoming the most diffuse form of chemical pollution in the world, and one which is also very difficult to control. In the last 20 years the number of publications relating to pesticides has doubled to up to one thousand per year, and the majority of these articles concern their negative effects on human health, and focus, in particular, on neurological diseases in children^[26-31]. Recently, *Science*, a high impact factor journal, has addressed the problems relating to pesticides in a special issue entitled "Smart pest control"^[32]. The articles in this issue focus on many topics including the increasing use of pesticides in Asia, South America and Europe, their widespread diffusion and transformation in the environment, their involvement with neuro-degenerative diseases and cognitive deficits in developing age, new strategies to reduce their negative impacts, the development of new synthetic chemicals with less collateral damage, and above all the major role that science could play in guiding policy changes in this field.

STATE OF THE ART IN CHEMICAL DETECTION AND DEGRADATION

The detection and monitoring of toxic compounds has improved significantly in the last few years. Technological advances, such as Surface-Activated Chemical Ionization^[33,34] which increases the sensitivity and accuracy of GC-LC-MS measurements, in association with methodologies, such as Quick Easy Cheap Effective Rugged and Safe^[35], allow the identification of hundreds of these substances in a single shot. However, their detection in the environment still remains difficult, because toxic chemicals are so widely distributed in the world and can be found in locations as diverse as pristine forests and

the blood of arctic animals, wind, rain and other weather phenomena contributing to their diffusion.

The use of biomarkers in combination with sampling from water and soil will facilitate the tracking of the amount of chemicals in the environment^[36-38]. However, any analysis using the technology currently available, although it is very powerful, generally requires several weeks to complete. In fact, it is necessary to collect many samples in order to have an accurate representation of an environment (randomly from air, soil, water, foods and human and animal fluids or tissues, such as the blood and urine). These samples must be transferred and stored for the necessary time for their analysis in authorized centers, without considering the possibility that very often an extraction and/or derivatization of the samples is also required. All this infrastructure has too high a cost in terms of money and time to allow a continuous monitoring of the environment or of the population.

In agreement with the United States Environmental Protection Agency^[39], *in vitro* screening using biological pathway-based approaches has become central to 21st century toxicity testing. Furthermore, I would suggest that the establishment of a capillary network of environmental sensors for the on-line and real-time monitoring of toxic chemicals and the development of new eco-sustainable methodologies for the removal of these toxic compounds from the environment should now be considered among the most important challenges for the future.

In fact, the current chemical remediation methodologies are unsuitable because most of them use chemical or physical approaches which either simply shift the problem into the future (such as the storage of nerve agents in military bases, or the sequestration and storage of obsolete pesticides in inadequate facilities, particularly in third world countries) or introduce other harmful compounds into the environment, such as through the incineration of pesticides or the inactivation of reactive groups of chemicals using strong alkaline (sodium hydroxide) or acidic solutions^[40-43]. Moreover, due to the wide heterogeneity of chemical pollution, none of the strategies currently employed is totally satisfactory. Therefore, the development of new environmentally-friendly strategies to support the current methodologies has become crucial.

BIOCHEMICAL STRATEGIES TO DETECT CHEMICALS

The research into new technologies to replace conventional GC- and LC-MS methodologies for the environmental detection of chemicals has been intensifying in recent years. In particular, the development of sensors and biosensors for the precise detection and estimation of hazardous chemicals in different samples (water, human fluids and tissue, *etc.*) has been gaining momentum.

The advantages of biosensors compared to the current technological approaches could be summarized in a few words: Easiness, cheapness, and speediness.

Biosensors are characterized by a "bioreceptor",

which is the biological part recognizing the substance, a “transducer”, that transforms the biological interaction into a measurable signal, and a “reader/recorder”, that displays the results. Sometimes an “amplifier” can be used to amplify the signal improving the sensitivity of the biosensor^[44,45]. Biological scientists can play an important role in the development of biosensors, in that biosensors for toxic chemicals can be based on animals, microorganisms, antibodies, enzymes, and nucleic acids^[46-48]. The limit of detection (LOD) of a number of these biosensors is comparable with, if not greater than, standard technologies^[49-52]. Further details on the characteristics of the most recent biosensors and on the advances in the development of these technologies can be found in several recent articles^[44-46,48,53-55]. We can distinguish between two different applications of these biosensors, namely for diagnostic use in clinical settings and for the environmental monitoring of chemicals. The recent development of diagnostic biosensors permits us to hypothesize about their possible use in the near future for the measurement of chemicals in routine clinical analysis^[56-58].

New emerging biosensors for the analysis of environmental chemicals have been proposed in order to offer a simple alternative means of assessment approach, such as VHH antibodies (the antigen binding fragment of heavy chain antibodies) that combine the comparable performance of conventional antibodies with the affinity for small molecules^[59], or genetically engineered microbial whole-cells, that respond to target chemicals and produce detectable output signals^[60]. However, these advances in the field of environmental chemical monitoring are still far from producing a continuous real-time and on-line system for their detection.

In conclusion, it is necessary to highlight the fact that the use of biosensors is crucial not only for the detection of the presence of chemicals, but also for the detoxification monitoring which should always be used to evaluate the efficiency of a treatment technique.

DETECTION OF NEUROTOXIC CHEMICALS

Excluding neurotoxic poisons produced by certain fish, insects and reptiles (such as Bungarotoxin, Chlorotoxin, Conotoxin, and Tetrodotoxin), or by certain plants, algae and bacteria (such as Anatoxin-a, Tetanus and the Botulinum toxins), as well as some metals, such as lead and mercury, that can affect the activities of the nervous system, the most diffuse synthetic chemicals that impair the central nervous system are nerve agents, certain pesticides (for example, the organophosphates) and some organic solvents, such as hexane. These neurotoxins affect the transmission of chemical signals between neurons, causing a number of disorders and even fatality. In particular, the majority (the nerve agents and organophosphate pesticides) act as inhibitors of acetylcholinesterase activity^[61]. Acetylcholinesterases are enzymes belonging to the carboxylesterase family,

involved in the regulation of nerve signal transmission at the chemical synapses, by hydrolyzing acetylcholine and other choline ester neurotransmitters. The inactivation of this enzyme causes paralysis and even death. As this result, indeed, is the specific target of the above mentioned toxic chemicals, this family of enzymes remains the one most extensively studied for use as bioreceptors in the development of biosensors for neurotoxic chemicals^[62-64]. The principles of acetylcholinesterase biosensors are based on the measurement of the residual activity of the enzyme using different substrates, such as acetylcholine and thiocholine, that can be monitored using potentiometric, amperometric and optical devices^[62-64]. The LODs that can be obtained using acetylcholinesterase activities, in particular from insects, for the detection of these neurotoxic compounds, remain among the highest obtainable^[65].

Recently, new alternative bioreceptors have been proposed, including new enzymes, microorganisms, antibodies, and aptamers^[66-69], which represent a possible alternative to overcome the limitations involved in the use of acetylcholinesterases.

BIOREMEDIATION OF CHEMICALS

Exploiting the bioremediation lessons that the ecosystem teaches us, we advance to the development of strategies which use organisms (microorganisms, plants and animals) or metabolic pathway-based approaches (single enzymes or more complex enzymatic solutions) for the fixation, degradation and detoxification of chemicals in the environment.

In particular, microbial remediation strategies for soil ecology have attracted increasing interest since they are environmentally friendly and cost-effective^[70]. Several examples of such strategies can be found in literature, but none are totally satisfactory. A limitation in the use of strains isolated from the environment or genetically engineered in the laboratory, for the treatment of soil contaminated with chemicals, is related to their reduced growth capacity when sharing the environment with other microorganisms that use better substrates. Indeed, differently from results obtained in the laboratory where these microorganisms grow in optimized conditions and in the absence of competitors, the yields in chemical remediation are weak in operative conditions. A valid alternative is represented by the use of plants, but the introduction of new species into the environment requires considerable time before any significant results are obtained.

A different approach that overcomes the limitation consisting in the length of time required for decontamination using organisms could be the use of enzymes, which can bind with a high affinity the chemical compounds and catalyze their hydrolysis in hours if not minutes. Unfortunately, the use of enzymes in soil remediation is often handicapped by the large amount of enzyme required, by the absence of a sufficient quantity of water, generally needed for the solubilization and the hydrolysis

of substrates, by the low stability of enzymes outside cells, and by the presence of microorganisms, that inactivate them. For these reasons the bioremediation of ground represents one of the most important challenges of our century, but, unfortunately, we are still far from achieving important results.

A different situation applies in relation to the treatment of contaminated water, since it is possible to extract a large quantity of water from watercourses or lakes, transferring it into bioreactors containing enzymes and reducing the number of contaminants in a short time. The use of very stable enzymes, genetically engineered to be resistant to proteases or to the presence of organic solvents and detergents, and produced in a large quantity at a low cost by over-expression in an appropriate host, could be the right answer to the problem. A formulation of different enzymatic activities or a number of bioreactors containing different enzymes and connected in a series could treat several chemicals at the same time. In particular, as proposed for the detoxification by ligninolytic enzymes of wastewater from the textile industry with a yield from 69% up to 87%^[71], the enzymatic detoxification and degradation of toxic agents in wastewater from the chemical industry could possibly be used to reduce drastically the quantity of chemicals released into the environment.

BIOREMEDIATION OF NEUROTOXIC COMPOUNDS

For the bioremediation of neurotoxic compounds, such as organophosphates, other and efficient enzymes have recently been studied. Differently from the activities of acetylcholinesterase that are inhibited by neurotoxins, some enzymes belonging to the *Aryldialkylphosphatase* family, such as the *Pseudomonas diminuta* phosphotriesterase^[72] and *Sulfolobus solfataricus* paraoxonase^[73,74], are able to hydrolyze these chemicals. Considering the importance of this field, in particular in relation to the possible use of enzymes in the decontamination of nerve agents in military actions, all the enzymes are under patent, and some have already been commercialized individually or in formulation. Despite the great potential of these enzymes, several limitations still remain. Indeed, mesophilic enzymes, that show a high activity towards several nerve agents, have at the same time a very low stability and lose their activity in minutes in the environment. Differently, alternative enzymes isolated from thermophilic organisms, showing a high stability not only towards temperature but also towards organic solvents, detergents and proteases, present a lower catalytic performance, if compared to the mesophilic ones^[74].

Although the production of nerve agents should be discontinued, large amounts already produced during the cold war are still stored and require degradation into less dangerous chemicals. Moreover, some of these enzymes are also active on a number of pesticides, and therefore

developments in this field are undoubtedly much needed.

CONCLUSION

Human activity has resulted in severe environmental pollution, which has now emerged as a major global issue. However, it is a utopian idea to hope for a world without any chemical pollutants, as technological and scientific progress requires an extensive use of chemicals. Therefore, the requirement is not to reduce industrial activities, but to realize that we must optimize current technologies in order to reduce the quantity of contaminants released. An example could be the amount of pesticides used on crops, which could be significantly diminished simply by monitoring the quantity still present in the field, so avoiding an excessive use. On the other hand, we need to develop new eco-sustainable processes of production in order to avoid a massive introduction of harmful chemicals into the environment. Meanwhile, the use of metabolic pathways present in or extracted from microorganisms and plants could be the right approach for future strategies of bioremediation. To achieve this goal, it will be necessary to strengthen the current scientific engagement, enabling scientists from different fields to collaborate in the development of innovative strategies to address the problems related to toxic chemicals.

To put it simply, we should arguably have had an antidote before introducing a poison into the environment. That antidote may well be enzymes, which catalyze almost all of the reactions on the Earth.

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Biochemical changes in the injured brain

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Abstract

Brain metabolism is an energy intensive phenomenon involving a wide spectrum of chemical intermediaries. Various injury states have a detrimental effect on the biochemical processes involved in the homeostatic and electrophysiological properties of the brain. The biochemical markers of brain injury are a recent addition

in the armamentarium of neuro-clinicians and are being increasingly used in the routine management of neuro-pathological entities such as traumatic brain injury, stroke, subarachnoid haemorrhage and intracranial space occupying lesions. These markers are increasingly being used in assessing severity as well as in predicting the prognostic course of neuro-pathological lesions. S-100 protein, neuron specific enolase, creatinine phosphokinase isoenzyme BB and myelin basic protein are some of the biochemical markers which have been proven to have prognostic and clinical value in the brain injury. While S-100, glial fibrillary acidic protein and ubiquitin C terminal hydrolase are early biomarkers of neuronal injury and have the potential to aid in clinical decision-making in the initial management of patients presenting with an acute neuronal crisis, the other biomarkers are of value in predicting long-term complications and prognosis in such patients. In recent times cerebral microdialysis has established itself as a novel way of monitoring brain tissue biochemical metabolites such as glucose, lactate, pyruvate, glutamate and glycerol while small non-coding RNAs have presented themselves as potential markers of brain injury for future.

Key words: Biomarkers; Brain injuries; Brain ischemia; Epilepsy; Subarachnoid hemorrhage

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Core tip: The biochemical markers of brain injury are being increasingly used to assess the severity and prognosis in the injured brain. While S-100, glial fibrillary acidic protein and ubiquitin C terminal hydrolase have been used as early biomarkers to aid in clinical decision-making and initial management, other biomarkers help in long-term prognosis. Cerebral microdialysis is a novel way of monitoring brain tissue biochemical metabolites and each component gives an idea about the severity and type of pathologic process in the brain. In addition, small non-coding RNAs have presented themselves as potential markers of brain injury for future research.

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INTRODUCTION

The brain is one of the most energy intensive organs of the body, utilizing around 60% of the available energy for the fulfillment of electrophysiological function, and the remaining 40% is expended in the homeostasis of the internal milieu of the brain cells^[1]. Brain metabolism is an energy intensive phenomenon involving a wide spectrum of chemical intermediaries and their consequent usage in brain energy production.

The evolution of techniques to monitor brain metabolism started in the late 19th century^[2]. However, major strides in the understanding of the cerebral metabolic processes have happened only in the last 50 years and have greatly contributed to our understanding of the processes governing the myriad and complex activities of the central nervous system in general and the brain in particular.

In this editorial we focus on the basics as well as perturbations of brain metabolism in the different clinical scenarios of neurological injury such as traumatic brain injury (TBI), stroke and subarachnoid hemorrhage (SAH). The aim of this review is also to discuss the means at our disposal to monitor such deviations and the practical clinical applications of such techniques^[2].

BRAIN METABOLISM AND BIOCHEMISTRY

As mentioned earlier, brain metabolism is peculiar for being a highly energy intensive process. Although it contributes approximately (only) 2%-2.5% of the total body weight, it receives approximately 20% of the total blood supply and 25% of the total oxygen supply^[3].

The biochemical processes in the brain exhibit various peculiarities with ramifications in brain injury. First is the presence of a blood brain barrier formed by endothelial cell layers of the brain vessels^[4-6], which plays an important role in the maintenance of homeostasis in relation to the electrolytes and energy substrates such as glucose, glutamate and ketone bodies^[7-9]. Nerve impulse propagation is the key function within the brain and is basically an amalgamation of electrical and chemical processes. The electrical processes are responsible largely for impulse propagation within a neuron whereas chemical reactions influence signal transmission from one neuron to another as well as at the effector cells and axon ends in the synapse^[10]. The synapses perform the critical function of transferring electrical impulses across the synaptic cleft or for further impulse propagation on to another neuron or muscle for a particular desired

action. Impulse transmission through a synaptic cleft is a complex biochemical process involving neurotransmitters like glutamate and γ -aminobutyric acid as well as the activation of various ion channels. Sodium and potassium are the major ions involved in the generation of action potentials, especially in the process of hyperpolarization and depolarization of neurons^[11-14]. The enormity of the biochemical processes involved in the signal transduction of neural impulse can be gauged from the fact that while a single neuron has 1000 to 20000 synapses, there are around 90 billion neurons in an adult human brain^[15]. Brain injured states such as stroke and head injury have a detrimental effect on the biochemical processes involved in the aforesaid homeostatic and electrophysiological properties of the brain.

BIOCHEMISTRY OF THE INJURED BRAIN

The biochemical basis of brain injury can be explained on the basis of either one or a combination of the following broad pathological mechanisms^[16]: Ischemia; traumatic brain injury; epileptogenesis.

Ischemic brain injury

Ischemia and resultant hypoxia lead to the derangement of energy intensive processes critical to homeostasis in the brain. Dysfunctional ATP dependent ion pumps result in consequent disequilibrium in sodium, calcium and potassium ion homeostasis, culminating in the release of excitatory amino acids such as glutamate^[17,18]. Glutamate plays a pivotal role in the ensuing excitotoxicity by activating α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, N-methyl-D-aspartic acid (NMDA) and metabolic receptors. Calcium, free radicals and phospholipase activation also contribute significantly in the cellular damage of the brain.

An important aspect of ischemic injury in the brain is the nature of ischemia. Global ischemia of the brain follows events such as cardiac arrest, whereas focal ischemic changes are seen after events such as episode of stroke. In focal ischemia there exists a penumbra region which is responsive to brain resuscitation measures albeit within a critical time frame of a few minutes. In the scenario of ongoing global ischemia, the severity of brain damage is dependent on the time until re-establishment of brain circulation as well as the differential susceptibility of the various regions of the brain to hypoxia^[19,20].

Traumatic brain injury

Primary injury following trauma to the brain consists of direct concussional neuronal damage, herniation of important structures as well as ischemic injury because of damage to blood vessels. Reversal of primary injury is impossible. However, amelioration of secondary effects is possible. The biochemical processes detailed previously play a pathologic role in traumatic brain injury and calcium is an important ion implicated in traumatic brain injury at the cellular level^[21,22].

Epileptogenic injury

Epilepsy is defined as sudden and excessive electrical discharge from neurons and occurs from a plethora of causes such as electrolyte and metabolic perturbations, temperature disturbances, and structural insults such as tumors, trauma and infections. The mechanism of epileptiform damage resembles ischemia and involves the previously detailed sequences culminating in glutamate excitotoxicity and NMDA and metabotropic nerve activation^[23,24].

The ongoing process of cellular injury in the injured brain leaves in its wake a multitude of biochemical markers. An ideal marker for injury should be specific to the brain, pick up brain injury within a reasonable and defined time frame and exhibit low variation with age and sex^[25,26]. However, the search for such a marker remains elusive till date.

BIOCHEMICAL MARKERS OF BRAIN INJURY

The biochemical markers of brain injury are a recent addition in the armamentarium of neuro-clinicians and are being increasingly used in the routine management of neuro-pathological entities such as traumatic brain injury, stroke, SAH, and intracranial space occupying lesions. The use of such markers in the brain *via-a-vis* their use in the heart had been limited by various factors such as the heterogeneity of different cell types in the brain, the differential integrity of the blood brain barrier as well as the multimodal mechanisms contributing to neuronal death. However, they are recently being increasingly used in assessing severity as well as in predicting the prognostic course of neuropathological lesions. S-100 protein, neuron specific enolase (NSE), creatinine phosphokinase isoenzyme BB (CPK-BB) and myelin basic protein (MBP) are some of the biochemical markers which have been proven to have prognostic and clinical value in the brain injury and are dealt henceforth in a detailed perspective.

S-100 PROTEIN

S-100 is a calcium binding protein with a molecular weight of 21 kDa and is present in two isoforms - "α" (25%) and "β" (75%). While S-100 "α" protein is found in melanocytes, S-100 β isoform is found predominantly in glial cells and Schwann cells of the peripheral nervous system and central nervous system. Although the β isoform is found in adipocytes and chondrocytes, the concentration of S-100 β in non-neural tissue (100-200 ng/mg of soluble brain protein) is minimal as compared with glial and Schwann cells (3500 ng/mg of brain protein)^[26,27].

S-100 β protein is metabolized and excreted by the kidneys, has a $t_{1/2}$ of 2 h and a mean serum level of 0.050 ± 0.081 g/L^[28]. S-100 β protein levels have been found to increase especially following brain tissue injury in various experimental models^[29].

S-100 β in head injury: Elevated levels of S-100 β have been found in patients after minor and major head injury^[26,30-36]. In patients with mild head injury (GCS 13-15) where initial computed tomography (CT) scans of their brain do not exhibit any abnormality, S-100 β levels have been found to be high, especially in the golden hour following trauma^[26]. Elevated levels of S-100 β in serum following head injury have also been associated with impaired cognition score^[37].

In severe head injury an increased serum S 100 β level of > 2 g/L just after and during evolution of TBI has been found to be associated with a high mortality rate. Persistent elevations of S-100 β have shown an association with ongoing secondary brain damage following the primary insult. S-100 β has exhibited correlations with CT pathologies, with lower values being more common in diffuse type I and type II injuries. As a marker of clinical outcome following TBI, S-100 β has shown promising results^[33-36,38-42].

Hence S-100 β in TBI can be concluded to be of clinical utility in assessing the extent of primary and secondary brain injury. It also has a role in predicting the time course of recovery and probability of an improved clinical outcome.

S-100 β protein in SAH: Plasma concentration of S-100 β in patients with SAH has shown a correlation with the severity of hemorrhagic affliction in the early phase of the disease as well as with the incidence of delayed cerebral ischemic events. There is also evidence correlating S-100 β levels with the severity of long-term neurological impairment as well as Glasgow outcome scores. Similar results have been observed with ventricular cerebrospinal fluid (CSF) S-100 β concentrations. There is significant evidence to suggest that S-100 β in CSF may show a superior correlation with CT and single-photon emission CT findings in addition to being predictive for outcome in patients with cerebral aneurysm^[43-46].

NSE

As an isoenzyme of enolase enzyme involved in glycolysis, NSE was thought to be a relevant marker of neuronal injury^[47]. However, it has also concurrently evolved as a marker for neuro-endocrine malignancies such as small cell lung cancer and neuroblastoma and hence its specificity for neural tissues is doubtful^[48]. Serum levels are in the range of 5-12 ng/mL and CSF levels normally are less than 2 ng/mL^[49].

NSE in TBI: In experimental model studies on cortical contusion, the highest concentration of NSE was observed at around 7.5 h following injury. This coincides with the primary mechanism of injury to the brain parenchyma and could be explained on the basis of extrusion of the cytoplasmic protein into the CSF from damaged neural and glial tissue. A secondary peak in the NSE levels was observed at around 1.5 d and in all probability reflects secondary ischemic damage to the contused

brain^[29]. An experimental TBI model in rats clearly demonstrated that CSF NSE is a more accurate motor of ongoing neuronal damage than serum NSE levels^[50].

There have been a plethora of studies on the correlation of serum and CSF NSE levels with head injury as well as their prediction of long-term outcome^[33,37,39,40,51-54]. Serum NSE levels showed a significant correlation with an identifiable contusion on CT scan and also predicted the incidence of long-term mortality and persistent vegetative state in patients with TBI^[51].

NSE in SAH

NSE in SAH patients had been found to be an excellent predictor of delayed cerebral ischemic events and poor perioperative outcome. However, the correlation of serum NSE levels with the clinical grade of SAH patients at the time of admission is a contentious issue with various studies giving different levels^[55-57].

NSE in stroke

Experimental studies in cerebral ischemia models and animal studies have unequivocally demonstrated that NSE levels in CSF correlate with the degree of severity of cerebral ischemia. In addition they have been found to be increased before irreversible brain cell damage, hence offering the promise of being used as a marker of guidance of cerebro-protective measures in stroke^[58-60]. In human studies examining the correlation of CSF with serum NSE levels, NSE has been found to have a positive correlation with infarct size and volume^[61-66]. In a study by Cunningham *et al.*^[67], serum NSE levels in patients with ischemic stroke were higher when compared with hemorrhagic stroke, and the highest levels in ischemia was observed at 48 to 96 h. NSE had also been found to correlate with and help in differentiation between reversible and irreversible brain damage in survivors of cardiac arrest^[68-71]. In such patients, serum NSE levels post resuscitation care are a reliable predictor of neurologic outcome and they also aid in prognostication of such patients.

CPK-BB

Of the three isoenzymic forms of creatinine phosphokinase, the CPK-BB isoform is found in the brain^[48]. CPK-BB levels in various pathological entities of brain injury such as stroke, TBI, post cardiac arrest and SAH have shown a correlation with the extent of injury and have also shown to be able to predict outcome^[72-78].

MBP

MBP originates from oligodendroglial cells and binds with myelin^[79]. In TBI it is released into CSF and serves as a useful marker predicting the clinical course and outcome^[52,80-84].

In addition there are various other proteins which are less established *via-a-vis* their role in predicting severity and outcome in the brain injured states.

TAU PROTEIN

Tau is a protein arising from the microtubules, which offers theoretical promise as a marker of brain injury and has been especially studied in TBI states^[85,86]. However, recent evidence has been very conflicting and the evidence on the diagnostic and prognostic value of tau protein and its correlation with abnormal CT findings in TBI has been very limited^[87-90].

GLIAL FIBRILLARY ACIDIC PROTEIN

As a major component of astroglia, glial fibrillary acidic protein (GFAP) offers the promise of exclusivity to the central nervous system^[91-93]. There have been numerous studies in TBI sub-population such as severe or moderate TBI wherein GFAP concentration has shown a positive correlation with severity of injury, outcomes as well as CT and MRI findings^[94-98]. In a study comparing GFAP and S-100 β , GFAP exhibited characteristics of being a more sensitive marker of neural injury. It also had higher value for predicting return to work *via-a-vis* S-100 β especially in patients with severe head injury^[99].

UBIQUITIN C TERMINAL HYDROLASE

Ubiquitin c terminal hydrolase (UCH-L1) is a neuron specific protein comprising 1%-5% of total brain protein, which has been implicated in neuron repair in pathological and degenerative conditions of the brain^[100-102]. There is a release of UCH-L1 into CSF and blood in brain injury and elevated levels have exhibited a correlation with severity and outcome in TBI populations^[103].

WHICH BIOMARKER TO CHOOSE AND WHEN?

The preceding discussion indicates that the different biomarkers in brain injury do not exactly fit into the "one size fits all" algorithm. Evidence in the field is an evolving process and it seems increasingly probable that neuro-clinicians will rely more and more on a combination of different biomarkers as an aid in diagnosis, severity scoring, prognostication and interventional decisions in brain injured patients^[101,104]. S-100, GFAP and UCH-L1 are early biomarkers of neuronal injury and have the potential to aid in clinical decision-making in the initial management of patients presenting with an acute neuronal crisis such as stroke, TBI and SAH. The other biomarkers are of value in predicting long-term complications and prognosis in such patients.

INTRICACIES OF SAMPLE COLLECTION AND ANALYSIS

While CSF levels of biomarkers reflecting CNS injury are more accurate, in acute settings such as TBI and stroke, collection of blood samples represents a more convenient

Table 1 Serum and cerebrospinal fluid biomarkers of cerebral injury

Structure effected		Findings in brain injury	
		Cerebro spinal fluid	Blood/serum
Tau protein	Axon	Levels peak 4-8 d after injury ^[111,112]	Elevated levels in hypoxic injury ^[113,114]
Myelin basic protein	Axon	Precise measurement difficult ^[115]	Elevated levels in brain injury ^[116]
γ -enolase	Neuron	Confounded by blood contaminated CSF ^[117]	Serum levels are very sensitive to lysis of RBC in blood contaminated CSF ^[117] , elevated levels in brain injury ^[116]
S-100 β	Astroglial cells	Elevated levels but less sensitive ^[108]	Confounded by release from extracerebral tissue ^[118]
GFAP	Astroglial cells	Elevated levels but less sensitive ^[107,108]	Serum levels correlate with changes in brain imaging ^[119] , no extracerebral sources detected ^[120]
UCH-L1	Neuron	NA	Only one pilot study ^[98]

GFAP: Glial fibrillary acidic protein; UCH-L1: Ubiquitin c terminal hydrolase; NA: Not available; CSF: Cerebrospinal fluid; RBC: Red blood cell.

Table 2 The components monitored by cerebral microdialysis and their clinical implications

Variable	Normal levels (at a flow rate of 0.3 μ L/min)	Clinical implications
Lactate	2.9 $\pm$ 0.9 mmol/L	Increased levels seen in ischemia and hyperglycolysis ^[121-123]
Pyruvate	166 $\pm$ 47 μ mol/L	Decreased levels seen in ischemia and hypoxic conditions ^[124,125]
L/P ratio	Normal value-20	Value > 25 - metabolic crisis ^[124]
		Type 1-lactate increased, pyruvate decreased, signifying ischemia
		Type 2-raised LPR due to primarily decreased pyruvate level, seen in glycolysis failure or shunting of glucose to alternative metabolic pathways ^[125]
Glycerol	82 $\pm$ 4 μ mol/L	One of the constituents of the cell membranes
		An increase in levels signifies cell damage ^[124]
Glutamate	16 $\pm$ 16 μ mol/L	Marker of excitotoxicity ^[124]
Glucose	1.7 $\pm$ 0.9 mmol/L	Changes in blood flow or metabolism cause disproportionate changes in brain glucose
		Affected by ischaemia, hyperaemia, hyperglycaemia, hypermetabolism and hypometabolism ^[124]

and practical approach. In recent times there have been enormous strides in the field of standardization of methods by which samples are being collected for the measurement of the neuronal biomarkers^[105,106]. Recently there have been attempts to isolate the aforementioned biomarkers from urine and saliva of patients to preclude non-invasiveness and ease collection^[107].

LIMITATIONS

The widespread use of neuro-pathological markers is limited by variability and discrepancies in the values indicating significant levels of these biomarkers. The results of various studies paint a very inconsistent picture and this could be attributed to flaws and variation in study design as well as non-standardization of techniques in collection, handling and assay of such biomarkers. To summarize, the data till date on biomarkers of the injured brain can be described as a work in progress. There is a need for robust multicentric studies which will go a long way in the determination of reference points for guidance of care in patients presenting with neurological injury.

NEW DEVELOPMENTS

In addition to serum and CSF assays of biomarkers of brain injury, there has been a variety of neuro-chemical methods which have been of use in brain tissue bio-

chemistry. These methods have gradually progressed from analysis of post mortem samples to advent of newer and sophisticated methods such as cerebral microdialysis (CMD).

CMD was a modification of the push-pull cannula technique and was invented by Delgado *et al.*^[108] with subsequent modifications and popularization by Ludvig *et al.*^[109] and Ungerstedt *et al.*^[110]. It is a novel way of monitoring brain tissue biochemical metabolites such as glucose, lactate, pyruvate, glutamate and glycerol wherein the monitoring of each component gives an idea about the severity and type of pathologic process in the brain. Table 1 summarizes all the commonly used serum and CSF biomarkers of cerebral injury with their clinical implications^[111-120]. Table 2 summarizes components monitored by cerebral microdialysis and their clinical implications^[121-125].

CMD is being increasingly used as a research tool and as a component of multi-modality monitoring in the brain injured states such as TBI, SAH, brain tumors, stroke and epilepsy. Table 3 illustrates the clinical implications of cerebral microdialysis in various scenarios^[126-151].

Proteomic analysis of potential new CSF biomarkers for TBI has not yet identified any such markers that can be used in clinically useful tests^[152]. A number of proteomic studies on potential biomarkers of TBI in peripheral blood have been published. These studies have replicated the findings from targeted analyses of specific candidate biomarkers, but as yet none of

Table 3 Cerebral microdialysis implications in clinical scenarios

Clinical condition	CMD implications
Traumatic brain injury	Helpful in optimising therapy in neuro-ICUs as a component of multi-modality monitoring Helpful in individualising management on the basis of cerebral perfusion pressure targets and assessment of response to medical and surgical interventions ^[126,127] Predictor of severity, neurological outcome and long-term anatomical aberrations in the injured brain ^[128-130] Detection and management of glycemic perturbations of the injured brain ^[131,132] Predicting long-term anatomical alteration ^[133]
Subarachnoid haemorrhage	Detection of ischemic changes during aneurysm clipping ^[134] Specific for the detection of delayed ischaemic neurological deficit ^[135-138] Prognostication of SAH patients ^[139,140]
Acute ischaemic stroke	Detecting development of oedema of the infarcted tissue ^[141] Monitoring effects of decompression hemicraniectomy and hypothermia in stroke patients ^[142,143]
Brain tumours	Neuro-biochemistry of brain tumours ^[144,145] Biochemical changes during treatment Drug pharmacokinetics study ^[146] Monitoring of drug effect Development of tumor drug delivery systems ^[147,148]
Epilepsy	Study of biochemical milieu of epileptic focus ^[149]
Other applications	Study of the perihemorrhagic zone in intracranial hemorrhage ^[150,151] Study of biochemical changes and novel therapeutic options in neurodegenerative diseases such as Parkinson's and Alzheimer's disease

CMD: Cerebral microdialysis; SAH: Subarachnoid hemorrhage; ICU: Intensive care unit.

the novel biomarker profiles identified in these studies as being associated with TBI has been validated in independent studies using unrelated, non-proteomic or genomic techniques^[153]. Exciting preliminary data on the expression profiles of small non-coding RNAs in peripheral blood mononuclear cells from military personnel exposed to mild TBI have been reported; three small RNAs seem to be primarily associated with mild TBI, but the results require replication^[154].

CONCLUSION

To conclude, biochemical markers of brain injury have witnessed major developments in acquisition and processing of samples, with cerebral microdialysis and expression of non-coding RNAs being the most recent modality to analyze such changes. Use of such biomarkers, while not as popular as their cardiac counterparts, is slowly but surely being established both in the realms of basic research as well as in management, severity scoring and prognostication of patients with neurological injury. There is abundant potential in the regular use of such biomarkers and efforts are underway to integrate such biomarkers into clinical practice in TBI, SAH and stroke.

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Retroviral integrase protein and intasome nucleoprotein complex structures

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Abstract

Retroviral replication proceeds through the integration of a DNA copy of the viral RNA genome into the host cellular genome, a process that is mediated by the viral integrase (IN) protein. IN catalyzes two distinct chemical reactions: 3'-processing, whereby the viral DNA is recessed by a di- or trinucleotide at its 3'-ends, and strand transfer, in which the processed viral DNA ends are inserted into host chromosomal DNA. Although IN has been studied as a recombinant protein since the 1980s, detailed structural understanding of its catalytic functions awaited high resolution structures of functional IN-DNA complexes or intasomes, initially obtained in 2010 for the spumavirus prototype foamy virus (PFV). Since then, two additional retroviral intasome structures, from the α -retrovirus Rous sarcoma virus (RSV) and β -retrovirus mouse mammary tumor virus (MMTV), have emerged. Here, we briefly review the history of IN structural biology prior to the intasome era, and then compare the intasome structures of PFV, MMTV and RSV in detail. Whereas the PFV intasome is characterized by a tetrameric assembly of IN around the viral DNA ends, the newer structures harbor octameric IN assemblies. Although the higher order architectures of MMTV and RSV intasomes differ from that of the PFV intasome, they possess remarkably similar intasomal core structures. Thus, retroviral integration machineries have adapted evolutionarily to utilize disparate IN elements to construct convergent intasome core structures for catalytic function.

Key words: DNA integration; 3-dimensional structure; Integrase; Intasome; Mouse mammary tumor virus; Retrovirus; Rous sarcoma virus; Prototype foamy virus; Human immunodeficiency virus/acquired immune deficiency syndrome

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Core tip: This review examines the history of retroviral integrase structural biology and covers the currently available high-resolution structures of retroviral intasomes in detail. We in particular focus on the similarities and differences among the intasome structures of prototype foamy virus, Rous sarcoma virus and mouse mammary tumor virus.

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INTRODUCTION

Retroviral replication requires the incorporation of the viral genetic information into the host cellular genome, which occurs *via* two main steps: (1) the reverse transcription of single-stranded viral RNA into linear double-stranded DNA; and (2) the integration of this DNA into a host chromosome. These steps occur in the context of two subviral nucleoprotein complexes: The reverse transcription complex (reviewed in^[1]) and the pre-integration complex (PIC)^[2], each of which consists of a variety of cellular and viral proteins including reverse transcriptase (RT) and integrase (IN)^[3-7]. In the cytoplasm, RT mediates the synthesis of a linear viral DNA (vDNA) molecule that harbors a copy of the viral long-terminal repeat (LTR) at each end^[8-10]. In the confines of the PIC, vDNA is trafficked toward the nucleus, where its integration into host cell target DNA (tDNA) is promoted by IN. Here, we discuss the current knowledge of IN structural determinants and intasome function, highlighting both key similarities and differences among the retroviruses.

REACTIONS CATALYZED BY IN

Retroviral IN performs two biochemically and temporally distinct bimolecular nucleophilic substitution (S_N2) reactions^[11]: 3'-processing and strand transfer (Figure 1). During 3'-processing, a di- or trinucleotide is hydrolytically cleaved from each 3' vDNA end^[12-14], exposing reactive hydroxyl groups of invariant CA dinucleotides. These groups act as nucleophiles for subsequent strand transfer whereby the newly processed 3' vDNA ends are covalently inserted into a major groove of tDNA in a staggered fashion. The product of the second reaction is an integration intermediate that is characterized by unjoined 5' vDNA overhangs^[15,16]. Following disassembly of the associated strand transfer complex (STC, Figure 1), a DNA polymerase, 5' flap endonuclease, and DNA ligase are required to fill in the single-strand gap regions in tDNA, excise 5' vDNA overhangs, and join the vDNA 5' ends to host DNA strands, respectively (reviewed in^[17]). During this process, short target site

duplications are generated, which flank the integrated provirus. Depending on the genus of retrovirus, the size of these target site duplications ranges from 4-6 base pairs (bp). Whereas spumavirus prototype foamy virus (PFV)^[18,19] and lentivirus human immunodeficiency virus 1 (HIV-1)^[20,21] integration yield 4 bp and 5 bp target site duplications, respectively, mouse mammary tumor virus (MMTV)^[22] and Rous sarcoma virus (RSV)^[23,24] INs cleave tDNA phosphodiester bonds that are separated by 6 bp.

RETROVIRAL IN DOMAIN

ORGANIZATION

Retroviral IN proteins consist of approximately 275-470 amino acid residues. The INs to be discussed in detail in this review amount to 288 (HIV-1), 392 (PFV), 286 (RSV), and 319 (MMTV) residues^[25-27]. Retroviral INs comprise three domains common to all genera: The N-terminal domain (NTD), the catalytic core domain (CCD), and the C-terminal domain (CTD)^[28-32], which connect to one another *via* flexible linkers that vary in length across the viruses (Figure 2). The NTD adopts a helix-turn-helix fold and harbors two pairs of Zn^{2+} -coordinating histidine and cysteine residues (HHCC motif), which are additionally conserved in retrotransposon INs and are involved in the recognition of the viral LTRs^[30,31,33-35]. Accordingly, Zn^{2+} binding triggers HIV-1 IN multimerization and increases its catalytic activity^[36,37]. The CCD adopts an RNase H fold and coordinates two Mg^{2+} ions *via* the invariant Asp and Glu amino acid residues that comprise the D, DX₃₅E catalytic triad motif^[28,29,38-40]. The coordination of Mg^{2+} ions chemically activates the nucleophiles for 3'-processing (water) as well as for strand transfer (3'-OH groups of vDNA) and destabilizes the respective scissile phosphodiester bonds^[41-43]. The CTD is the least conserved among the shared IN domains, however, the tertiary structures of resolved CTDs show similar characteristics: They adopt a Src homology 3 fold^[44,45], are involved in DNA binding^[46], and promote IN multimerization^[47,48]. Some retroviruses, namely spuma-, ϵ - and γ -retroviruses, harbor an additional NTD extension domain (NED) that precedes the NTD^[26,40,49] and engages vDNA in the context of the intasome structure^[40]. These IN proteins accordingly are larger than their lenti-, α -, β - and δ -retroviral cousins that lack the NED.

MULTIMERIZATION OF IN

Numerous biochemical studies revealed that the active form of retroviral IN is a multimer that engages vDNA and tDNA in the confines of a nucleoprotein complex^[50-58]. Bacteriophage Mu-mediated PCR footprinting of PICs extracted from infected cells revealed the protection of several hundred bp at the vDNA ends, and the associated complex was termed "intasome" to distinguish it from the larger PICs^[59,60]. Subsequently, the intasome term was adopted by structural biologists who constructed and purified distinct, functional IN-DNA complexes capable

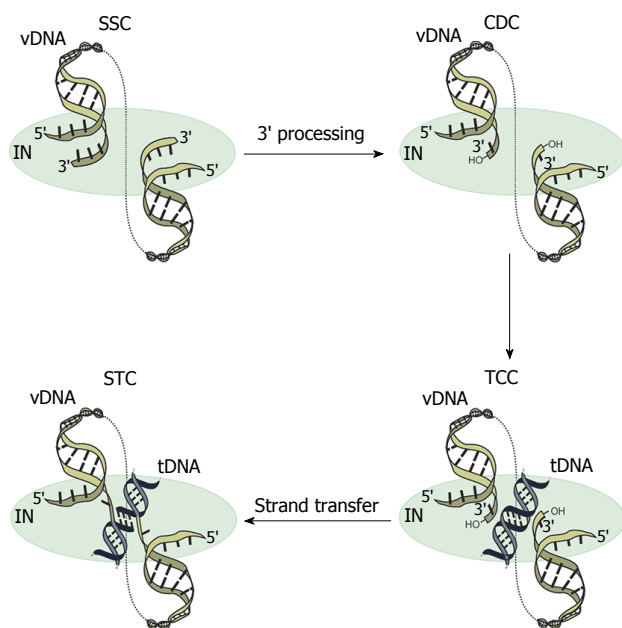


Figure 1 Integrase catalytic functions and intasome complexes. A multimer of integrase (IN) (depicted simply by blue oval) engages the end regions of the linear vDNA molecule (yellow), forming the stable synaptic complex (SSC). During 3'-processing, IN hydrolyzes the vDNA ends adjacent to invariant CA dinucleotides, revealing a set of reactive 3'-hydroxyl groups in the confines of the cleaved donor complex (CDC). After nuclear localization, the target capture complex (TCC) is formed upon tDNA (black) capture. Strand transfer, whereby IN employs the 3' hydroxyl groups as nucleophiles to attack the tDNA, marks the transition to the strand transfer complex (STC).

of efficient concerted integration of two synapsed oligonucleotide vDNA ends, the structures of which were solved by X-ray crystallography^[40,43,61-63] or single particle cryo-electron microscopy (EM)^[26]. Although retroviral INs have been studied for decades, the 3-dimensional structures of PFV intasomes greatly aided the elucidation of the details of 3'-processing and strand transfer reaction mechanisms^[40,43,62].

The "intasome" term today applies to the family of nucleoprotein complexes that are known to mediate retroviral DNA integration (Figure 1), which encompasses the stable synaptic complex (SSC)^[43], the cleaved donor complex (CDC) or cleaved intasome^[40,43], the target capture complex (TCC)^[43,62], and the STC^[62,63] (Figure 1). The SSC forms upon IN binding to the vDNA ends^[5,40,55,57-60,64,65]. The hydrolytic cleavage of a di- or trinucleotide from each 3'-end marks the transition to the CDC. The TCC forms when the CDC engages tDNA, whereas the STC is formed when the vDNA ends are inserted into tDNA and thus strand transfer is completed^[57,58,61-64,66]. The PFV system has importantly afforded high-resolution structures for each of these complexes^[40,43,62,63].

APPROACHES TO STUDY THE THREE-DIMENSIONAL STRUCTURES OF RETROVIRAL IN PROTEINS

Mechanistic studies of retroviral DNA integration began

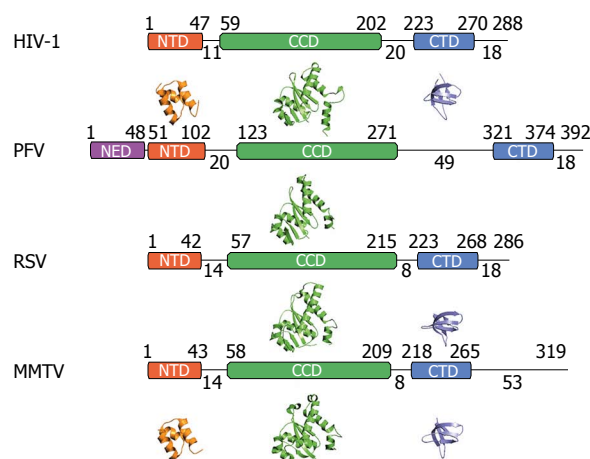


Figure 2 Integrase domain organization and representative secondary structures. Starting and ending residues for integrase (IN) domains are indicated above the boxes, and interdomain linker lengths as well as C-terminal tail lengths are indicated below the lines. Crystal structures of N-terminal domains (NTDs), catalytic core domains (CCDs), and C-terminal domains (CTDs) are provided underneath the corresponding schematic IN representation. Crystal structures in the absence of DNA are not available for the PFV NTD extension domain (NED), NTD, or CTD, as well as for the RSV NTD. PDB accession codes: HIV-1 (NTD, 1K6Y; CCD, 1BIU; CTD, 1EX4), PFV (CCD, 3DLR), RSV (CCD, 1C0M; CTD, 1C0M) and MMTV (NTD, 5CZ2; CCD, 5CZ1; CTD, 5D7U). HIV: Human immunodeficiency virus; PFV: Prototype foamy virus; RSV: Rous sarcoma virus; MMTV: Mouse mammary tumor virus.

in earnest in the late 1980s and early 1990s when PICs partially purified from infected cells were shown to promote correct DNA integration *in vitro*^[5,14,60,67-70]. Nearly parallel biochemical studies, which utilized purified avian, murine, and human retroviral proteins, importantly showed that IN alone was sufficient to catalyze both the 3'-processing and strand transfer of recombinant vDNA substrates^[71-77]. The results of these experiments opened up a new field dedicated to the structural and functional analysis of retroviral INs. Initial work on HIV-1 IN quickly revealed the relatively poor solubility of the full-length enzyme *in vitro*, which limited further biochemical and thus structural characterization^[48]. To date, there is no high-resolution structure of a full-length retroviral IN in the absence of DNA, which is likely attributable to the inherent flexibility of the interdomain linkers^[78-80]. In essence, the intertwined architecture of IN with vDNA in the context of the intasome complex necessarily "locks down" the inherently flexible enzymes, which afforded platforms for their detailed structural analyses.

STRUCTURES OF INDIVIDUAL DOMAIN AND TWO-DOMAIN IN CONSTRUCTS

Initial structures of individual IN domains or two-domain constructs in the absence of DNA (reviewed in^[81,82]) (Figure 2) turned out to be challenging in many cases, and were only possible for the HIV-1 IN protein with the help of solubility-enhancing mutations. By systematically replacing hydrophobic residues in the HIV-1 IN CCD, Phe185 was identified as a solubility-limiting residue^[83]: Substituting either Lys or His for Phe185 dramatically

improved HIV-1 IN protein solubility^[48,84]. Although the F185K change enabled the determination of the HIV-1 CCD X-ray structure^[85], it conferred a lethal viral phenotype due to deficiencies in virus particle assembly and reverse transcription in addition to defective vDNA integration^[48]. Indeed, the vast majority of IN mutations elicit such pleiotropic defects on HIV-1 replication (reviewed in^[86]). In contrast, the F185H change was tolerated by the virus, and importantly enabled crystallization of the CCD^[84,87]. In both cases, the positively charged substituent residue reaches across the CCD dimerization interface and makes a hydrogen bond contact with the main chain carbonyl of Ala105 of the partner IN monomer. The F185K and F185H changes are therefore likely to dramatically increase HIV-1 IN solubility by removing a surface exposed aromatic residue that may nucleate protein aggregation, as well as by enhancing multimerization by adding two hydrogen bonds per IN dimer. Even greater solubility of the HIV-1 CCD was achieved by mutating the tryptophan at position 131 to glutamic acid in the context of the F185K change^[88]. Simultaneous to the work on the HIV-1 IN CCD, the X-ray structure of the avian sarcoma virus (ASV) IN CCD was solved^[89]. Of note, ASV IN harbors His at the position analogous to Phe185 in HIV-1 IN, and solubility-enhancing mutations accordingly were not required to crystallize the ASV IN CCD. Succeeding structures of HIV-1^[88,90-93] and ASV^[94-97] IN CCDs elucidated binding sites for metal ion cofactors as well as for early inhibitors. NTD and CTD structures of HIV-1 IN and HIV-2 IN, which were solved by nuclear magnetic resonance spectroscopy^[34,35,44,45,98,99], suggested functions of the NTDs in metal ion coordination and the binding of the CTD to vDNA, each of which is required for integration.

Two-domain IN constructs were initially studied as an approach to understand how individual IN domains might interact to form an active nucleoprotein complex. Three different CCD-CTD two-domain structures for HIV-1, simian immunodeficiency virus (SIV), and avian sarcoma-leukosis virus (ASLV) were reported in 2000^[100-102]. Each construct harbored at least one solubility-enhancing mutation: C56S, W131D, F139D, F185K, and C280S for HIV-1, F185H for SIV, and F199K for ASLV, the latter two of which are analogous to the F185K change in HIV-1. The HIV-1 CCD-CTD structure revealed an extended α -helix for the CCD-CTD linker region^[100]. Whereas the SIV IN CCD-CTD linker could not be traced^[101], the RSV IN CCD-CTD linker had a rather extended, non-helical form compared to the HIV-1 IN CCD-CTD linker^[102]. The appearance of three different linker configurations in three different IN CCD-CTD constructs led some to suggest that such configurations may result from crystal packing and therefore represent limited physiological relevance^[82].

Crystallization of a HIV-1 IN NTD-CCD construct was achieved in 2001 by including W131D, F139D and F185K solubility-enhancing mutations^[103]. The resulting X-ray structure revealed possible interactions between two of the NTDs with two CCDs of opposing NTD-CCD molecules, which was of potential physiological relevance

due to the fact that it was known from prior biochemical studies that the NTD functioned *in trans* with the CCD^[51-53]. However, the inability to trace the NTD-CCD linker regions limited the confidence of this interpretation. Importantly, the domain sharing arrangement suggested by this structure was later confirmed by additional NTD-CCD structures and mutagenesis^[104], and ultimately through the elucidation of intasome structures (see below).

LENTIVIRAL IN-LEDGF CO-CRYSTAL STRUCTURES

In the early 2000s, a host factor implicated in the nuclear retention of HIV-1 IN, lens epithelium-derived growth factor/transcriptional co-activator p75 (LEDGF/p75), was reported to increase the solubility of HIV-1 IN through its tight binding interaction^[105-107]. LEDGF/p75 is a lentiviral-specific IN-binding protein^[105,108,109] that tethers vDNA integration to transcriptionally active regions of the host genome (reviewed in^[110,111]). LEDGF/p75 engages lentiviral IN *via* its C-terminally located IN-binding domain (IBD)^[112]. Although the HIV-1 IN CCD was sufficient for LEDGF/p75 binding, the NTD was required for the high efficiency interaction^[107]. LEDGF/p75 binding stabilizes lentiviral IN tetramerization^[104,113], which is likely related to its ability to enhance the solubility of the viral proteins.

Crystal structures of lentiviral INs in complex with LEDGF/p75 include an HIV-1 IN F185K CCD construct^[114] as well as HIV-2^[115] and maedi-visna virus (MVV) IN NTD-CCD two-domain fragments^[104]. Though HIV-2 and MVV INs harbor hydrophobic residues at the positions analogous to Phe185 in HIV-1 IN (Phe and Ile, respectively), the favorable solubility properties of lentiviral IN-LEDGF/p75 complexes likely dispelled the need for solubility-enhancing mutations for the crystallization of these constructs. The LEDGF/p75 IBD is a PHAT domain composed of two helix-hairpin-helix motifs^[116], with Asp366 at the tip of the N-terminal hairpin nestling into a binding cleft at the HIV-1 IN CCD dimerization interface and contacting the main chain amides of IN residues Glu170 and His171 *via* hydrogen bonds^[114]. A novel class of potent anti-HIV compounds, known as LEDGINS (LEDGF-IN inhibitors) or ALLINIs (allosteric IN inhibitors), structurally mimic the role of Asp366 in their binding to HIV-1 IN, which accounts for their abilities to compete for LEDGF/p75 binding to IN (reviewed in^[117]). The two domain NTD-CCD constructs revealed the structural basis for the IN NTD interaction with the LEDGF/p75 IBD, which was ionic in nature. Interestingly, the polarities of the participating salt bridges were functionally reversible, such that HIV-1 particles carrying NTD reverse charge substitutions that were otherwise dead regained partial activity in the presence of the complementary reverse charge LEDGF/p75 partner protein^[115,118].

PFV INTASOME STRUCTURE

Although the aforementioned individual and two-domain

constructs provided insight into retroviral IN function, the field sorely required the structural determination of a functional intasome. The sole class of clinically approved HIV-1 IN inhibitors, known as IN strand transfer inhibitors (INSTIs), displays little if any binding affinity for free IN protein; their clinical target is the IN-vDNA complex^[119]. Fortuitously, INSTIs are active against most types of retroviruses^[120-122], so intasome structures derived from basically any retroviral genus would have in theory provided a backdrop for understanding the structural basis for INSTI action and the clinical emergence of drug resistance.

Due to the poor solubility of HIV-1 and other early studied retroviral INs, the search for an enzyme with more favorable biochemical properties for *in vitro* experimentation and crystallography was initiated. Though early work had revealed that relatively short oligonucleotide substrates, which modeled the vDNA ends, supported IN 3' processing and strand transfer activities^[72-77], not all enzymes behaved similarly. Most critical for intasome structural biology was the ability for the IN multimer to coordinate the binding of two vDNA ends, and insert these in concerted fashion into opposing strands of tDNA. Critically, PFV IN was discovered to promote efficient concerted integration of oligonucleotide vDNA ends^[121]. By contrast, HIV-1 IN had revealed the tendency to insert just one vDNA end at a time^[77]. Subsequent modifications of HIV-1 IN expression systems, including protein purification under relatively dilute conditions to prevent IN aggregation^[123], or by fusing the small Sso7d DNA binding domain from *Sulfolobus solfataricus* to the IN N-terminus to mimic the NED that naturally exists in PFV IN^[124], yielded proteins that supported efficient concerted integration activity. Such modifications might eventually prove useful to characterize HIV-1 intasomes structurally^[123,124].

Functional PFV-vDNA complexes assembled by differential salt dialysis migrated as a distinct species on gel filtration columns, and remained intact and active following challenge with high salt concentrations^[40]. The initial X-ray crystal structure of the PFV intasome, representing the CDC, was reported in 2010^[40] (Figure 3). To date, 37 PFV intasome structures composed of wild-type IN or mutant variants that contain clinically relevant amino acid substitutions have been solved by X-ray crystallography or cryo-EM, representing complexes in the presence of divalent metal ion cofactors, tDNA/nucleosomes, and INSTIs^[40,43,62,63,125-130]. The INSTI-bound structures elucidated the mechanism of drug action: The halo-benzyl chemical group common to these compounds assumes the position of the invariant 3' deoxyadenylate in vDNA with its critical hydroxyl group, thus ejecting the strand transfer nucleophile from the enzyme active site and disarming the nucleoprotein complex^[40].

The PFV intasome consists of a tetrameric assembly of IN arranged around a dimer-of-dimers architecture^[40] (Figure 3). The inner dimer is composed of two inner monomers (green and pink in Figure 3A), whereas each

outer dimer is composed of an inner IN monomer and an outer IN monomer (cyan-green and pink-light grey in Figures 3 and 4). The inner IN monomers make all contacts with vDNA and thus are the catalytic subunits, with each of their constitutive domains mediating vDNA in addition to IN-IN contacts. As previously alluded to, the catalytic subunits are established *via* a domain sharing mechanism whereby the NTD of each inner IN monomer interacts intimately with the CCD of the opposing IN monomer. The outer IN dimers center around the extensive CCD dimeric interface observed in prior retroviral IN CCD crystal structures^[85,87-97]. The CTDs, NTDs, and NEDs of the outer IN monomers are not resolved in the electron density maps, and it is currently unclear what precise role(s) they may play in the catalysis of vDNA integration^[131]. It is generally thought that the outer IN monomers mainly play a supportive architectural role to truss the inner IN monomers and vDNA together. As the outer CTDs can contribute to nucleosome binding *in vitro*^[130], it seems possible they might play a role during vDNA integration into chromatinized templates, as occurs during virus infection. The NTD-CCD and CCD-CTD linkers, which are only visible for the inner IN monomers in the crystal structures, adopt extended conformations and contact the vDNA^[40].

MMTV AND RSV INTASOME STRUCTURES

MMTV and RSV intasome structures were recently solved using single-particle cryo-EM^[26] and X-ray crystallography^[61], respectively. The MMTV intasome was assembled using pre-processed 22 bp vDNA, and thus represents the CDC^[26]. The RSV intasome structure by contrast is the STC, which was assembled using a so-called X-mer disintegration substrate^[132] where three oligonucleotide strands were annealed together to yield a synapsed complex composed of two 22 bp vDNA branches covalently linked through a central 6 bp stagger to a 38 bp palindromic tDNA^[61]. The crystal structure of the PFV STC assembled with its analogous X-mer DNA substrate^[63] was virtually identical to the structure that was solved when the CDC integrated into tDNA during crystallogenesis^[62], validating the X-mer substrate design approach for RSV STC crystallography.

Although the tetrameric IN₄-to-vDNA₂ stoichiometry represented by the PFV intasome was generally thought to be evolutionarily conserved across the retroviruses^[54,55,57,58], the intasome structures of MMTV and RSV strikingly revealed octameric IN assemblies^[26,61] (Figures 3 and 4). The MMTV and RSV intasomes comprise a core density region consisting of IN dimers A and B, as well as flanking density regions that consist of IN dimers C and D (Figure 3). Analogous to the PFV structure, core inner IN monomers IN1 and IN3 intimately contact the vDNA ends and are catalytically active, with their NTDs reaching out and contacting the CCDs of the opposing inner monomer (Figure 4). The core structure moreover is primarily

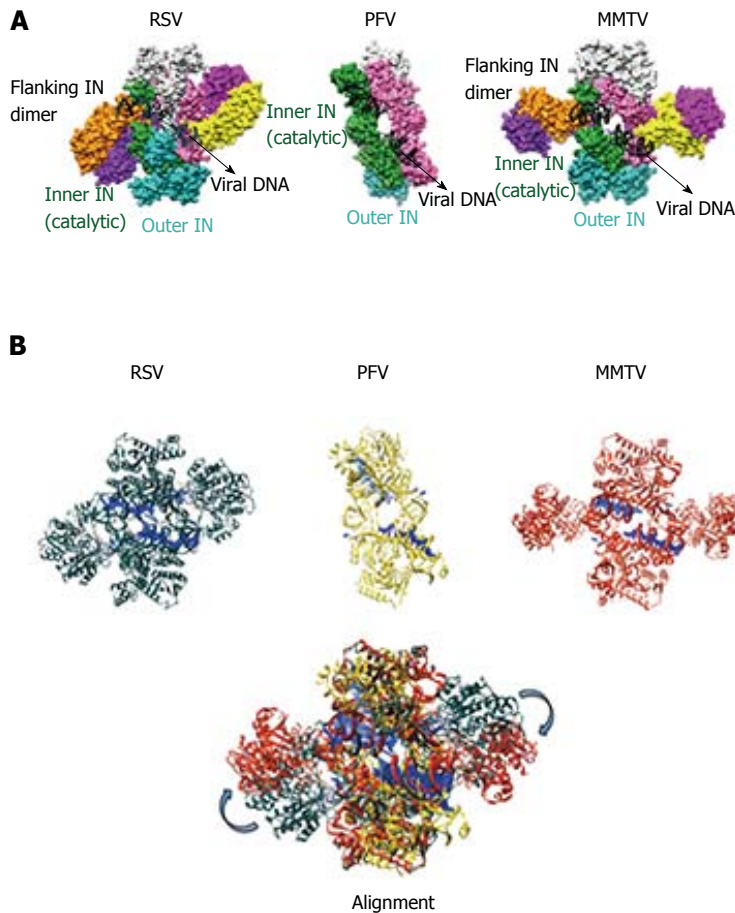


Figure 3 Comparison of prototype foamy virus, Rous sarcoma virus and mouse mammary tumor virus intasomes. A: The PFV intasome comprises two catalytic inner subunits (green and pink) and two outer supportive INs (cyan and light grey). Only the CCDs of the outer subunits are discernable in crystallographic electron density maps. RSV and MMTV share the PFV intasome core architecture and employ two additional flanking IN dimers (orange-purple and yellow-dark pink) to complete the intasome structures; B: Three-dimensional alignment of RSV (grey, PDB accession code 5EJK), PFV (yellow, PDB code 3L2Q), and MMTV (red, PDB code 3JCA) intasome structures was performed using Chimera. For the MMTV intasome, flanking dimers were unambiguously positioned into the intasome core of the cryo-EM map *via* rigid-body docking. The alignment reveals a high degree of flexibility (approximately 30-40 Å) for the flanking RSV and MMTV dimers relative to the common intasome core structures (arrows). PFV: Prototype foamy virus; RSV: Rous sarcoma virus; MMTV: Mouse mammary tumor virus.

supported through CCD dimerization interfaces with outer IN monomers IN2 and IN4. In contrast to PFV, flanking structures in the MMTV and RSV intasomes constitute additional IN dimers, which on their own multimerize primarily through the familiar CCD dimer interface^[26,61]. The NTD-CCD linker that is extended in IN1 and IN3 to contact the opposing CCD *in trans* is contracted in the other IN monomers^[26]. This observation highlights the necessity for NTD-CCD linker flexibility: Though principally contracted, it must also possess the ability to extend when situated at the IN1 and IN3 positions to support IN catalytic function. In hindsight, it is not surprising that the linker regions in the original HIV-1 IN NTD-CCD structure, which lacked LEDGF/p75 or DNA binding partners, were untraceable^[103]. Of note, whereas the crystallographic PFV intasome structure is rather rigid, the flanking dimer regions in the MMTV and RSV intasome structures reveal significant flexibility (Figure 3B). As small angle X-ray scattering analysis of the PFV intasome revealed significant conformational flexibility for the outer subunit NEDs, NTDs and CTDs of the IN tetramer^[79], it is tempting to describe retroviral

intasomes as common rigid core structures surrounded by extraneous elements that, although likely to play physiologically relevant roles during virus infection, display marked movement as purified biochemical entities.

The most striking difference between the tetrameric and octameric assemblies is the unique function attributed to the CTDs of the flanking dimers in the MMTV and RSV intasome structures. While contacts with vDNA in the PFV structure are restricted to the inner IN1 and IN3 subunits, MMTV and RSV INs donate their CTDs *in trans* to the core region of the intasome^[26,61]. The locations of six CTDs, including those of the flanking IN dimers, are conserved in the MMTV and RSV intasomes (Figure 4). The exclusive conformation of the CTDs allows them to tightly associate near the vDNA and assume positions resembling those of the inner PFV CTDs (Figure 4). Biochemical complementation assays revealed that the flanking MMTV IN dimers are crucial for IN catalytic function^[26]. Intriguingly, the length of the CCD-CTD linker is quite variable among retroviral INs, amounting to about 50 residues for PFV, but only to 8 residues for MMTV and RSV IN^[26] (Figure 2). The extended conformation of the

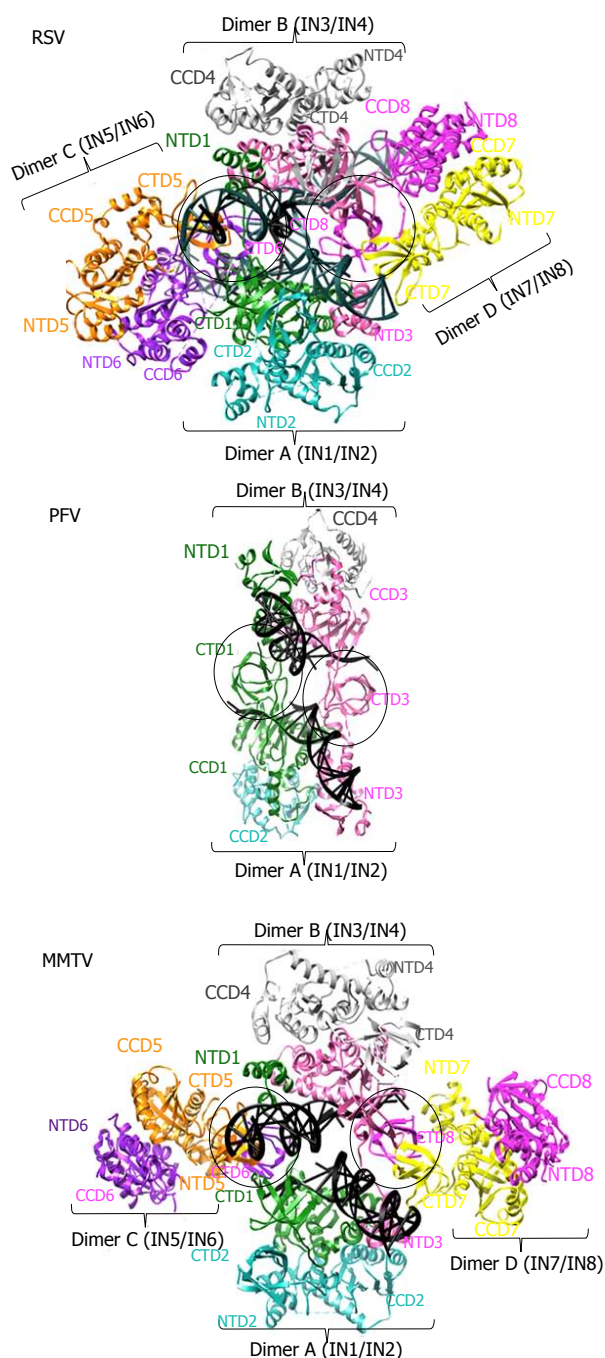


Figure 4 Integrase domain organizations within the prototype foamy virus, Rous sarcoma virus and mouse mammary tumor virus intasome structures. Separate integrase (IN) domains are labeled, with IN monomer coloring code retained from Figure 3. The green IN1 and pink IN3 monomers donate their active sites for catalysis of 3' processing and strand transfer across the structures. Circled areas represent similarly positioned CTDs. While these emanate from inner IN1 and IN3 monomers in the PFV structure, they originate from flanking MMTV and RSV IN monomers IN6 and IN8. PDB accession codes same as in Figure 3. PFV: Prototype foamy virus; RSV: Rous sarcoma virus; MMTV: Mouse mammary tumor virus; CCD: Catalytic core domain; NTD: N-terminal domain; CTD: C-terminal domain.

PFV IN 49-mer CTD-CCD linker affords the positioning of IN1 and IN3 CTDs to enable critical contacts with vDNA and tDNA during integration^[40,62]. The analogous HIV-1 IN linker, composed of 20 residues (Figure 2), could be stretched to similarly position inner IN monomer CTDs in

a molecular model of the HIV-1 intasome based on the PFV structure^[133]. However, it is physically impossible for an 8 amino acid region to span the required distance. MMTV and RSV accordingly solve this conundrum by employing additional IN molecules to donate their CTDs to the required positions in the intasome core structure (Figure 4). Hence, retroviral IN CTD-CCD linker length is suggested to be a key determinant for the higher-order architecture of the respective intasome structures^[26,61].

TARGET DNA BINDING AND STRAND TRANSFER

The integration of vDNA into tDNA does not occur randomly in the host genome, with integration site selection preferences varying among retroviruses (recently reviewed in^[134]). Whereas HIV-1 and other lentiviruses favor integration into highly expressed genes that are rich in introns^[135-139], the spumavirus PFV avoids active gene regions^[130,140,141]. Interestingly, α - and β -retroviruses such as RSV and MMTV are the least selective in integration site selection, displaying patterns that much more closely approach random^[138,142,143].

The co-crystallization of the PFV intasome with an oligonucleotide tDNA^[62] derived from the PFV consensus integration sequence^[121,140] elucidated the mechanism of strand transfer. Whereas crystallization in the absence of Mg^{2+} or the presence of a dideoxy viral 3' end led to high-resolution TCC structures, the addition of Mg^{2+} with the normal vDNA end afforded integration during crystallography, yielding the first high-resolution structure of a retroviral or bacterial transposon STC^[62] (Figure 5). The tDNA adopted a highly bent conformation at the PFV DNA insertion sites, with the major groove widened to 26.3 Å and the minor groove compressed to 9.6 Å^[62]. This conformation enables the accommodation of the inner IN1 and IN3 D₁DX₃₅E catalytic triads to the scissile phosphodiester bonds of tDNA, thus promoting integration^[62]. As S_N2 transesterification reactions are iso-energetic, they have the potential to reverse direction if chemical leaving groups remain associated with the catalytic active site. Following strand transfer, the newly formed phosphodiester bonds are displaced by 2.3 Å from the IN active sites, effectively suppressing the probability for strand transfer reversal^[62]. A similar displacement is described for the tDNA in the RSV intasome^[61].

Early studies revealed that retroviral INs prefer chromatinized tDNA templates over naked DNA for integration *in vitro*^[144-147], and subsequent work revealed the propensity to similarly target nucleosomes during virus infection^[135,148]. PFV IN prefers relatively flexible tDNA sequences for integration^[62], and a cryo-EM structure of an intasome-nucleosome complex revealed the same degree of local tDNA distortion can occur on the nucleosome surface during PFV integration^[130]. Since spumaviral INs cleave tDNA with a 4 bp stagger, it has been suggested that the degree of tDNA kink

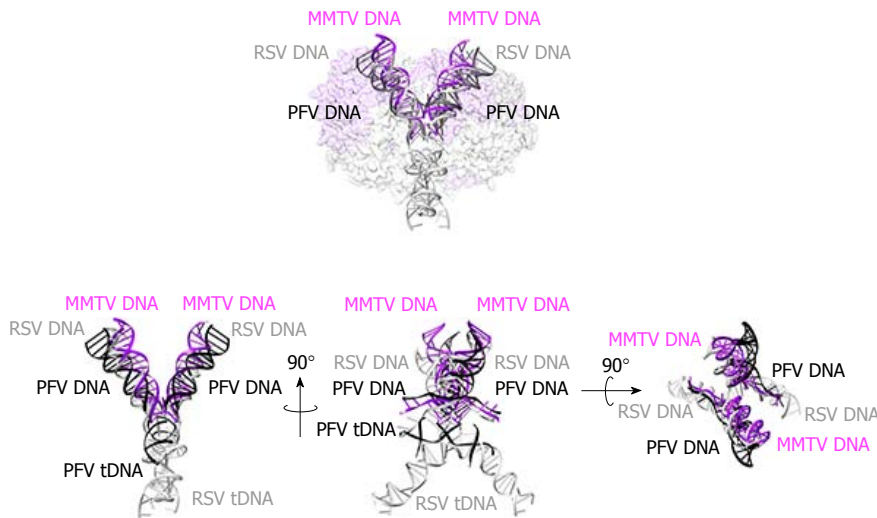


Figure 5 Superposition of prototype foamy virus strand transfer complex, Rous sarcoma virus strand transfer complex, and mouse mammary tumor virus cleaved donor complex structures with respect to vDNA and tDNA. For simplicity, integrase content is either partially transparent or omitted. Color-coding is as following: PFV DNA: Black; RSV DNA: Grey; MMTV DNA: Purple. 90° rotations show different angles of the intasomes. PDB accession codes: PFV STC: 3OS0, RSV STC: 5EJK, MMTV CDC: 3JCA. PFV: Prototype foamy virus; RSV: Rous sarcoma virus; MMTV: Mouse mammary tumor virus; STC: Strand transfer complex; CDC: Cleaved donor complex.

has to be greater for spumaviral integration than for viruses that cleave target DNA with a 6 bp stagger, such as the α - and β -retroviruses^[149]. Although the IN1 and IN3 catalytic triads of the PFV and MMTV intasomes are superimposable, modeling of tDNA into the MMTV CDC revealed a relatively unbent conformation to accommodate a 6 bp staggered cut as compared to the highly bent tDNA conformation for the 4 bp stagger in the PFV TCC^[26] (Figure 5).

The RSV STC harbors a highly bent tDNA conformation despite the fact that RSV IN cleaves the DNA with a 6 bp stagger (Figure 5). Kinks located at the vDNA/tDNA junctions of the RSV intasome provoke a 20 Å shift in the helical axis, leading to an overall tDNA twist^[61]. Hence, the tDNA conformation in the RSV STC differs significantly from the tDNA conformation in the PFV structure (Figure 5) and also from the relatively unbent tDNA conformation in the MMTV TCC model^[26]. These observations suggest potentially different modes of integration into nucleosomal DNA among the studied viruses. Whereas PFV and RSV are predicted to target chromatinized DNA during virus infection, MMTV was unique among a study of 10 exogenous retroviruses for its apparent avoidance of nucleosomal DNA *in vivo*^[149]. Elucidation of high-resolution MMTV STC/TCC structures will help to illuminate the degree of tDNA bending that occurs during MMTV integration.

Based on the variety of tDNA structural properties that influence target site selectivity, including bendability^[62,149-152], major groove widening, and nucleosomal packaging^[144-147,153,154], retroviral INs can be classified as shape-readout DNA binding proteins^[155]. DNA minicircles, which mimic nucleosome-induced tDNA circularization in the absence of histones, represent a relatively new tool to tease out physiologically-relevant influences of tDNA structure on integration site selectivity *in vitro*, and the

roles of IN-binding cofactors such as LEDGF/p75^[156].

CONCLUSION

The study of retroviral integration has come a long way since its beginnings in the late 1970s. The relatively large repertoire of individual and two-domain retroviral IN structures that were solved initially has since expanded to a set of high-resolution intasome structures, including those from the spumavirus PFV, β -retrovirus MMTV, and α -retrovirus RSV. To date, the plethora of PFV intasome structures represents a remarkable advance for the field of retroviral integration. Not only have they elucidated the mechanism of INSTI action, they provide high-resolution structures of the entire set of complexes (SSC, CDC, TCC and STC) that mediate retroviral DNA integration^[40,43,62,63].

The recently emerged octameric intasome structures of MMTV^[26] and RSV^[61] reveal an unexpected evolutionary diversity among retroviruses. As the intasomal core is conserved among the three studied retroviruses, the utilization of flanking dimers to complete the functional MMTV and RSV intasome structures represents a remarkable example of convergent evolution of the DNA integration apparatus^[26,61]. Considering CCD-CTD linker length as a predictor of the state of IN multimerization within functional intasomes^[26], it remains to be investigated whether retroviral INs with intermediary linker lengths, including those of HIV-1 and the δ -retrovirus human T-cell lymphotropic virus, which harbor 20 and 19 residues, respectively^[26], will reveal tetrameric, octameric, or perhaps even higher-order IN assemblies.

Motivated by the recent advances in the intasome field, new IN-DNA complexes are currently being investigated in various laboratories, including those derived from lentiviruses. The emergence of new three-

dimensional intasome structures will help to model novel interactions between HIV-1 IN and DNA, and thus should reveal new insights into the mechanisms of emergence of drug resistance to clinical INSTIs.

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Role of microRNAs in translation regulation and cancer

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Abstract

MicroRNAs (miRNAs) are pervasively expressed and

regulate most biological functions. They function by modulating transcriptional and translational programs and therefore they orchestrate both physiological and pathological processes, such as development, cell differentiation, proliferation, apoptosis and tumor growth. miRNAs work as small guide molecules in RNA silencing, by negatively regulating the expression of several genes both at mRNA and protein level, by degrading their mRNA target and/or by silencing translation. One of the most recent advances in the field is the comprehension of their role in oncogenesis. The number of miRNA genes is increasing and an alteration in the level of miRNAs is involved in the initiation, progression and metastases formation of several tumors. Some tumor types show a distinct miRNA signature that distinguishes them from normal tissues and from other cancer types. Genetic and biochemical evidence supports the essential role of miRNAs in tumor development. Although the abnormal expression of miRNAs in cancer cells is a widely accepted phenomenon, the cause of this dysregulation is still unknown. Here, we discuss the biogenesis of miRNAs, focusing on the mechanisms by which they regulate protein synthesis. In addition we debate on their role in cancer, highlighting their potential to become therapeutic targets.

Key words: MicroRNA; Translation; Cancer; OncomiR; Tumor suppressor

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Core tip: MicroRNAs (miRNAs) are short non-coding RNAs (19-25 bp in length) which negatively regulate gene expression at the mRNA and protein level. By binding coding transcripts, miRNAs cause degradation or translation inhibition of their target genes and affect a multitude of biological processes, such as proliferation and tumor growth. In this review we critically analyze the mechanism of action of miRNAs and their potential role in cancer, opening a window on future perspectives for their use as novel therapeutic targets.

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INTRODUCTION

Recent advances in transcriptome analysis and high-throughput technologies highlighted an impressive complexity in the RNA world. The most studied RNA regions are protein-coding genes, mRNAs, accounting for around 1.5% of the human genome^[1]. The importance of coding mRNAs is undisputable as they have been for years the building brick of experimental biology, culminating in the systematic deletion of coding genes in several species. Not less important are retrotransposons, specific genetic elements which are known to regulate gene expression^[2,3]. Since RNA was identified as the crux of genetic regulation, the idea that it carries fundamental information has been extended to novel classes of RNA. More recently, the non-protein coding portion of the genome gained attention due to its unexpected role in regulating development and disease^[4]. Nowadays, most scientists agree in stating that transcription of the human genome is pervasive, therefore raising questions on the function of many uncharacterized RNAs.

The discovery of non-coding RNAs (ncRNAs) has changed the way we look at the human genome and led the scientific world to characterize the different types of ncRNAs transcribed in human cells. Although there is not a clear delineation of ncRNA classes, they are usually classified, according to their nucleotides length, in three main groups: Short ncRNAs, mid-size ncRNAs and long ncRNAs^[5]. Among short ncRNAs we can distinguish between microRNAs (miRNA) and piwi-interacting RNAs (piRNAs), respectively 19-25 base pairs (bp) and 26-31 bp long. miRNAs are involved in the regulation of gene expression at the translational and stability level^[6-8], while piRNAs are involved in DNA methylation and transposon repression^[9-11]. Small nucleolar RNAs (60-300 bp) are part of mid-size RNAs and act as guides for rRNA modifications^[12], Promoter Associated RNAs (22-200 bp) belong to the same group but their function is obscure^[13]. Last but not least, long non-coding RNAs (lncRNAs) comprise all ncRNAs longer than 200 nucleotides and include the largest portion of the non-coding transcriptome^[4]. lncRNAs are involved in several biological and pathological processes, such as genomic imprinting, telomere regulation, X-chromosome inactivation, development, stem cell pluripotency, immune regulation, cancer progression and in metastatic potential^[14,15]. In particular, a subset of lncRNAs, the T-UCR, themselves target specific miRNAs. The binding between these lncRNAs and miRNAs prevents target transcription degradation determining an intricate co-regulation between lncRNAs and miRNAs^[16-18] and strictly linking these two different types of ncRNAs. It should

be however stressed out that the definition of ncRNA relies mainly on bioinformatic tools that are likely to be challenged in the next future. In particular, open reading frames (ORF) shorter than 100 nucleotides and/or lacking a strong ATG consensus sequence for translational start are considered noncoding. In view of the emergence of alternative translational start sites^[19], we may discover that at least some ncRNAs are indeed "coding" for small peptides.

The relevance of the non-coding transcriptome in the comprehension of human diseases is highlighted by the impressive number of ncRNAs that are abnormally expressed in cancer, in neurological and heart diseases or in immune disorders. In this context, short RNAs have attracted the attention of most researchers. Here, we focus on miRNA and on their role in translation regulation and cancer. In particular we zoom in the known mechanisms of miRNA-regulated translation, after a brief elucidation of their discovery and biogenesis. Finally, we account for the aberrant expression of miRNAs in cancer and for their therapeutical potential as new drugs.

miRNA: DISCOVERY AND BIOGENESIS

A brief history

miRNAs are endogenous, non-coding single stranded RNAs of approximately 19-25 nucleotides in length, found both in animals and plants and involved in post transcriptional regulation^[7,20]. Two decades ago the existence of miRNAs was obscure and the scientific community was focused largely on protein-coding genes.

However in 1993 the discovery of the first small ncRNA *lin-4*, in *C. elegans*, has totally changed the scientists' point of view^[21]. At the time of the first discoveries, two main questions were raised: (1) what is the role of *lin-4*; and (2) what is its mechanism of action? Genetic studies showed that *lin-4* is one of the most relevant genes involved in the control of temporal development of larval stages^[22,23]. Almost simultaneously, Lee and collaborators discovered that null mutations of the *lin-14* gene were able to cause an opposite phenotype to null *lin-4* mutations, suggesting that *lin-4* could regulate *lin-14*^[23,24]. How was this regulation taking place? Several groups unequivocally demonstrated that the introduction of mutations in the putative ORF of the *lin-4* gene, did not affect its function, concluding that *lin-4* did not encode for a protein. Mature *lin-4* was found to be present in two small transcripts with different lengths, 22 and 61 nucleotides^[24]. Furthermore, mutations in the 3'UTR of *lin-14* mRNA and gene fusion experiments showed that *lin-14* was downregulated posttranscriptionally by *lin-4*, delineating the 3'UTR of *lin-14* as necessary for the regulation of LIN-4 protein levels^[25,26]. These data led to a unified conclusion: *lin-4* transcripts were complementary to the 3'UTR of the *lin-14* gene and regulated its expression by annealing to its 3'UTR. With a similar approach, seven years later another miRNA was discovered, *let-7*, which was able to regulate *lin-41* expression by binding to its 3'UTR^[27,28]. Further, the sequence of *let-7* was found conserved among species,

from flies to humans. A new era in transcriptomics was now open for study by the entire scientific world!

miRNA biogenesis and function

miRNA biogenesis occurs in two main steps that take place in the nucleus and in the cytoplasm. miRNA genes are transcribed by RNA polymerase II and processed through both a canonical and a non-canonical biogenesis pathway. During canonical biogenesis primary miRNAs (pri-miRNAs) are processed into the nucleus by the RNase III Drosha generating an approximately 70 nucleotide-long precursor miRNA (pre-miRNA) product. In the non-canonical pathway pre-miRNAs are instead generated by the mRNA splicing machinery, avoiding Drosha digestion^[29]. The subsequent steps are identical in both the canonical and non-canonical pathways. Pre-miRNAs are recognized by the Ran-GTP dependent transporter Exportin 5, which mediates their translocation to the cytoplasm. Here, Dicer, an other RNase type III enzyme, cleaves the pre-miRNA hairpins and the mature miRNAs generated by this mechanism are loaded into miRISC (miRNA associated RNA induced silencing complex), where, with the help of Argonaute proteins, they act as post-transcriptional regulators^[30]. It is clear that, due to its complexity, the system of miRNA biogenesis requires a tight control. Transcriptional regulation remains the preferential process of miRNA expression control^[31]. Knockout of Drosha causes the entire ablation of canonical miRNA production, suggesting its essential role in miRNA biogenesis^[32]. DGCR8 is able to stabilize the Drosha complex by binding to Drosha itself. Drosha reduces DGCR8 expression^[33,34]. It has also been shown that high levels of DGCR8 compromise Drosha activity^[35]. Thus, complex networks may regulate Drosha complex activity. Dicer-deleted cells, instead, show some detectable canonical miRNAs, even if at reduced levels. What is more, Dicer is destabilized by low expression of TRBP. These data reveal the important, but not essential contribution of Dicer in the miRNA biogenesis pathway^[32,36,37].

miRNA biogenesis is characterized by a physical separation between Drosha (nucleus) and Dicer (cytoplasm). Nevertheless, several mature miRNAs are located in the nucleus, like miR-29^[38,39], or in the mitochondria, such as miR-1 and miR-181^[40-42], or in small vesicles, suggesting non-canonical roles for miRNAs. Particularly, several studies reveal that miRNAs are transported into the nucleus, where they regulate the maturation of other miRNAs, by targeting their primary transcript, or control their own expression. Here, they can also bind long ncRNAs and thus regulate their expression and maturation^[43]. In conclusion, it would be important for miRNA characterization to explore potential roles in non-canonical functions.

The function of miRNAs was first defined 20 years ago. A mature miRNA loaded into the RISC, is capable to bind and regulate the expression of target mRNA *via* base-pairing. In particular, miRNAs bind the 3'UTR of target mRNAs through a sequence of 2-8 nucleotides

in their 5'end, termed seed region (Figure 1A). The partial or perfect complementarity between miRNAs and target mRNAs causes repression of translation or mRNA degradation, respectively^[44] (Figure 1B). Owing to the short base pairing between a miRNA and the 3'UTR of its target mRNA, the interaction is dynamic: One miRNA can bind sequentially to hundreds of target mRNAs and a single mRNA can be targeted by several miRNAs^[20]. miRNAs are able to select and interact with their targets based on I) their expression levels or II) expression levels of their mRNA targets. Since the expression and function of some miRNAs are species- and/or tissue-specific, the co-localization of a miRNA with its mRNA target is clearly necessary for its functionality^[45]. Moreover, tissue-specific miRNAs can localize both in intragenic and intergenic regions, and consequently they could be under the control of host gene promoters or, alternatively, they could hold their own promoter. Hence, for the intragenic miRNAs, expression could also be dependent on the transcription of host genes^[46], suggesting the latter to be able to influence miRNA function.

Summarizing, by selecting their targets in a dose-dependent manner, miRNAs could control the balance of specific cellular processes.

Recent reports have suggested that in addition to the classical binding of miRNAs to the 3'UTR of mRNAs, they are able to bind also the 5'UTR region and ORF^[47,48]. Sites located in coding regions and in 5'UTRs appear to be less robust than those in 3'UTR and, surprisingly, determine translational activation, and not repression, of miRNA-targeted mRNAs. This situation has been described upon growth arrest conditions^[49,50] (Figure 1C). However, these models are not universally accepted because ribosomes that scan the 5'UTR and the ORF are expected to remove annealed miRNAs.

Nowadays, the miRNA landscape is very tangled as the number of miRNA genes is exponentially growing^[51], rendering it much more difficult to clearly define their function. miRNA genes have been clustered into different groups, known as miRNA families, based on the sequence of mature miRNAs or on the structure of pre-miRNAs. This clusterization is really relevant for studying miRNA functions, since miRNA genes belonging to the same family co-localize and take place in the same specific mechanism, *e.g.*, immune system regulation, development or cancer^[52]. Moreover, the increasing number of novel miRNAs reveals that some of them are evolutionarily conserved whilst others are species- and/or tissues-specific. The expression levels of both the newly discovered and the long-known miRNAs are different from tissue to tissue, unraveling a differential tissue- and cell specific-functional impact of miRNAs^[53].

miRNAs AND TRANSLATION REGULATION

mRNA translation is a cellular process finely regulated during growth and development, and its control is

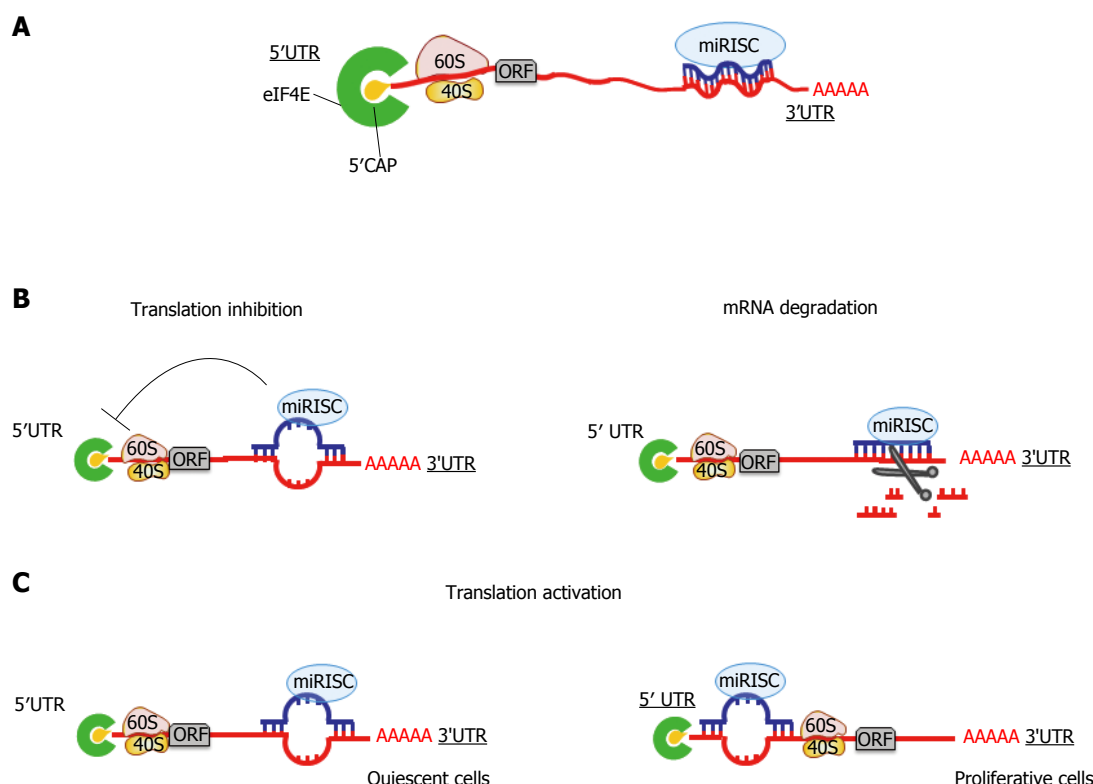


Figure 1 Mechanisms of action of microRNAs. A: miRNAs bind the 3'UTR of a target gene by base pairing. The binding between the miRNA seed sequence (nucleotides 2-8 at the 5'end of the miRNA sequence) and the miRNA regulatory element (MRE) at the 3'UTR of a target gene determines the specific type of regulation; B: miRNAs act as inhibitors of translation when the binding at the 3'UTR of target genes is only partially complementary. Instead, when the binding complementarity is perfect, miRNAs induce mRNA degradation; C: miRNAs can also function in an unconventional manner: Under specific conditions, particularly during quiescence, they can activate translation by binding non canonical sites in the 5'UTR of target genes. ORF: Open reading frames.

essential to maintain physiological processes in the cell. Translational control plays the major role in regulation of gene expression^[54] and miRNAs take part in the regulation of mRNA translation.

Since a miRNA binds the 3'UTR of a target mRNA, how can it inhibit its translation? To date it is very clear that miRNAs contribute to the regulation of protein synthesis in two ways, mRNA destabilization or translational repression. Unfortunately, to date, a general mechanism for the translational inhibition by miRNAs has not been widely accepted; we rely on several different models that will be critically presented^[55]. mRNA translation is divided in four phases: Initiation, elongation, termination and recycling. Here we will review in detail how miRNAs can repress translation at the initiation, post-initiation and elongation steps (Figure 2).

Initiation of translation

Initiation is the rate-limiting step in translation of a given mRNA and leads to the formation of the ribosome-tRNA-mRNA complex. The golden method to analyze the step at which the translation of a specific mRNA is blocked is measuring the localization of the same mRNA in a sucrose polysome gradient. The general assumption is that a translated mRNA associates with multiple ribosomes (polysomes) and co-sediments to the heavy part of the gradient. Several *in vitro* studies reveal that repressed

mRNAs shift to the lighter region of the sedimentation gradient, indicating reduced ribosome loading of the repressed mRNAs^[56]. The mechanistic effect of miRNAs at initiation is confirmed by studies in which Ago was found bound to the translational machinery. Briefly, mammalian Ago2 is able to bind the m⁷G-cap of mRNA directly, suggesting that Ago2 and the cap binding protein eIF4E compete for association with the cap structure^[57]. In this model miRNAs prevent translation of capped but not Internal Ribosome Entry Site (IRES) containing mRNAs. The discovery of a specific Ago2 domain responsible for the interaction with the cap structure supports the above hypothesis. Mutation of two key amino acids in Ago2 disrupts cap-Ago2 binding, and abolishes also the association between Ago2 and GW182, the latter being an important factor in miRNA mediated repression^[58,59].

In vitro studies suggest other mechanisms of miRNA repression at the initiation step. For some studies, the presence of the m⁷G-cap is necessary for translational inhibition. Other studies demonstrated that miRNA-mediated repression impairs also cap-independent, IRES-initiated translation, and exclude eIF4E-cap recognition as a target for miRNA function^[60,61]. This consideration is complicated by kinetic issues, as IRES-containing mRNAs are in general less efficiently translated, but also by the fact that some mRNAs have both a m⁷G-cap and an IRES.

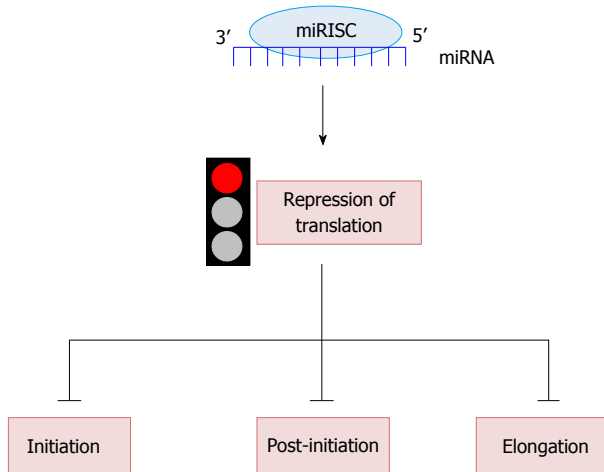


Figure 2 Schematic representation of microRNAs role in translation. miRNAs can inhibit protein synthesis at three different stages of translation: Initiation, the rate limiting step, post initiation and elongation. For details refer to text.

Studies performed on *D. Melanogaster* and mouse cells indicate that miRNAs impair the association of mRNAs to 40S or 80S ribosomes, probably disrupting the mRNA-40S complex^[62]. In this study an excess of eIF4F ameliorates the miRNA mediated inhibition of a specific mRNA. Other *in vitro* studies confirmed that translational repression exists only in the presence of both the m⁷G cap and poly-A tail, indicating that polyadenylation itself may have a role in miRNA mediated repression^[63-66]. Several hypotheses were proposed: (1) the CCR4-NOT deadenylation complex is sufficient to mediate silencing and could inhibit mRNA translation independently of its deadenylation activity^[67,68]; (2) miRISC is able to inhibit 43S scanning by impairing eIF4F function^[69,70], in particular NOT1 interaction with eIF4A2 could block eIF4A2 function and consequently 43S scanning; and (3) in contrast to hypothesis 2, a very recent study suggests that eIF4A activity and 43S ribosomal scanning are not required for miRNA silencing. In this context AGOs, GW182, CCR4-NOT and DDX6 complexes are able to repress and degrade mRNAs in a 43S scanning-independent manner^[65]. It is evident that conflicting reports may be due to difficulties in the analysis of the fast translation inhibition driven by miRNAs.

In addition, in the intertwined scenario of mRNA translational inhibition and mRNA degradation driven by miRNAs, the recurrent question is which step precedes the other. Recent studies performed in human cells, zebrafish, and *D. Melanogaster*, show that miRNAs reduce translation just before mRNA deadenylation and decay. Kinetic analysis monitoring in parallel the level of mRNAs, proteins and poly-A tail lengths coupled with ribosome profiling data, revealed that protein levels are affected prior to mRNA stability and poly-A tail length^[63,64,71]. A recent work presents another interesting hypothesis: miRNAs destabilize mRNAs when they are in a ribosome free state, but at the same time mRNAs targeted by miRNAs are fully polysome associated. The authors demonstrate that while mRNAs are associated with

polysomes, the decapping mechanism occurs proceeding in a 5' to 3' direction following the last translating ribosome^[66]. According to this model miRNA mediated mRNA decay occurs cotranslationally, providing a solution to this complex mystery.

Finally, it has been also suggested that miRNAs act at the level of active 80S complex formation, by affecting 60S joining. Eukaryotic Initiation Factor 6 (eIF6) associates with the 60S ribosomal subunit and is able to coimmunoprecipitate with the Ago2-Dicer-TRBP complex. Studies in human cells and *C. Elegans* led to the conclusion that miRISC, when associated with eIF6, abolishes polysome formation, disrupting 80S ribosomes assembly^[72]. However, these results were not confirmed in *D. Melanogaster* cells, where depletion of eIF6 had no effect in miRNA-mediated inhibition^[73]. Furthermore all published data converge on the idea that eIF6 acts as an anti-association factor that, by binding the 60S ribosomal subunit, prevents the formation of active 80S^[74,75]. This mechanism is regulated by post-translational modifications. Indeed eIF6 is activated downstream of the RACK1-PKC β II axis^[76,77] through phosphorylation on residue Ser235, an event which is found deregulated in several types of cancer^[78-80].

Post-initiation and elongation of translation

At the end of the initiation step, the mRNA is positioned on the ribosome and amino acids are bound together to form a polypeptide chain, thus determining the intermediate step of translation. Since 1999, by sedimentation velocity ultracentrifugation in a sucrose gradient, it has been reported that some miRNAs fully associate with polysomes^[81,82]. The copurification of miRNAs with polysomes, confirmed by many studies in the last years, not only proves that miRNAs are involved in translational repression, but suggests that miRNA targets are actively translated. Taken together, these data suggest that mRNAs could be silenced by miRNAs at the post initiation step. Some examples will clarify the situation. Most miRNAs are shown to be associated with polysomes and in particular *let-7* is capable to cosediment with polyribosomes^[82,83]. When translation is blocked, by either hypertonic stress or puromycin treatment, miRNAs are no longer associated with polysomes, differently from their mRNA targets. The latter in fact dissociate from polyribosomes only partially, suggesting a reduction of translational elongation or impairment of the post initiation step. The capability of miRNAs to repress translation could also depend on their strength in associating with polyribosomes, *i.e.*, the amount of a specific miRNA in polysomes relative to its total quantity. Molotski and coworkers quantified the association of miRNAs with polysomes^[83], and, in line with this study, we discovered that in a Mesothelioma cellular model, only 8% of the miRNAs analyzed is stable and enriched on polysomes (data not published), suggesting that the preference for a microRNA to bind polyribosomes might depend on: (1) the specific seed sequence of the microRNA; (2) the level of

Table 1 List of selected tumor suppressor microRNAs and oncomiRs in cancer

Cancer type	
OncomiRs	
miR17-92 ^[108]	B-cell lymphoma, small cell lung cancer, colon cancer, gastric cancer
miR-21 ^[107]	Breast, colon and lung cancer, glioblastoma
miR-106 ^[133]	Gastric cancer, colorectal cancer
miR-10b ^[113]	Breast cancer
miR-191 ^[134,135]	Human colorectal and breast cancer
Tumor suppressor miRNAs	
let-7 ^[105,106]	Lung cancer, Burkitt lymphoma
miR-15a, miR16-1 ^[103,104]	CLL, prostate cancer, mesothelioma
miR-29 ^[136]	Lung cancer, breast cancer
miR-34a ^[116]	Prostate cancer, mesothelioma, HCC
miR-126 ^[114]	Lung and breast cancer
Both O and TS	
miR-24 ^[137,138]	Breast cancer, glioma (O) Laryngeal carcinoma (TS)
miR-125 ^[122,123]	Pancreatic and prostate cancer (O) Melanoma, osteosarcoma, ovarian cancer (TS)
miR-155 ^[120,121]	Lymphoma, breast cancer (O) Melanoma, ovarian and gastric cancer (TS)
miR-221/222 ^[139]	Glioblastoma, HCC, breast cancer (O) Tongue squamous cell carcinoma (TS)

CLL: Chronic lymphocytic leukemia; HCC: Hepatocellular carcinoma; O: OncomiR; TS: Tumor suppressor miRNA.

pairing energy between the miRNA and its target mRNA; and (3) the fact that target mRNAs are being translated or not. Overall what these data suggest is that the miRNA ability to repress translation could also depend on the rate of association with polysomes. However, how these small non-coding RNAs are capable to impair elongation or termination of translation is unclear. It has been also proposed that proteins synthesized from miRNA-targeted mRNAs are not able to accumulate because they are degraded by certain proteases employed by microRNA ribonucleoprotein complexes (miRNPs)^[81], thus proposing another mechanism on how miRNAs might function.

miRNA “surprise” role: miRNA-dependent activation of translation

The dynamic interactions between miRNAs and mRNAs open new frontiers in the field of miRNome studies. Most miRNAs negatively regulate gene expression and led scientists to deeply characterize the binding mechanism of miRNAs seed sequence to mRNAs. It is clear that miRNAs bind 3'UTRs and repress translation of target mRNAs. The demonstration that miR-369 has the capacity to either activate or repress protein translation^[84] raised the question on why and how miRNAs activate translation of their target mRNAs. When cells are grown in normal growth factor conditions, target mRNAs are translationally inhibited or decayed. Instead, in the absence of growth factors, *i.e.*, in serum starved conditions, the same miRNAs are able to activate translation and increase the protein levels of their target mRNAs, as it happens with miR-369 and its target TNF

alfa^[84-86]. Nevertheless this is not true for all miRNAs. For example, when miR-16 targets TNF alfa in a different 3' UTR region from that targeted by miR-369, it inhibits translation also in quiescent conditions^[86,87]. This suggests that, when a cell exits the cell cycle, activation of translation depends on miRNAs seed sequences and on miRNA-mRNA base pairing. It has also been demonstrated that the repression or activation of translation requires the FXR1 protein and Ago2, and that other miRNAs, among which *let-7*, respond to serum starvation upregulating translation of their target mRNAs^[85,86].

New paradigms discovered more recently make the mechanism of miRNA regulation even more puzzling. In the recent years, non-canonical sites of binding have been reported. Such sites map to the 5'UTR and coding regions of mRNAs^[88,89]. Several studies reported that miR-122 and miR-103a-3p have their target sites in 5' UTR^[90,91], and that some miRNAs are even able to target both 3' and 5'UTRs. Moreover, under cellular stress miR-10a activates translation by binding the 5'UTR of its target ribosomal protein coding mRNA^[48]. All of these mechanisms, and probably several others yet unknown, render the landscape of miRNAs mode of action even more difficult to assess. In conclusion, it is essential to study the function of each miRNA singularly and in a specific cellular context in order to understand its precise function.

miRNAs AND CANCER

Cancer is a pathological condition in which gene expression is dramatically deregulated. miRNAs affect all steps of tumor progression including tumor growth, invasion, metastatic capability and angiogenesis. The relevance of miRNAs in cancer has been highlighted by alterations in their expression (Table 1) and consequently by the deregulation of the expression of their target mRNAs^[92,93]. The first evidence of the involvement of miRNA in cancer derived from studies on chronic lymphocytic leukemia (CLL). Croce's group discovered that two miRNAs, miR-15a and miR-16-1 derive from the same polycistronic RNA which is transcribed from a specific region of chromosome 13, frequently found deleted in CLL. Analyzing a set of CLL patients, they found that 69% of them presented the deletion of miR-15-a and miR-16-1^[44]. Moreover, they realized that a significant percentage of miRNA genes localizes in fragile sites and/or in genome regions which often show chromosomal alterations, including amplifications or deletions. This last finding suggested that miRNAs are a new class of genes important in regulating cancer pathogenesis and development. These relevant and preliminary observations implemented the need for investigation with new advanced technologies. All known miRNAs are now mapped and the development of several new platforms is helpful to study the miRNome in both normal and pathological tissues and for the establishment of tumor classification, diagnosis and prognosis by miRNA profiling^[94-96].

Just like classical protein-coding genes, also miRNA

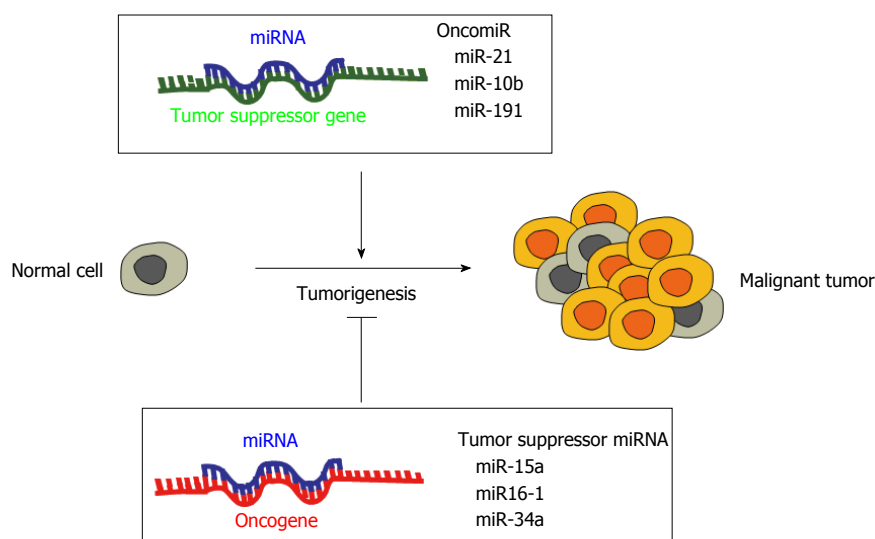


Figure 3 Roles of microRNAs in cancer. miRNAs suppress the expression of their target genes. An oncogenic miRNA, termed oncomiR, can repress the translation of a tumor suppressor gene, stimulating tumorigenesis and leading to tumor formation. Conversely, a tumor suppressor miRNA is able to inhibit the expression of oncogenes, blocking the tumorigenesis process and consequently the development of cancer.

genes can be altered by promoter methylation, chromosomal amplifications, deletions and transcriptional activation. Genetic alterations may involve the miRNA machinery^[97] or alter the target binding site^[98], the processing of miRNAs and their post-transcriptional editing^[99]. In cancer, dysregulated miRNAs can act as oncogenic miRNAs (oncomiRs) or tumor suppressor miRNAs, based on their capability to repress the expression of tumor suppressor genes or oncogenes, respectively (Figure 3). The inhibition or stimulation of oncomiRs or tumor suppressor miRNAs modulates cancer cell proliferation, tumor growth, metastasis formation and cell survival^[100]. Generally, oncomiRs, which modulate tumor suppressor proteins, are overexpressed in cancer, whilst tumor suppressor miRNAs, which target oncoproteins, are downregulated or deleted. Tumor suppressors miR-15a and miR-16-1, whose target is Bcl-2^[101,102], are downregulated in several cancers, such as mesothelioma^[103], CLL and prostate carcinoma^[104], tumor suppressor let-7 targets RAS and Myc^[105,106], while oncomiR miR-21 is overexpressed in breast cancer, colon cancer and glioblastoma and targets PTEN in non-small cell lung cancer^[107]. Furthermore, the most studied miRNA cluster, miR17-92 is able to induce lymphomagenesis in a B-cell specific transgenic mice^[108], and miR-19, miR-20a and miR-92, which are part of this cluster, promote T cell ALL development in mouse models^[109]. It has been established that knocking down the upregulated oncomiRs reduces cell proliferation and tumor growth both *in vitro* and *in vivo* tumor systems^[110-112]. In addition to classical tumor suppressor or oncogene functions, miRNAs are also involved in cell migration and metastasis formation. In breast cancer miR-10b modulation increases cell invasion and migration by targeting HOXD10 and eliciting the expression of the pro-metastatic gene RHOC^[113]. Other examples are miR-335 and miR-126, which act as negative regulators of metastasis and tumor invasion in lung

and breast cancer^[114,115]. miR-34a, instead, is lost in several tumors and is involved in the p53 pathway^[116]. Moreover, miR-34a is able to inhibit migration and invasion downregulating MET expression in HCC cells^[117].

Several miRNAs cannot be clearly and unequivocally categorized as tumor suppressors or oncomiRs given that the data in our hands are quite intricate and conflicting since they could act as tumor suppressors in one scenario or as oncomiRs in the other (Table 1). This is not surprising considering that the same miRNA may regulate from ten to hundreds of genes involved in completely different cellular pathways. If we consider miR-155, it works as an oncomiR in solid and hematological malignancies, such as lymphoma and breast cancer^[118,119], but in melanoma, as well as in ovarian and gastric cancer, it shows a tumor suppressor role^[120,121]. Another relevant example is represented by miR-125, which shows tumor suppressor properties in several cancers, like melanoma, osteosarcoma, ovarian and breast cancer, and tumor promoting functions in pancreatic and prostate cancers^[122,123]. It is clear that the dual role of miRNAs could be due to the heterogeneity and variability of cancer, causing the same miRNA to carry out different effects in different tumors.

miRNAs have also an important role in the clinic where they are useful in terms of diagnosis, prognosis and prediction of therapy response. In this context miRNAs expression can be used as a tool to predict tumorigenesis and overall survival, but also to classify malignant and non-malignant tissues. To date, the clinical importance of miRNAs has been demonstrated for several types of cancers and by using also biopsies or surgery specimens^[124].

To avoid the invasiveness of surgery techniques, several studies focused their attention on analyzing miRNA expression levels in human fluids, such as plasma/serum, saliva and urine, speculating the idea that circulating

miRNAs could be stable and therefore useful clinical biomarkers. To support this idea, to date, miRNA deregulation in serum of cancer patients has been described for several types of tumors such as leukemia, lymphoma, gastric, lung, ovarian, prostate, pancreatic and breast cancer^[125]. Most miRNAs found outside of cells, particularly in body fluids, are stable, and this is quite surprising since most RNA molecules in the extracellular environment are subjected to ribonucleases. These observations suggest that secreted miRNAs could be protected by degradation possibly by being packed in particular extracellular vesicles^[126].

Among extracellular vesicles, exosomes turn out to be the most studied membrane bound vesicles released from cells into the extracellular space^[127]. Exosomes play an important role in exchanging information between cancer cells, and such cell-to-cell communication is essential for tumor survival and progression and for metastases formation. Several studies identified exosomes as the key components of this process, and the idea that extracellular miRNA are among the mediators used by exosomes for this inter-cell communication makes this model even more attractive. In line with these data, several studies showed that exosomal miRNA expression is altered in cancer^[128,129]. The function of exosomal miRNAs is poorly understood, but some reports showed that in this context they carry out their conventional role of negative regulators of gene expression. One example is miR-105 which, once released from breast cancer cell lines, reduces ZO-1 gene expression and promotes metastases formation in the lung and brain^[130]. Recently, a novel and peculiar function of exosomal miR-21 and miR-29a was demonstrated: Such miRNAs are capable to activate immune cells, by acting as toll-like receptors ligands^[131].

These observations and future progresses in the miRNA research field will be very helpful for the development of new therapeutical strategies to fight cancer. Indeed, when a cancer is characterized by the overexpression of specific miRNAs, the use of anti-miRs as drugs could help restoring the non-pathological condition. On the contrary, the same results could be obtained by the use of miRNA mimics in cancers in which specific miRNAs are downregulated. A similar approach was described by Kota *et al.*^[132]: The restoring of miR-26a in hepatocellular carcinoma is able to reduce cancer cell proliferation by triggering apoptosis. These data widely show that miRNAs have a precious potential to act as therapeutical targets.

CONCLUSION

In conclusions, the miRNA world is fascinating and mysterious. The number of miRNA genes that are being discovered is increasing and novel mechanisms of action might reveal possible new therapeutic strategies. The fact that miRNAs use non-canonical target sites to perform their function opens a puzzling scenario that could lead

researchers to discover completely new miRNA functions and modes of action. Although great strides have been made in the recent years, the comprehension of the global miRNome and the establishment of functional therapeutic strategies in miRNA cancer research are yet far from being achieved. The discovery and development of miRNA inhibitors or miRNA mimics as novel drugs will offer new hopes in the fight against cancer.

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Challenges in the chemotherapy of Chagas disease: Looking for possibilities related to the differences and similarities between the parasite and host

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Abstract

Almost 110 years after the first studies by Dr. Carlos Chagas describing an infectious disease that was named for him, Chagas disease remains a neglected illness and a death sentence for infected people in poor countries. This short review highlights the enormous need for new studies aimed at the development of novel and more specific drugs to treat chagasic patients. The primary tool for facing this challenge is deep knowledge about the similarities and differences between the parasite and its human host.

Key words: *Trypanosoma cruzi*; Trans-sialidase; Trypanothione reductase; CYP51; Cruzipain; Tubulin

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Core tip: Chagas disease remains the most neglected parasitic illness the world. Here, we note that detailed knowledge of both the differences and similarities between the *Trypanosoma cruzi* parasite and the human host's biochemical targets may be the key to developing novel effective drugs to treat patients who are suffering with this severe and debilitating sickness.

Sueth-Santiago V, Decote-Ricardo D, Morrot A, Freire-de-Lima CG, Lima MEF. Challenges in the chemotherapy of Chagas disease: Looking for possibilities related to the differences and similarities between the parasite and host. *World J Biol Chem* 2017; 8(1): 57-80 Available from: URL: <http://www.wjgnet.com/1949-8454/full/v8/i1/57.htm> DOI: <http://dx.doi.org/10.4331/wjbc.v8.i1.57>

INTRODUCTION

American trypanosomiasis (or Chagas disease) is a parasitic illness that results from infection by the hemoflagellate protozoan *Trypanosoma cruzi* (*T. cruzi*). The discovery of this parasite was made in 1908 by Chagas^[1], and it was followed by his complete description of the disease's pathology^[2], as well as diagnostic methods^[3]. This sequence of events has established Carlos Chagas as the only scientist in the entire history of medicine to elucidate all aspects of an infectious disease completely, from the etiological agent to the vector, transmission, and its hosts and clinical manifestations^[4]. Carlos Chagas' discoveries were characterized by their unusual deductive reasoning steps: The initial identification of the etiologic agent in primates from Minas Gerais State (and not the disease itself), followed by the identification of vectors and their association with the occurrence of recurring infestations caused by triatomines in the region [predominantly blood-feeding insects such as *Triatoma infestans* (*T. infestans*)]. The confirmation of the disease, which is usually the first step in the elucidation of an infectious disease process, was subsequently performed by observing the protozoa in the blood of individuals who were living under the precarious health conditions of the people in the region; they presented the lethargic symptoms of some well-known parasitic infections^[5]. This unusual sequence of stages to understanding Chagas disease is the result of a process of social stratification in which the poorest individuals, who lived in wattle-and-daub houses in rural areas, were more vulnerable to *T. infestans* exposure. Almost one hundred years after an important speech by Carlos Chagas for his inaugural lecture as the Professor of the Tropical Medicine Course^[6] in which Chagas said that tropical diseases should not be analyzed through a simplistic approach, but as a biologically, culturally and economically complex phenomenon, Chagas disease remains neglected in all its aspects. At present, there is no effective drug that can cure chagasic patients.

After the publication of the first studies on Chagas disease in the early twentieth century, there was a series of disparaging campaigns against the relevance of Carlos Chagas' work that were led by members of the Brazilian National Academy of Medicine^[7]; it undermined the interest of Brazilian researchers in the disease during the years that followed. However, studies on Chagas disease continued to be performed around the world, and we highlight the contributions of the Argentine re-

searcher Cecilio Romãña as one of the most important. Romãña made a great contribution to the classification of vectors as well as knowledge of the transmission and diagnosis of Chagas disease. Romãña described the first pathognomonic symptom associated with Chagas disease as follows: A one-sided bipalpebral inflammatory edema called unilateral conjunctivitis, which became known as the "mark of Romãña" in accordance with the recommendations of Evandro Chagas (Medical Doctor and son of Carlos Chagas), in recognition of Cecilio Romãña's contributions to our knowledge of the disease^[8].

The transmission of Chagas disease occurs primarily through the bite of an infected triatomine bug on an individual. Triatomines are insects that usually belong to the genera *Triatoma*, *Rhodnius* or *Panstrongylus*, which are commonly known as "barbeiros" in Brazil and "kissing bugs" in the United States, due to their preference for biting the faces of sleeping people. These insect genera include more than 140 species, of which 61 are endemic to Brazil^[9]. The insect's bite itself does not cause the transmission of viable forms of *T. cruzi*. However, the triatomine's physiology is characterized by a short digestive apparatus, leading these insects to defecate upon blood suction, releasing the infective trypomastigote forms of the parasite, which are present in high quantities in their feces. The itching caused by the bite causes the individual to move the infective forms to the wound, where the parasite enters the bloodstream^[10], causing infection. Despite the fact that the primary form of contamination is due to vector bites, there are other clinically relevant transmission pathways, with blood transfusion and organ transplantation among them^[11]. Vertically, transmission can occur *via* the placenta or breastfeeding^[12] or less commonly by oral contamination due to the consumption of fresh infected food^[13]. After infection, the disease in the human host has two phases: Acute and chronic. The acute phase occurs during the first months after infection, and it is characterized by a high parasitic load in the host's bloodstream. It may be asymptomatic, or it may present moderate symptoms that are of low diagnostic value. These characteristics hinder drug intervention at this stage. Although the acute phase is asymptomatic, sometimes it leads to the enlargement of the liver and lymph nodes, rashes, a loss of appetite, a swelling at the bite site (chagoma), and, occasionally, the Romãña's mark^[14]. After the acute phase, infected individuals spend long periods without symptoms, after which some patients evolve to the chronic phase. This stage of the infection is characterized by the appearance of severe degenerative disorders in the host's vital organs including megacolon, megaesophagus and cardiomegaly^[15].

SOCIO-ECONOMIC IMPACT OF CHAGAS DISEASE

Damage to vital organs such as the heart contributes

Table 1 Estimated number of Disability-Adjusted Life Year ($\times 1000$) by cause and by region (excluding the Europe)¹ 2004

Neglected disease	World ²	Region (OMS criteria)				
		Africa	Americas	East of Meditterran	Southeast Asia	Pacific West
Sleeping sickness	1673	1609	0	62	0	0
Chagas disease	430	0	426	0	0	0
Schistosomiasis	1707	1502	46	145	0	13
Leishmaniasis	1974	328	45	281	1264	51
Filariasis	5941	2263	10	75	3525	65
Onchocerciasis	389	375	1	11	0	0
Leprosy	194	25	16	22	118	13
Dengue	670	9	73	28	391	169
Trichomoniasis	1334	601	15	208	88	419
Ascariasis ³	1851	915	60	162	404	308
Trichiuriasis ³	1012	236	73	61	372	269
Ancylostomiasis ³	1092	377	20	43	286	364

¹Source: The global burden of disease: 2004 update. Geneva, World Health Organization^[18]; ²Europe was omitted, so the sum of the regions will not be equal to the total value; ³Soil-transmitted helminthiasis.

greatly to the reduced economic capacity of a population of individuals and influences their economic and social conditions. This approach is currently used by the World Health Organization, through the use of a modern indicator for measuring the economic impact of diseases over certain regions using a number called the Disability-Adjusted Life Year (DALY). This number corresponds to the number of productive years lost to death or disability resulting from an illness in a given population^[16]. This indicator has the advantage of accounting for two complementary factors as follows: Mortality, as measured by the number of years lost due to premature death [Years of Life Lost (YLL)]; and a new parameter for years lived with disability and economic output [Years Lived with Disability (YLD)]. The YLD indicator also indicates the burden to social security systems as a result of early retirement^[17]. The YLL values are calculated by multiplying the number of deaths for the life expectancy of a particular group of individuals; the YLD can be calculated as the product of the number of cases, the duration of the disease (a parameter that is particularly relevant for chronic diseases) and a constant for each disease [disability weight (DW)] that varies depending on the severity of the disability caused, ranging from zero (healthy) to one (dead). The resulting formula is shown below:

$$\text{DALY} = \text{YLL} + \text{YLD}$$

$$\text{YLL} = N \times L$$

$$\text{YLD} = I \times \text{DW} \times L'$$

Where N = number of deaths, L = life expectancy, I = number of individuals affected by the disease, and DW = disability weight; L' = duration of the disease. Table 1 shows the impacts of various neglected diseases on the economies of certain regions.

A closer look at Latin America shows that the geographical regions that are affected by higher rates of *T. cruzi* infection are also those in which the population is traditionally poorer. In countries such as Panama, Costa Rica, Bolivia and Venezuela and the hinterlands of northeastern Brazil and Northern Argentina^[19], there is

an estimated loss of 752000 d of work per year due to the early deaths of individuals with Chagas disease. In addition, United States \$1.2 billion is lost each year from Latin American countries, with at least United States \$ 5.6 million lost from Brazil^[20]. Taking into account that this financial loss is absorbed mostly by a specific group of people, it makes the discussion of Chagas disease even more complex since it is no longer a consequence of poverty but an agent that maintains poverty. These findings are due to decreasing productive capacities with a consequent reduction in the capital movement of a particular group of people in these geographical areas^[21].

The governmental programs aimed at both insect control and the quality of the blood used in transfusions in the countries where Chagas disease is endemic (primarily in Central and Latin America) led to an important decrease in the notifications of new cases. However, in non-endemic countries such as the United States and some countries in Europe, there was a significant increase in the number of infected individuals^[22]. First, this increase is associated with the increased migratory flux of people that has occurred in recent decades. Additionally, there is an expectation that global warming could also contribute to the advance of vector-transmitted tropical diseases, including American trypanosomiasis^[23]. There are several species of triatomine bugs that are capable of vectoring *Trypanosoma cruzi* in United States^[22]. In a recent study conducted in the metropolitan area of Tucson, Arizona (United States), investigators found that 41.5% of 164 triatomine bugs collected tested positive for *T. cruzi*^[24]. These data are alarming, and they show that the population of the southern part of the United States is exposed and at risk of infection by *T. cruzi*.

THE AVAILABLE TREATMENTS FOR CHAGAS DISEASE: OLD AND INEFFECTIVE DRUGS

The prevalence of Chagas disease in certain regions over

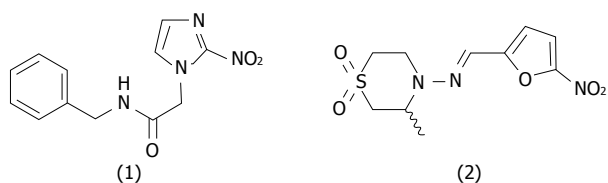


Figure 1 Chemical structures of benznidazole (1) and nifurtimox (2).

many years is primarily due to a lack of interest among pharmaceutical companies in developing drugs to treat Chagas disease. Despite the existence of a high demand, the potential consumers have no money to pay for medicines. In other words, there is demand, but there is no market. In this scenario, only two almost 100-year-old drugs are used to treat Chagas disease, namely the heterocyclic derivatives benznidazole (1) and nifurtimox (2), as shown in Figure 1. However, neither of these drugs is effective during the chronic phase of the disease, and both of them cause numerous toxic side effects.

Nifurtimox (2) is a 5-nitrofuran that is commercially available under the name Lampit[®], and it was initially described as a promising alternative for the treatment of Chagas disease. This drug was developed by Bayer[®] under the name Bay-2502. It was shown to be effective for treating sleeping sickness (or African trypanosomiasis), which is caused by the trypanosomatid *Trypanosoma brucei*^[25]. In 1969, this compound yielded a total of 11 publications in the *Bulletin of the Chilean Parasitology*, in which the clinical outcomes of patients with the disease were described, as well as the biological properties of the drug *in vivo*^[26]. Despite the fact that nifurtimox (2) is still on the list of essential medicines^[27], its distribution has been discontinued due to its low efficacy during the chronic phase of the disease, as well as its severe adverse effects, such as gastrointestinal problems, central nervous system disturbances and peripheral neuropathy^[28]. Its mechanism of action (Figure 2) involves the participation of the type I and II nitroreductases that are present in the parasite.

Type II nitroreductases cause electron transfer, converting nitrofuran (2) into the corresponding nitroanion radical through the conversion of molecular oxygen to a superoxide anion radical. These reactive oxygen species (ROS) are substrates of superoxide dismutase, which disproportionates superoxide into molecular oxygen and hydrogen peroxide (other ROS). Hydrogen peroxide is converted into water through the oxidation of the trypanothione in its reduced form, which is T(SH)₂. This process is reversed by the action of the trypanothione reductase (TR) enzyme. H₂O₂ can also oxidize ferrous ions from microsomal systems using the classical Haber-Weiss reaction to form hydroxyl radicals, which is harmful to the parasite^[29,30].

Through the action of type I nitroreductases, the transfer of two electrons takes place (provided by NADH), and nitrofuran (2) is reduced directly to the nitroso derivative that is sequentially reduced to N-furan-

2-yl-hydroxylamine. The hydroxylamine intermediate undergoes ring opening after losing water through the generation of an unsaturated nitrile, which is reduced again to the corresponding saturated derivative^[31]. The unsaturated nitrile is toxic to both the parasite and to the host cells. This high toxicity is due to the presence of a Michael-type acceptor that can bind bionucleophiles from both the host and parasite irreversibly^[32]. Although it has relevant activity against the intracellular form of the parasite, nifurtimox (2) is no longer marketed in Brazil due to the emergence of many resistant *T. cruzi* strains^[28] and the significant genotoxic effects^[33] caused by metabolites derived from the opening of its nitro-furan rings^[34].

Benznidazole (1), a 2-nitroimidazole, is the drug of choice for treating patients who are infected with *T. cruzi*, and it was introduced to the market by Roche under the name Rochagan[®]. The rights to the drug were given to the Brazilian government in 2003, allowing the Pharmaceutical Laboratory of the State of Pernambuco to prepare and market benznidazole. Despite having a nitro-heterocyclic fragment in its structure, the mechanism of anti-chagasic action of benznidazole (1) differs from the proposed mechanism for nifurtimox (2) because its 2-nitroimidazole subunit has a lower electrochemical potential for the reduction when compared to the 5-nitrofuran moiety. Thus, the concentration of superoxide anion radicals is sufficiently low for the parasite to perform the detoxification on its own^[35]. The selective toxicity shown by benznidazole (1) is due to the transfer of an electron to its nitro-aryl motif, which disproportionates^[36], then generating nitroimidazole and a nitrosoimidazol that binds irreversibly to trypanothione, which is an essential cofactor for the viability of parasite cells^[37]. The addition reaction may happen to the nitrous group, but the work of Trochine *et al.*^[38] showed that adducts are also formed by an aromatic electrophilic substitution at position 4 of the imidazole ring. Another possible mechanism of action for benznidazole is proposed by Patterson *et al.*^[31], in which the drug is converted to an *N*-aryl-hydroxylamine in the same way as it occurs with nifurtimox (2). Then, a number of non-enzymatic reactions take place, culminating in the formation of a metabolite containing a guanidine subunit and a glyoxal molecule, which has cytotoxic properties; these properties could explain the trypanocidal activity shown by benznidazole (1). Figure 3 shows the two possible mechanisms of benznidazole activation.

However, nifurtimox (2) and benznidazole (1) are active only during the acute phase of the disease, which is usually asymptomatic and has a short duration. During the chronic phase, the long-term administration of these two nitroderivatives leads to the development of severe side effects in patients, making treatment with these compounds non-viable.

Based on the information provided above, there is a clear need for new studies on the development of novel and more specific drugs with low toxicities to the host. An important point to highlight here is the lack

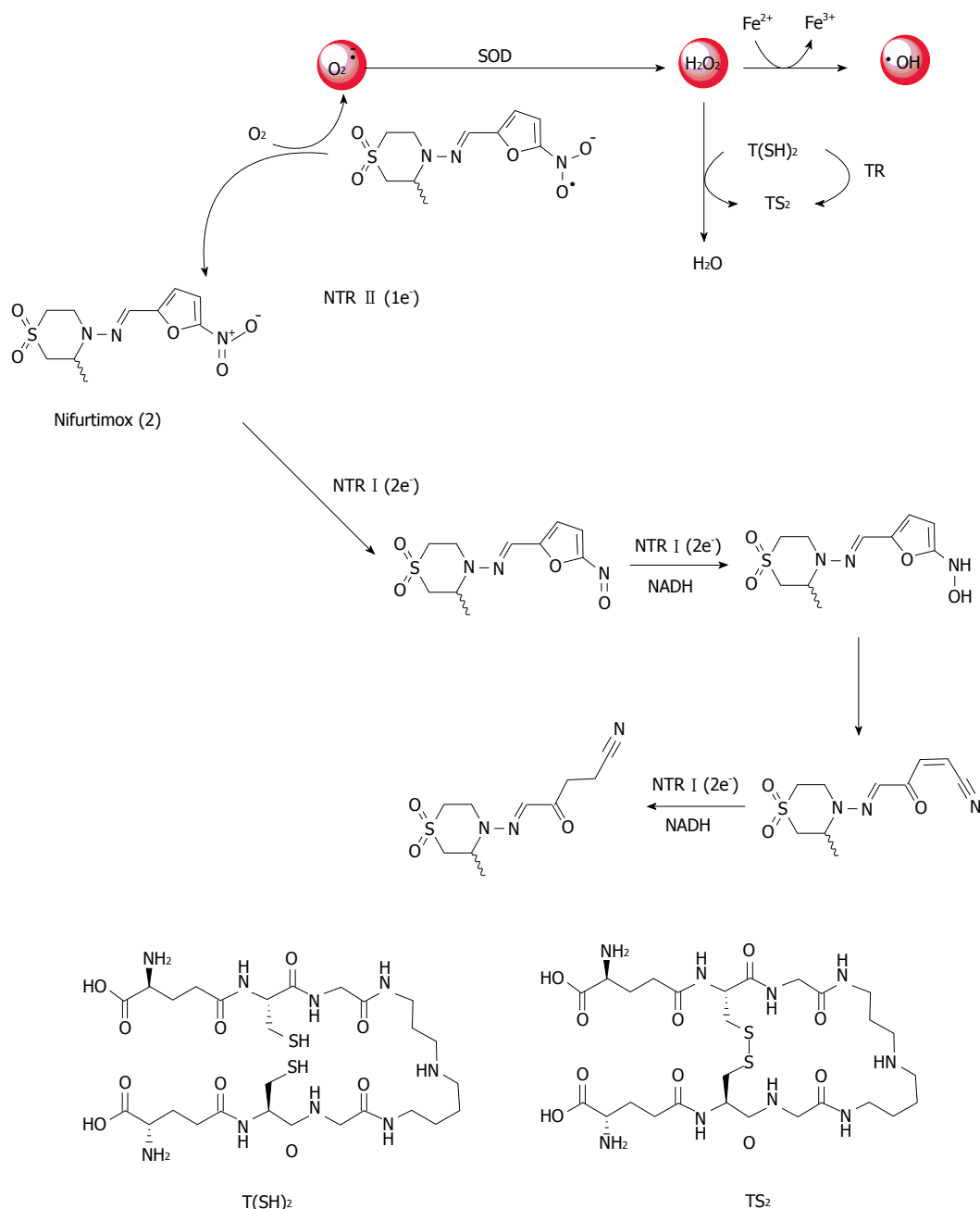


Figure 2 Nifurtimox (2) as a generator of reactive oxygen species. SOD: Superoxide dismutase; TR: Trypanothione reductase; NTR: Nitroreductases; $T(SH)_2$: Reduced trypanothione; TS_2 : Oxidized trypanothione.

of interest of big pharmaceutical corporations in the development of new and more effective therapeutic alternatives for the treatment of Chagas patients. This lack of interest is one of the most important factors that contributes to the fact that this disease remains a death sentence for infected people in poor countries. Chagas disease and other parasitic illnesses have left a huge mark of destruction and economic loss on humanity.

POTENTIAL TARGETS FOR TRYPANOCIDAL DRUGS: *T. CRUZI* VIRULENCE FACTORS AS A TARGET

The search for novel compounds that are selectively

harmful to the parasite without compromising the host's health is the primary paradigm in the search for new antiparasitic drugs. The approach to antiparasitic chemotherapy is usually based on two broad classes of targets, namely those targets that are specific to the parasite (such as trypanothione and cruzipain) and those shared between the parasite and the host (such as tubulin and sterol 14α -demethylase). When a common target is shared between the parasite and host cells, there should be some selectivity by the bioactive substance for the receptors/enzymes of the parasite, to the detriment of those related to the host cells, with the aim of exerting greater effects on the parasite and fewer effects on the host.

Some of the most common targets for Chagas

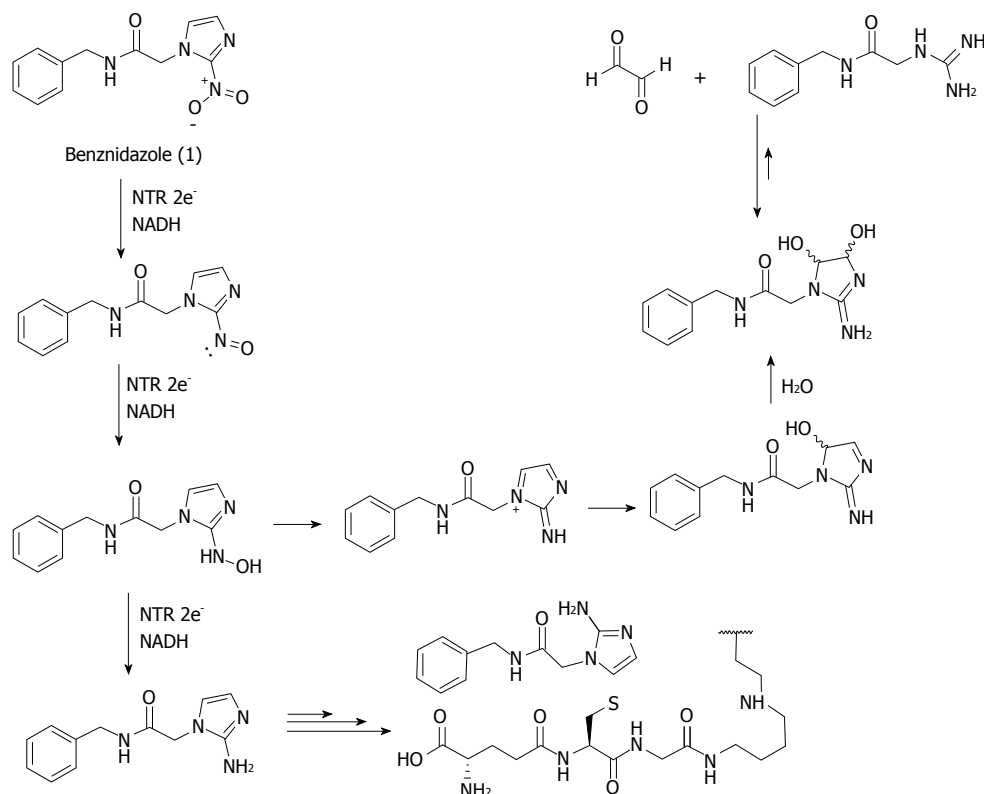


Figure 3 Benznidazole (1) is converted to nitroso metabolites and N-Aryl hydroxylamine, which can be enzymatically reduced to the corresponding 2-aminoimidazole derivative or nitrenio ion, which then is spontaneously di-hydroxylated generating a glyoxal molecule and guanidine derived.

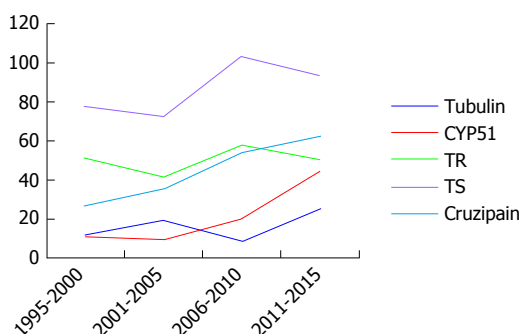


Figure 4 Search results of the terms “Trans-sialidase”, “Trypanothione reductase”, “Cruzipain and/or Cruzain”, “CYP 51 and/or sterol 14 α -demethylase” and “Tubulin” with “Trypanosoma cruzi”. The results show the evolution of the number of papers published since 1995, grouped in 5-year intervals. TS: Trans-sialidase; TR: Trypanothione reductase.

disease are shown below (Figure 4). These targets were chosen as some of the most relevant ones on the basis of research from the Scopus database, in which the term “*T. cruzi*” was searched together with the target’s name. The numbers of published papers available on the Scopus database (<https://www.scopus.com/>) were compared, resulting in the graph shown in Figure 4.

This result shows that some targets, such as trans-sialidase and trypanothione reductase, are well-known targets with a high average number of papers published in the last two decades. Cruzipain appears to have had a significant increase in the number of papers, possibly due

to the large number of deposits that contain crystalline structures with better resolution in databases such as the Protein Data Bank, which enhances the search for new substances that are capable of acting as enzyme inhibitors. Tubulin and CYP51 have a smaller number of published papers, despite their importance. These two targets, which also have distinct isoforms in human cells, have shown an increased growth trend in publications over the past five years. This trend could provide a possible increase in their relevance to antichagasic chemotherapy over the next decade. In this particular case, the selectivity needs to be considered because the modification of these substance dynamics in human cells can cause severe side effects in the host. Therefore, this review aims to present a literature review and critical analysis of the importance of each one of these targets in the development of substances with possible antichagasic activity.

CRUZIPAIN

Cruzipain^[39], which is also called GP 57/51 (recombinant cruzain), is a cysteine protease from the papain family; its primary feature is an atypical C-terminal segment that is highly glycosylated^[40]. Cruzipain is encoded by a polymorphic gene (*i.e.*, its expression is regulated differently at different developmental stages of the parasite), which suggests the existence of specific functions for the enzyme in each form of the parasite.

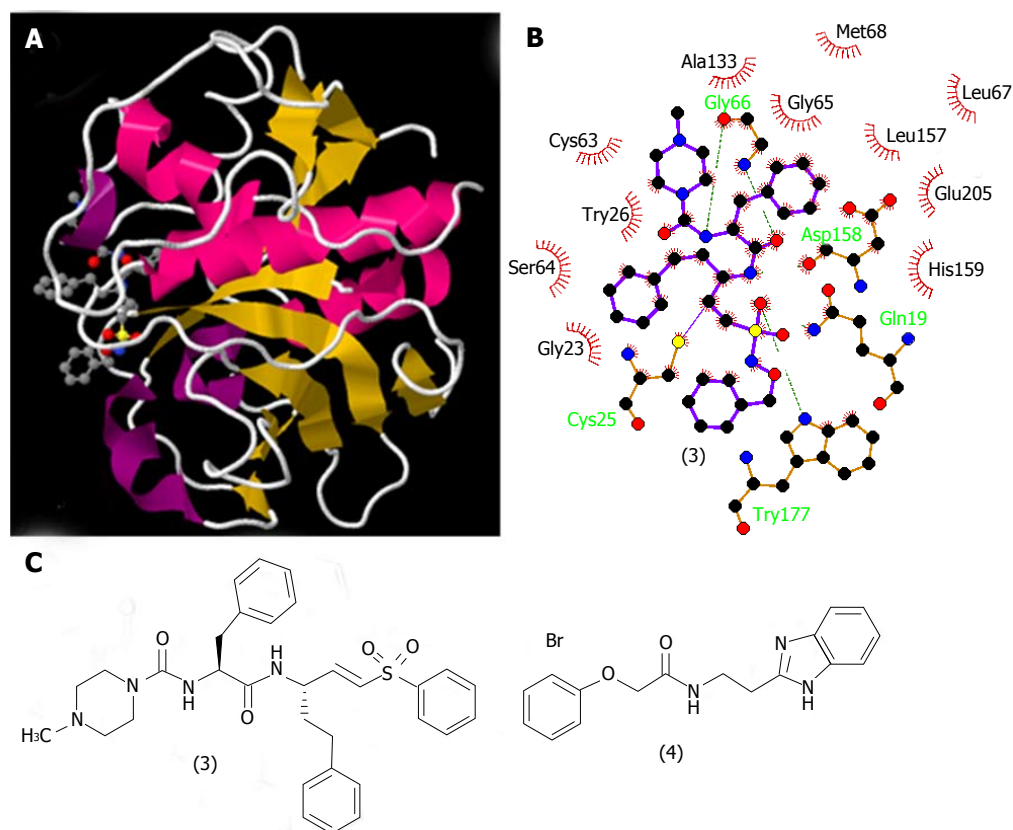


Figure 5 Structures of cruzain and its ligands. A: Three-dimensional structural representation of cruzain crystallized with vinyl sulfone derivative (3). Structure deposited in the Protein Data Bank under the code 1F2C^[45]; B: Schematic representation in two dimensions of the binding mode of (3) to cruzain generated by LigPlot[®] software upon the PDB file; C: Structures of the cruzain inhibitor derivatives (3) and (4).

In trypomastigotes, cruzipain is located in lysosomes, whereas in the amastigote form, it is present primarily at the cell surface. However, in the epimastigote form, cruzipain is compartmentalized in reservosomes, which are related to penetration processes in the host cell, intracellular nutrition and the escape mechanism from the cell for the trypomastigote form^[41-43]. Because they are cysteine proteases, the first cruzipain inhibitors were thought to be peptoids that were capable of binding irreversibly to the enzyme, *e.g.*, vinyl sulfone (3). Next, computational tools were used to design non-peptide derivatives, such as (4)^[44], which was a more potent inhibitor of the enzyme (Figure 5, entry A)^[45].

TRYPANOTHIONE REDUCTASE

TR, an enzyme that is present in many trypanosomatids (*e.g.*, *Trypanosoma*, *Leishmania* and *Crithidia* sp.), is responsible for the catalysis reaction shown in Figure 6. This enzyme maintains the redox balance of trypanothione and is responsible for maintaining an intracellular reducing environment by decreasing the concentration of ROS and other free radicals, which are consumed by the reduced form of trypanothione^[46].

The inhibition of this enzyme leads to the accumulation of ROS in the parasite cell, causing a potentially lethal oxidative stress in *T. cruzi*. The development of

TR inhibitors began in 1992 with the work of Benson *et al.*^[47], who isolated the TR and performed *in vitro* assays of enzymatic inhibition with some selected synthetic compounds. These studies identified clomipramine (5) as the first prototype for TR selective inhibition *in vitro* based on the redox potential of the glutathione system that is present in the vertebrate host^[47]. In the work of Benson *et al.*^[47], compound 5 has an inhibition constant of $K_i = 6.53 \pm 0.59 \mu\text{mol/L}$ for *T. cruzi* TR and no inhibition of human glutathione reductase at the maximum concentration of 1 mmol/L. In the early 90s, most rationally planned TR inhibitors were structurally related to the substrate, which usually led to irreversible inhibitions and little selectivity. The work of Zhang *et al.*^[48] made an important contribution to the development of TR inhibitors because it was the very first one that was planned through computational studies of non-peptidic inhibitors that were rationally designed with structural information from the target (Figure 7). Zhang *et al.*^[48] used the known crystallographic structure of trypanothione reductase from *Crithidia fasciculata* to create a homology model for the corresponding enzyme in *T. cruzi*. *C. fasciculata* is a kinetoplastid that is able to parasitize mosquitoes but is harmless to humans. TR from *C. fasciculata* shares 69% of its identity with the trypanosome TR, especially in terms of the active sites of the two enzymes^[49]. In the same year, Jacoby

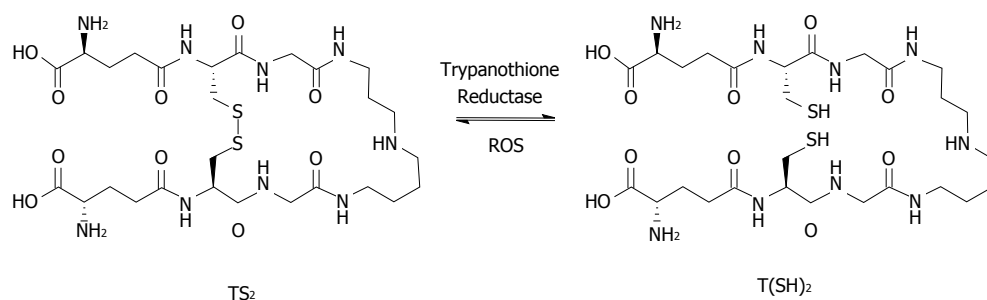


Figure 6 The equilibrium between the oxidized (TS₂) and reduced [T(SH)₂] forms of trypanothione. The reduction process takes place with catalysis of trypanothione reductase and the oxidation process occurs spontaneously by oxidative action of reactive oxygen species (ROS).

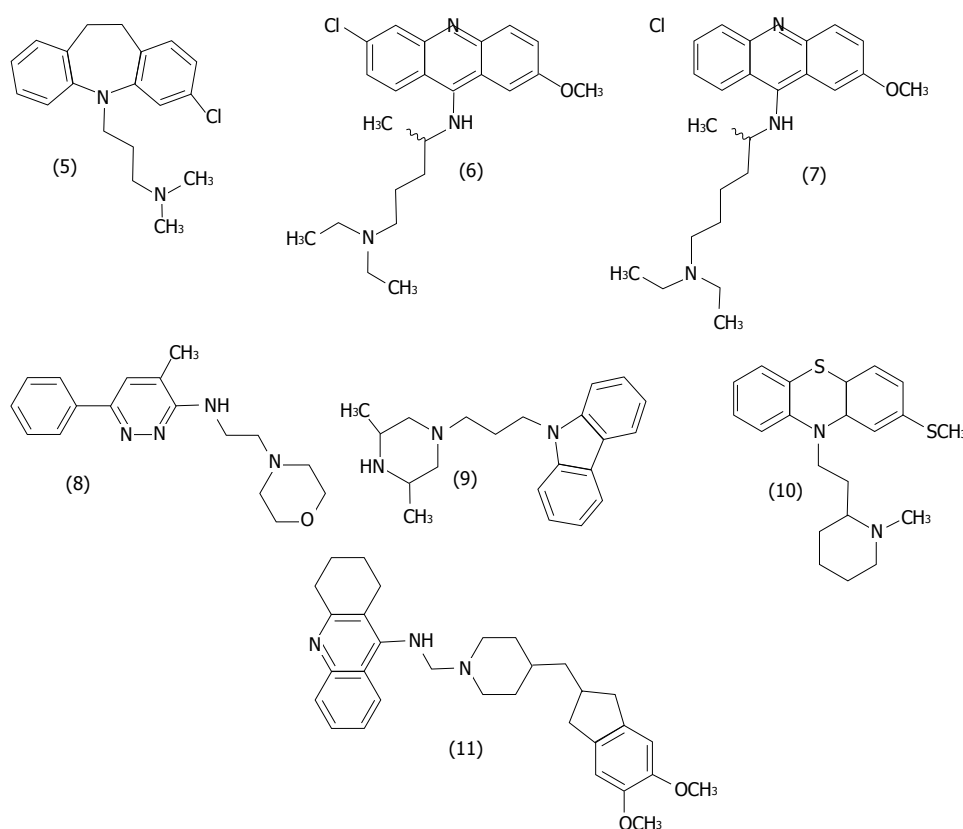


Figure 7 Structure of some non-peptidic *T. cruzi* trypanothione reductase inhibitors.

et al.^[50] elucidated the structure of the enzyme from the crystallography of TR that was co-crystallized with mepacrine (6). Since then, several TR inhibitors have been discovered by using the models of Zhang and Jacoby, especially the aminoacridine (7), a higher homolog of (6), as described by Bonse *et al.*^[51], and a pyridazine (8) and a carbazole (9) described by Horvath^[52]. More recently, the inhibitory activity of more potent derivatives such as thioridazine (10) and aminoquinoline (11) was described by Lo Presti *et al.*^[53] and Sola *et al.*^[54], respectively.

A series of twenty-one small peptides or peptide conjugates were assessed by McKie *et al.*^[55]. Among all the evaluated peptidic derivatives, two of them, namely *N*-benzyloxycarbonyl-Ala-Arg-Arg-4-methoxy- β -naphthylamide (12) and Bz-Leu-Arg-Arg- β -naphthylamide (13, Figure 8), showed good inhibitory activity against TR with

K_i values of 2.4 $\mu\text{mol/L}$ and 13.8 $\mu\text{mol/L}$, respectively. Additionally, the former derivative showed good selectivity for the parasitic enzyme (TR) compared to the host enzyme (human glutathione reductase).

TRANS-SIALIDASE

Another conspicuous target on *T. cruzi* is *trans*-sialidase (TcTS), an enzyme that was more often expressed on the trypomastigote forms of the parasite. *Trans*-sialidase is associated with the infective process, and it is a key component of the parasite's biology and its ability to evade both the innate and adaptive immune systems of the host^[56]. The cell recognition processes can occur in mammals, through sugars that are present in the cell glycocalyx. One of the sugars with fundamental

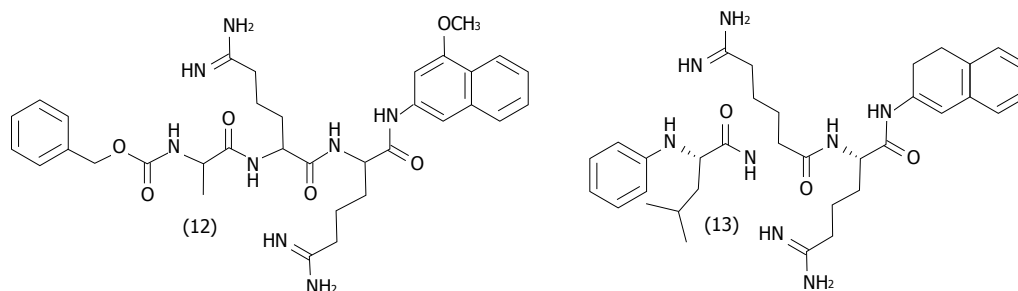


Figure 8 Structure of peptidic inhibitors of *T. cruzi* trypanothione reductase.

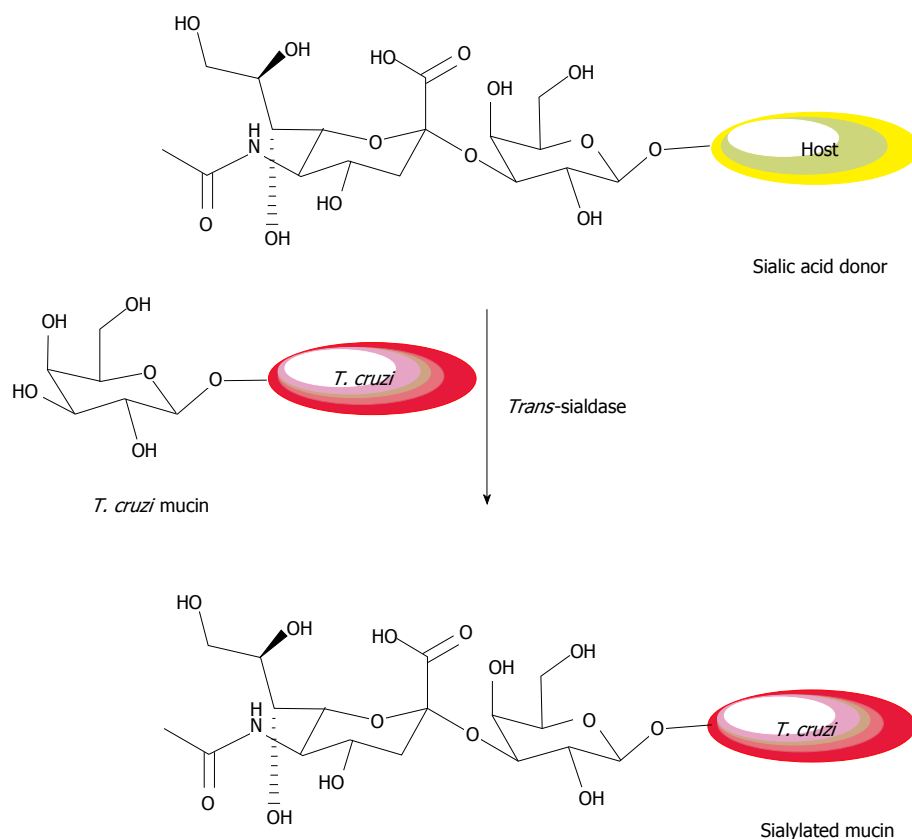


Figure 9 Transfer of sialic acid from a host glycoprotein to a β -galactopyranose from the parasite. This transfer occurs at the position 3 of the terminal sugar of the oligosaccharide which it is attached. Adapted from GIORGI and LEDERKREMER^[60].

importance in mammalian cell recognition is sialic acid, which is not produced by *T. cruzi*^[57]. The parasite has developed an enzyme that is capable of transferring sialic acid from the host cell to its own. Therefore, the parasite is no longer recognizable as a foreign agent and can then infect host cells without triggering the immune response^[58]. In trypomastigotes, *trans*-sialidase is anchored as a non-integral membrane protein to glycosyl-phosphatidyl inositol^[59]. This enzyme has the ability to sialylate mucin, a very abundant glycoprotein in the cell membrane of the parasite^[60], through the transfer of sialic acid from the host membrane to a β -galactopyranose that is present at the glycosylated hydrophilic site of the parasite's mucin (Figure 9). Thus, by using a specific carbohydrate in the host membrane, the parasite

can engage in a non-phagocytic invasion process without activating an immune response^[61].

To understand the *trans*-sialidase kinetic properties, Damager *et al.*^[62] performed studies on the enzyme catalytic properties using *in vitro* studies in which sialyllactose was used as a sialic acid donor, and *N*-acetylglucosamine was used as an acceptor. The kinetic isotopic effect studies led to the proposal of a so-called "ping-pong mechanism", in which the sialic acid donor binds first, followed by the acceptor, suggesting two near-lactose-binding sites leading to the chemical mechanism proposed in Figure 10.

The classical inhibitors of *trans*-sialidase are all weak and non-specific, with an inhibition constant (K_i) being at the millimolar order, and some of them are shown in

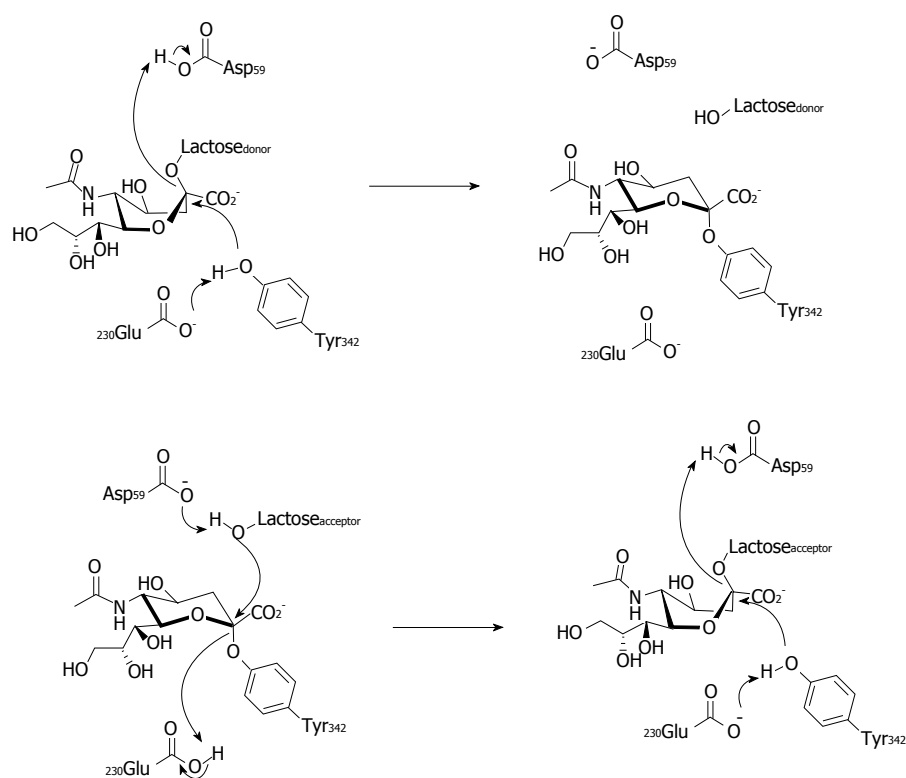


Figure 10 Proposed mechanism for the sialic acid transfer on the TcTS, showing Tyrosine 342 as the nucleophilic residue^[62].

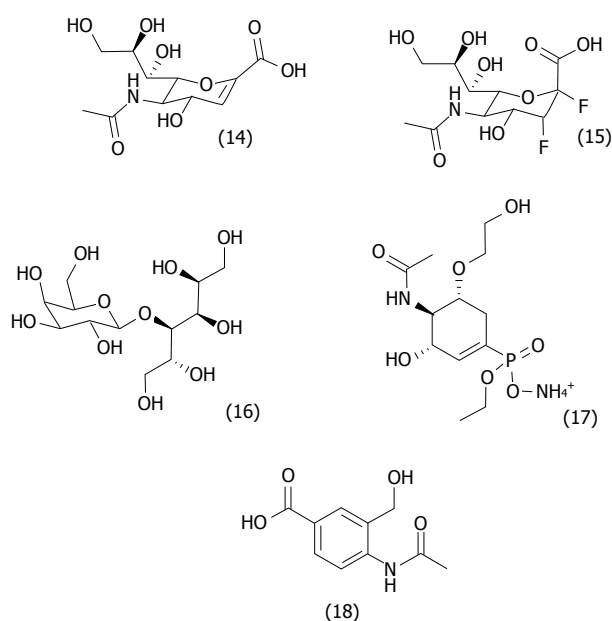


Figure 11 Chemical structures of the first *T. cruzi* trans-sialidase inhibitors (14-18). The more potent is (18), with a $K_i = 300 \mu\text{mol/L}$.

Figure 11. 2-Deoxy-2,3-didehydro-D-N-acetylneuraminic acid (DANA, 14) was used by Paris *et al.*^[63] to compare the inhibition of *T. cruzi* trans-sialidase and *Trypanosoma rangeli* sialidase (TrSA, an enzyme that hydrolyzes sialic acid but lacks transglycosidase ability). Despite being a potent inhibitor of TrSA ($K_i = 1.5 \mu\text{mol/L}$), DANA was reported to inhibit TcTS at a very high concentration, at

$K_i = 12.3 \text{ mmol/L}$ ^[63]. Watts *et al.*^[64] used 2,3-difluorosialic acid (15) to show the covalent binding of sialic acid with Tyr342 by mass spectra analysis. The TcTS complexed with (15) was readily subjected to peptide digestion. The LC-MS analysis of the hydrolysis product shows an m/z fragment of 1392, which corresponds to the peptide DENSAYSSVL+30HSial. The ESI tandem MS daughter ion spectrum of this fragment shows a pattern in which only the fragments with tyrosine included the 3-hydroxy sialil label, indicating that sialic acid would probably bind covalently to that area^[64]. Lactitol (16) also acts as an inhibitor, competing with the parasite's sugars (e.g., lactose) for the sialic acid, inhibiting TcTS activity toward conventional substrates. However, this inhibition demands a high concentration of (16), which shows that these lactose analogs are not suitable inhibitors for *in vivo* studies^[65]. With the aim of synthesizing analogs that can inhibit the transfer of sialic acid into different organisms, Streicher and Buse planned a series of pseudo-sialosides with a cyclohexene and a phosphonate ester moiety, and they tested it against some trans-sialidases, including TcTS^[66]. However, the most active compound (17) showed an $\text{IC}_{50} = 4.7 \text{ mmol/L}$, or the same magnitude as the previous inhibitors (14-16). With a different approach, Neres *et al.*^[67] designed a series of benzoic acid derivative analogs to pyridoxal phosphate, a well-known TcTS inhibitor with a $K_i = 7.3 \text{ mmol/L}$. This strategy was useful in the inhibition of the influenza virus neuraminidase, and it acted by replacing sialic acid with more simple structures such as benzene and pyridine.

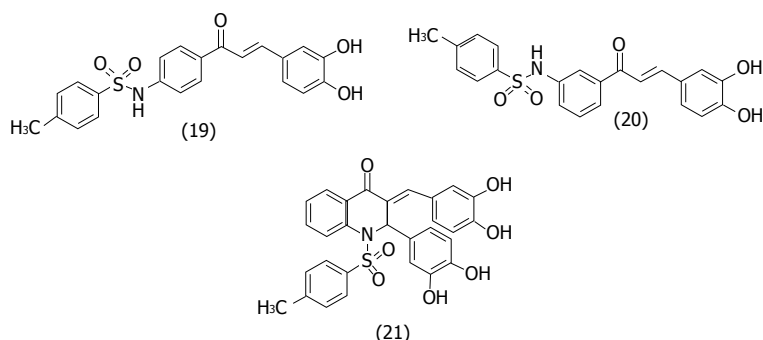


Figure 12 Chemical structures of the sulfonamide-chalcone derivatives designed by Kim *et al.*^[68] (19, IC_{50} = 0.9 μ mol/L; 20, IC_{50} = 2.5 μ mol/L; 21, IC_{50} = 0.6 μ mol/L).

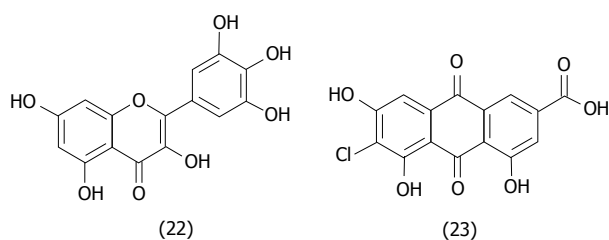


Figure 13 Chemical structures of myricetin (22, IC_{50} = 17 μ mol/L) and the anthraquinone (23, IC_{50} = 0.58 μ mol/L), TcTS inhibitors found by Arioka *et al.*^[69] from a natural products library.

The compound (18) was the most potent among the synthesized compounds, with a K_i = 300 μ mol/L.

Because the previous inhibitors described here were not very active against TcTS, a search for more potent scaffolds was led Kim *et al.*^[68] to explore novel chemical scaffolds by assessing the TcTS inhibition properties of non-sialyl derivatives. The capacity of sulfonamide chalcones to inhibit α -glucosidase was previously described, and since both enzymes have a sugar subtraction, Kim *et al.*^[68] planned and synthesized a series of chalcones based on the bioisosteric relationship between the carboxylic acid subunit of sialic acid with the sulfonamide moiety of the compounds (19-21, Figure 12). This synthesis resulted in the identification of the first TcTS inhibitors with an IC_{50} of lower than ten micromolar.

The need to find new chemical scaffolds of TcTS inhibitors led Arioka *et al.*^[69] to design a massive *in vitro* screening from a natural product library containing 2283 compounds, to search for more potent derivatives. The first trial selected the hit compounds that showed TcT inhibition above 40% at a 1 μ mol/L concentration, resulting in 103 compounds. Then, the second screening selected those compounds with IC_{50} < 100 μ mol/L, picking out a group of 50 compounds. The promiscuous inhibitors were excluded by a data analysis of the IC_{50} determination in the presence of 0.1% Triton X-100, and the resulting 16 selected compounds were critically evaluated using the Lipinski rules, culminating in the choice of two lead compounds called myricetin (22) and 6-chloro-9,10-dihydro-4,5,7-trihydroxy-9,10-dioxo-2-anthracenecarboxylic acid (23). These compounds

represented novel chemical scaffolds in the scope of TcTS inhibition, and they were submitted to structure/activity relationship (SAR) studies with the aim of optimizing these leads. Despite their great contributions to understanding the SAR of these scaffolds, none of the new derivatives were more potent than the natural prototypes shown in Figure 13. After a paper was published by Arioka *et al.*^[69], many groups synthesized TcTS inhibitors (all sialyl mimetics), but none of them were more potent than compound (23).

STEROL 14 α -DEMETHYLASE (CYP51)

In addition to those mentioned above, many other targets have been investigated for their antichagasic activities. One of the studied targets acts in regulating parasite cell membrane steroids, which play a fundamental role in cell division (since they are the primary components of the cell membrane) and cell maintenance (since its presence on the membrane is fundamental for maintaining selective permeability). As a class of molecules that are essential to the maintenance of cell viability, steroids are lipophilic biomolecules that act on the cell membrane, modulating its fluidity, integrity and permeability. The biosynthesis of steroids differs significantly between the Kingdoms^[70], and squalene oxide is a key intermediate in all eukaryotes. From this point, there is a divergence in the biosynthetic pathways that leads to different steroids; among animals, the major steroid is cholesterol (24). In fungi and protozoa, the primary steroid is ergosterol (25); and in plants, it is sitosterol (26), as shown above.

The differences shown in Figure 14 may be an advantage in the design of biologically active compounds that target enzymes involved in the biosynthetic pathways since this condition allows for the search for new derivatives possessing toxicity that is selective for parasites. The clinical use of the azole derivatives that modulate steroid biosynthesis is well established and clinically useful for fungal infection chemotherapy. In infections caused by trypanosomatids, the use of an azole derivative previously known as an antifungal called ketoconazole (28) was studied by McCabe *et al.*^[71], and the results led to a series of studies about the enzyme

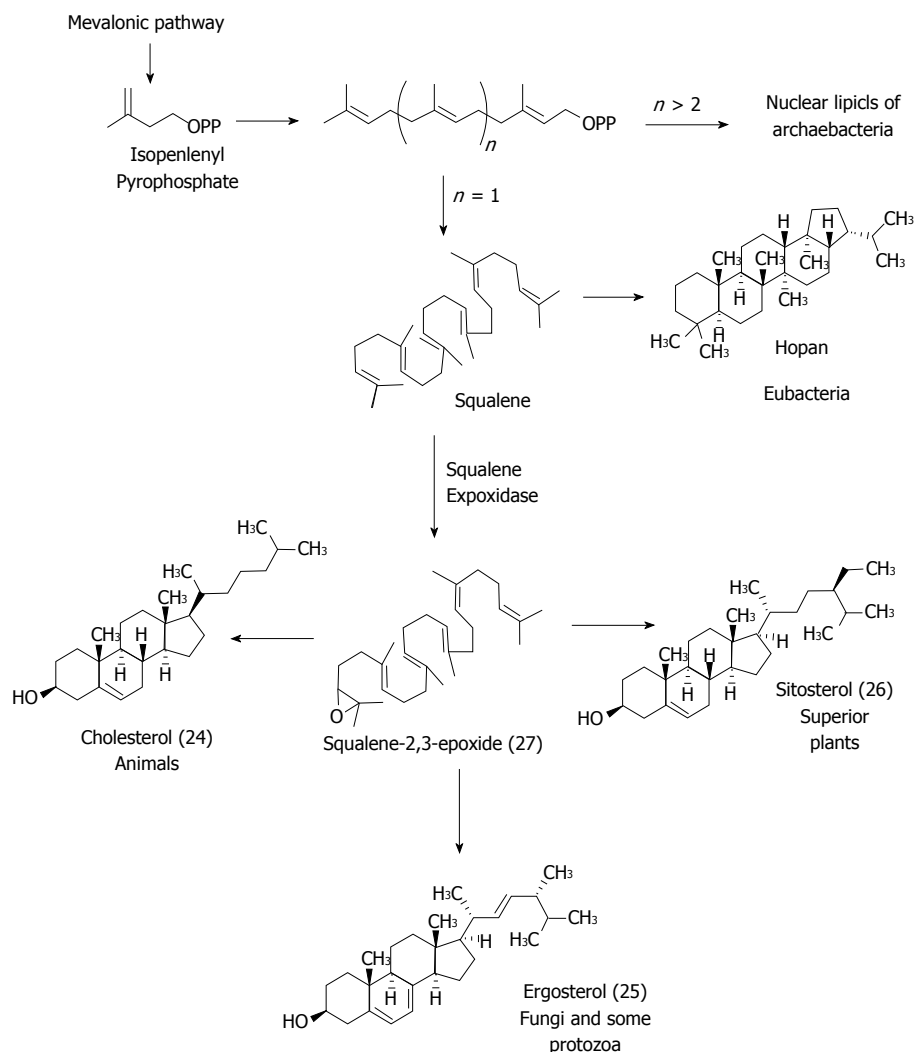


Figure 14 Comparative biosynthesis of natural membrane steroids of different organisms.

sterol 14 α -demethylase (or CYP51), a key enzyme in the regulation of sterol biosynthesis in eukaryotes^[72] (Figure 15).

The endogenous steroids in *T. cruzi* have a direct role in the cell viability and activity regulation of cell membrane enzymes^[73]. Although ergosterol is a final product of biosynthetic pathways that are common to both trypanosomatids and fungi, there are specificities regarding the synthesis of this lipid, principally during the demethylation step mediated by the CYP51 of each species. Despite the low similarity between the different isoforms of CYP51^[74], all the enzymes have both high regio- and stereoselectivity to the reactions they catalyze, which reduces the number of possible substrates. There are three known substrates in all the sterol-14 α -demethylases families; for example, lanosterol (29), eburicol (31) and obtusifolol (32)^[75]. However, this phenomenon diminishes the possibility that an azole is capable of inhibiting CYP51 from both protozoa and fungi since each isoenzyme possesses different affinities for ligands (Figure 16).

The inhibitors of *T. cruzi* CYP51 are the only class

of drug candidates that have reached clinical trials for Chagas disease chemotherapy^[76]. One example is the imidazole derivative VNI (33), which was found to be active during the chronic phase of the disease in *in vivo* experiments^[77]. The use of trypanocidal CYP51 inhibitors occurred before this enzyme was identified as a potential target. Antifungal agents such as itraconazole (34), fluconazole (35) and ketoconazole (28) were assessed *in vitro* and *in vivo* in Chagas models in the 80s, and they led to reductions in the parasite load in infected animals^[71,78]. The motivation for the first work involving ketoconazole (28) activity in a murine model of *T. cruzi* infection was derived from previous reports of its activity against *Plasmodium falciparum*^[79] and *Leishmania tropica*^[80]. The azole compounds act on *T. cruzi* CYP51 through interactions with the nitrogen heterocycles and the iron atom present in the central HEME (Figure 17) enzyme. This enzyme is responsible for the demethylation of eburicol, preventing the formation of a zymosterol intermediate (30) from lanosterol (29), thereby preventing the formation of ergosterol (25)^[73,81]. Thus, the consequence of inhibiting the final stages of ergosterol

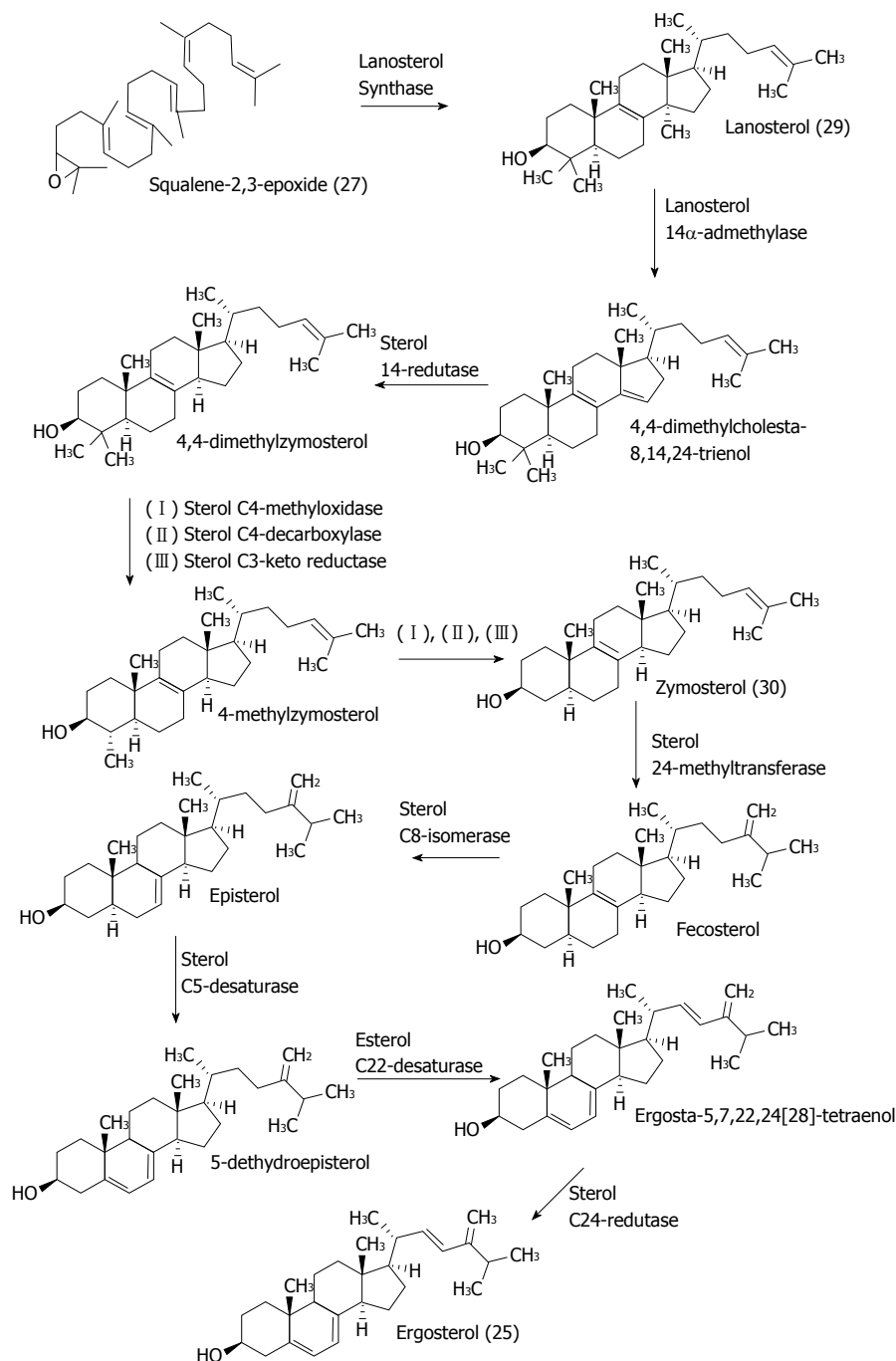


Figure 15 Ergosterol (25) biosynthesis in yeast (*Saccharomyces cerevisiae*), from squalene oxide.

biosynthesis is the accumulation of toxic biosynthetic precursors in the *T. cruzi* cell membrane, compromising its integrity, which is similar to what happens in yeast^[82,83] (Figure 18). This finding suggests that CYP51 inhibition as a promising approach to the development of new antichagasic molecules.

Recently, Franklim *et al.*^[84] described the synthesis of a novel series of triazole derivatives that were prepared from the natural amide piperine (36), and they were designed as CYP51 inhibitors of *T. cruzi* based on the bioisosteric relationship between the amide from (36) and the 1,2,4-triazole-3-thione from the antifungal drug

prothioconazole (37). Derivative (38), as shown in Figure 19, showed the best trypanocidal profile.

Despite their potential as trypanocidal agents, the new CYP51 inhibitors should be developed very carefully since these compounds can inhibit other enzymes that are involved in the hepatic microsomal system, leading to severe side effects such as hepatotoxicity and alterations in basal steroidogenesis. Long-term exposure to CYP51 inhibitors can cause deleterious effects on both the cellular biosynthesis of steroids and the phase I metabolism of drugs and xenobiotics, leading to a lack of clearance of toxic substances^[85]. One of the most relevant side effects

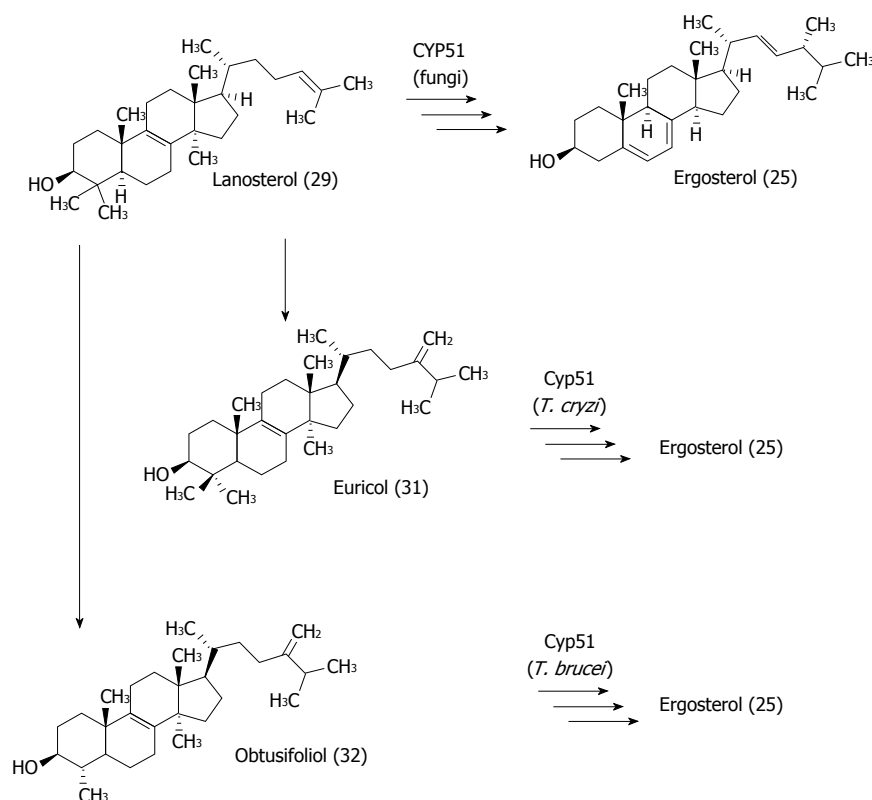


Figure 16 Structure of CYP51 preferential substrates in fungi (29), *T. cruzi* (31) and *T. brucei* (32).

of the administration of CYP51 inhibitors is the non-specific binding of these inhibitors to another important enzyme that is present in the hepatic microsomal system, which is known as CYP19 (aromatase). CYP19 is an enzyme that is located in the endoplasmic reticulum and is responsible for the demethylation of different steroids at position 10, *e.g.*, during the conversion of androstenedione (39) into estrone (40) or testosterone (41) to estradiol (42)^[86], as shown in Figure 20. The inhibition of CYP19 leads to the accumulation of (39) and (41) that impairs the balance between the steroid hormones, which is crucial for the development and maintenance of the reproductive system as well as for the differentiation of the sexual phenotype^[87].

TUBULIN

In addition to CYP51, tubulin is another promising target in the development of compounds that can modulate the cell cycle of *T. cruzi*. Tubulin is a class of globular protein whose isoforms comprise microtubules, which are cytoskeletal filaments that are responsible for maintaining the fundamental functions of eukaryotic cells. These functions include the segregation of chromosomes during cell division, the transport of intracellular components and the maintenance of the cell shape, cell motility and distribution of plasma membrane components^[88]. Microtubule formation occurs through the polymerization of two tubulin isoforms called α and β . Both subunits form a heterodimer of α and β -tubulin, which polymerizes^[89],

forming a filamentous cylindrical structure in the "head-to-tail" direction where the α subunit of a dimer binds to the β unit of the other. This polymerization leads to an initial polymer protofilament, which is grouped with other similar protofilaments to form the microtubule itself^[90], as shown in Figure 21.

Once the microtubule is formed, it becomes a dynamic structure in which the continuous processes of polymerization and depolymerization take place in equilibrium. This feature makes it possible for the microtubule to change its size and adapt to different situations, such as those that occur during the cell cycle. The α -terminal portion [or region (-)] is less dynamic, whereas the β -terminal [or region (+)] is more dynamic and can lengthen/shorten more quickly^[91]. This characteristic confers polarity to microtubules, which gives the different (+) and (-) regions different properties and causes them to be oriented in different directions. This characteristic is given by the fact that each tubulin subunit (both α and β) has a binding site for guanosine triphosphate (GTP), which binds more strongly to α than to β -tubulin. In this way, the GTP bound to β -tubulin is more easily hydrolyzed to guanosine-diphosphate (GDP) after polymerization^[92]. The kinetics of polymerization in this case are more favorable than the kinetics of GTP hydrolysis, allowing the growth of the microtubule. In that case, the increase or decrease of the microtubule length in the region (+) closely depends on the nucleotide linked to β -tubulin; a microtubule with a GTP molecule tends to polymerize, while those associated with GDP will try to

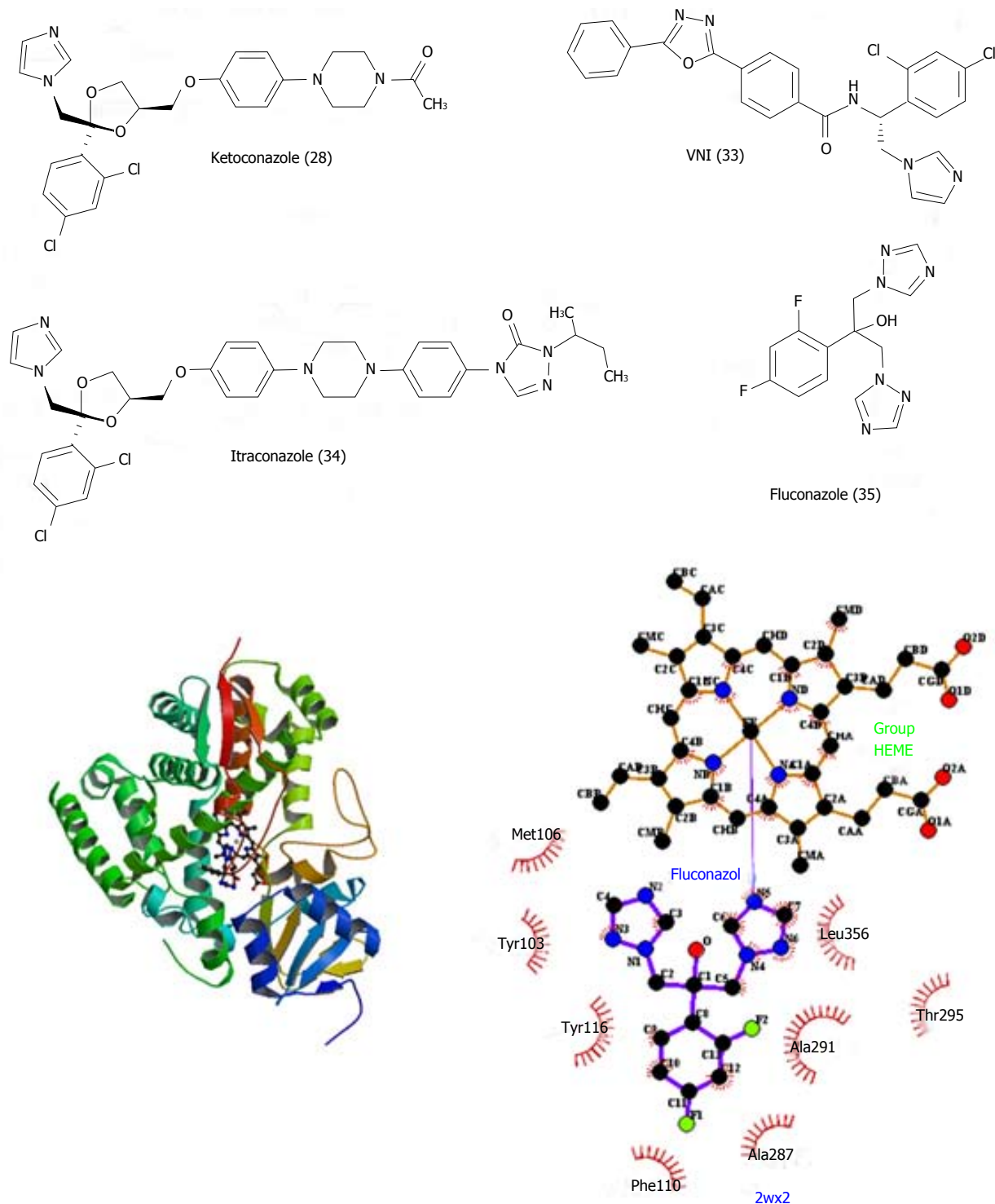


Figure 17 Structures of azole derivatives active in *T. cruzi* infection model: Ketoconazole (28), VNI (33), itraconazole (34) and fluconazole (35). Crystallographic structure of *T. cruzi* CYP51 with fluconazole (35) linked to the catalytic site of the enzyme [available at Protein Data Bank under the code 2wx2 (left)]. Bidimensional scheme of fluconazole (35) binding interaction with the catalytic site of the enzyme, generated by the program LigPlot[®] from the same code (right).

depolymerize^[93], as shown in Figure 22.

Since tubulin is a key component of cell proliferation, it is an important target in the development of cancer chemotherapy, and the tubulin inhibitors are some of the most effective anti-cancer drugs^[94]. Similarly, tubulin plays the same role in cell division in parasites such as *T. cruzi* that possess cell proliferation kinetics comparable to

those found in cancer cells. The cell division processes in parasites are strictly dependent on the polymerization/depolymerization equilibrium of tubulin^[95], and they act on the parasite motility process as well, which is essential for the maintenance of the host infection.

Although there is a strong shared identity between the sequences of tubulin amino acids from different

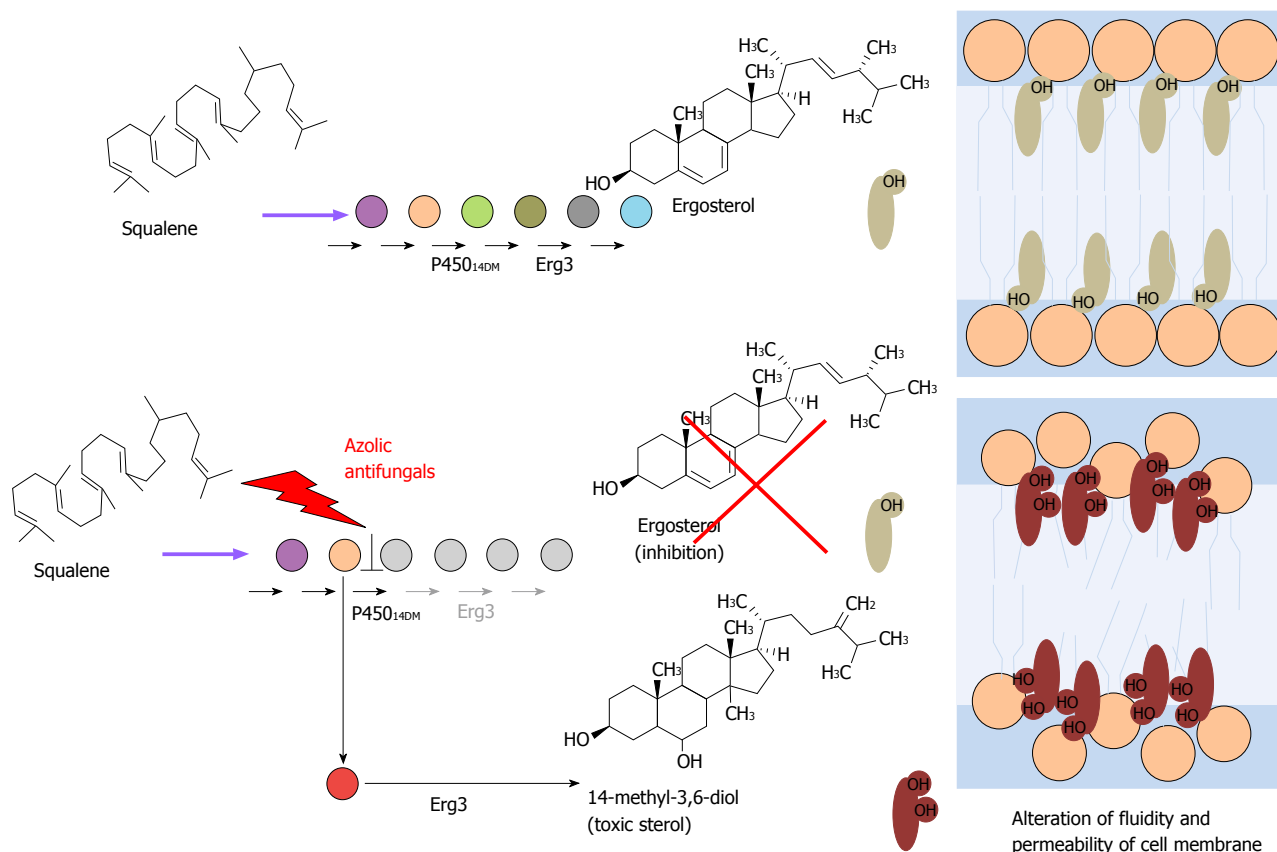


Figure 18 Schematic representation of the mechanism of actions of azolic compounds upon ergosterol (25) synthesis and subsequent alteration of composition and organization of cell membrane.

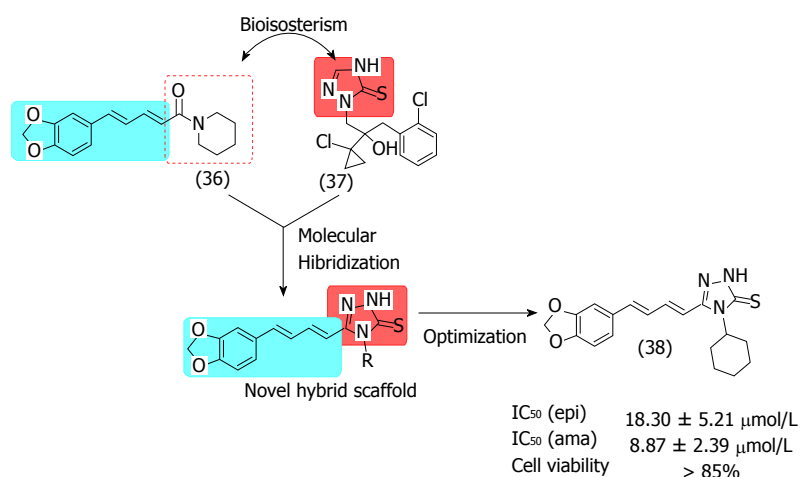


Figure 19 Structures of piperine (36), prothioconazole (37) and cycloexyltriazole (38), and its IC₅₀ values against epi- and amastigotes of *T. cruzi* and the toxic profile against murine macrophages.

organisms (*e.g.*, mammalian cells and yeast may have shared identities from 70% to 90% in their tubulin isoforms), there are several reports of tubulin inhibitor drugs being used in anti-parasitic chemotherapy in the literature. The antifungal benzimidazolic drug Benomyl® (43) has high selectivity for yeast tubulin; Kilmartin *et al.*^[96] showed that (43) is 300 times more potent at inhibiting *S. cerevisiae* tubulin than bovine brain tubulin. The deri-

vatives oxfendazole (44) and thiabendazole (45), as shown in Figure 23, are more selective for nematode tubulin than for mammalian tubulin^[97]. Thus, despite the high structural similarity between tubulins from diverse species, the small differences are probably responsible for the selective recognition of these compounds in different organisms, making tubulin an important target for Chagas disease chemotherapy.

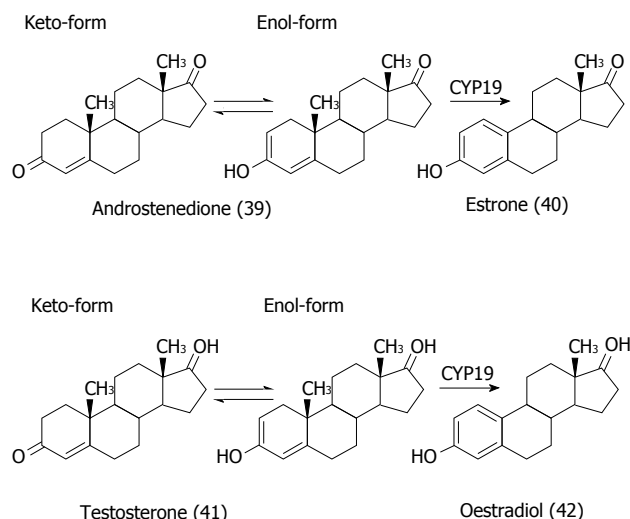


Figure 20 Reactions catalyzed by aromatase (CYP19): Conversion of androstenedione (39) to estrone (40) and testosterone (41) to oestradiol (42).

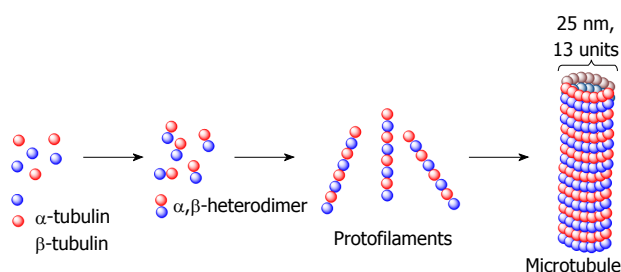


Figure 21 Schematic representation of protofilaments formed by polymerization of the heterodimers of alpha and beta tubulin.

The search for selective tubulin inhibitors is a current challenge in the development of new antichagasic drugs. The work of Werbovetz *et al.*^[98] identified some sulfonamide-dinitroaniline derivatives that were structurally analogous to oryzalin (46), an herbicide that acts by depolymerizing microtubules from plants and thereby prevents its anisotropic growth. In this study, compound GB- II -5 (47) was found to have greater potency against kinetoplastids than mammalian cells, as shown in Figure 24.

The sulfonamide derivatives (46-48) bind in a tubulin region called "the colchicine site"^[99], which is a region between the α and β tubulin subunits. This site is where colchicine (49, Figure 25) interacts with tubulin as a well-known tubulin inhibitor. Colchicine (49) is a natural product that is extracted from *Colchicum sp.* (e.g., *Colchicum autumnale* or meadow saffron), which is used to treat gout^[100]. When colchicine (49) binds to the region between two tubulin heterodimer subunits, it induces the depolymerization of microtubules by altering the conformation adopted by the β subunit after its binding. Once bound to (49), the dimer assumes a curved conformation that generates steric hindrance upon the formation of protofilaments that will generate the microtubules^[101].

Another binding site in the tubulin structure is located

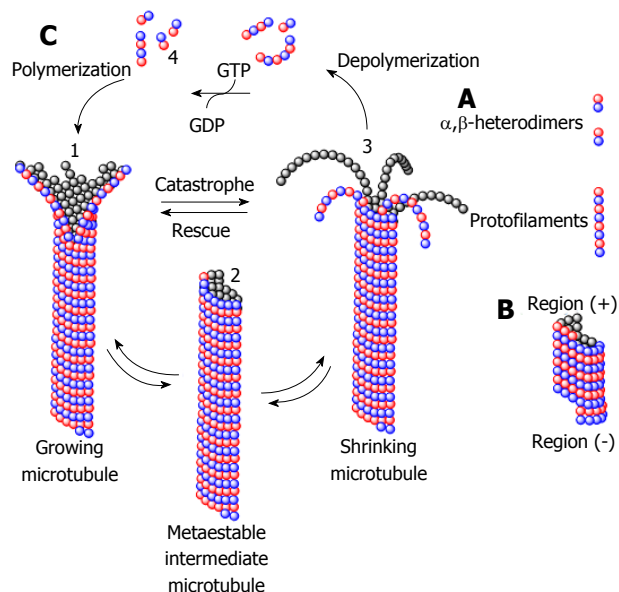


Figure 22 Schematic representation of polymerization/depolymerization dynamics of microtubules. A: Formation of protofilament with alpha e beta-tubulin heterodimers; B: Formation of microtubule with regions (+) and (-); C: Relationship between polymerization and GTP hydrolysis equilibrium, where is perceived: (1) the polymerization of the GTP-terminal microtubule; (2) an intermediate where the kinetics of hydrolysis and polymerization are equivalent; (3) the microtubule depolymerizing when the hydrolysis rate is superior; and (4) the microtubule salivation mediated by GTP consumption.

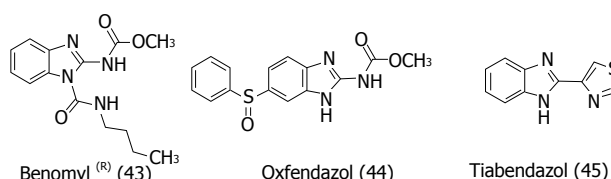
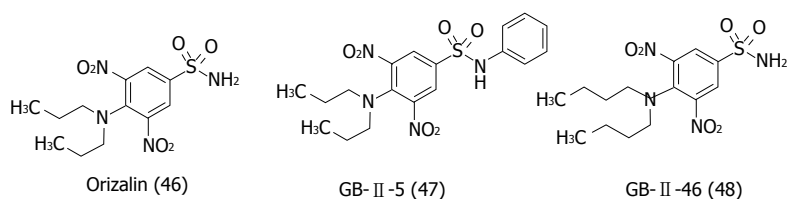


Figure 23 Structure of benzimidazolic compounds active on yeast (43) and nematoda (44, 45) but inactive against mammalian tubulin.

at the interface of two heterodimers, more specifically, on the β subunit^[102]. The vinca (*Catharanthus roseus*) alkaloids, e.g., vinblastine (50) and vincristine (51), bind to this site. Despite the fact that *C. roseus* had been used in popular medicine for a long time in various locations such as India, China and Hawaii, it attracted the interest of a group of Canadian scientists in the 1950s who wanted to study its popular use in a diabetes treatment from Jamaica^[103]. Although their efforts to pursue anti-diabetic substances did not succeed, strong cytostatic activity was identified in the crude extract of *C. roseus*, which led to the isolation of two alkaloids (50-51). These bis-indole monoterpene alkaloids are produced in very small quantities in the leaves of *C. roseus*^[104] through the reaction of two other alkaloids called catharanthine (52) and vindoline (53)^[105], as shown in Figure 26. The mechanism of action of these alkaloids involves the suppression of their polymerization in the positive region^[106] and the promotion of depolymerization in the negative region of the microtubules. This characteristic



Compound	<i>L. donovani</i> (amastigotes)	<i>T. b. brucei</i> (variant 221)	<i>T. b. brucei</i> (Lab 110 EATRO)	J774 (macrophages)	PC3 (prostate)
Orizalin (46)	72 ± 10	11 ± 0	6.6 ± 1.0	41 ± 5	57 ± 4
GB-II-5 (47)	5.0 ± 0.6	0.41 ± 0.02	0.73 ± 0.09	29 ± 1	35 ± 1
GB-II-46 (48)	20 ± 2	2.6 ± 0.3	1.9 ± 0.7	9.4 ± 2.0	23 ± 4

Figure 24 Structures and IC₅₀ values (micromolar) of orizalin (46) and the sulfonamide-dinitroanilin derivatives (47 and 48) against kinetoplastide and mammalian cells^[98].

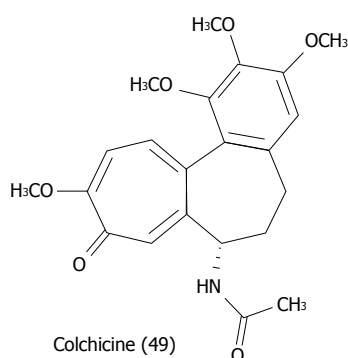


Figure 25 Chemical structure of colchicine (49).

allows these alkaloid derivatives to be able to change their microtubule dynamics during the formation of the mitotic spindle in the cell division process^[107]. In addition to the change in the polymerization/depolymerization dynamics, *Vinca* alkaloids also promote the fragmentation of the existing microtubules through the detachment of some regions near the (-) region of the biopolymer^[108].

The third-most common approach to the modulation of the microtubule dynamics does not involve increases in the depolymerization process, but instead involves its blockage. Thus, through the stabilization of microtubules, these organelles lose their dynamics, which is necessary for the maintenance of their functionality. This phenomenon occurs when paclitaxel (54, also known as Taxol®) binds to a specific site of the tubulin β subunit^[109]. Paclitaxel (54) is a natural compound that was initially identified as a secondary metabolite of the Pacific yew (*Taxus brevifolia*). Due to the small amount of taxol available in the plant, together with the difficulty of sustainably managing *T. brevifolia* cultures, a semisynthetic method was employed to manufacture paclitaxel (54) based on the isolation of 10-deacetylbaccatin III (55) from the leaves of *Taxus baccata*. Ojima *et al.*^[110] developed a method in which compound (55) is coupled with the lactam at C-13 (56), as shown in Figure 27. Subsequently, a great number of biotechnological approaches involving cell culture and gene expression in bacteria allowed for the preparation of appreciable amounts of

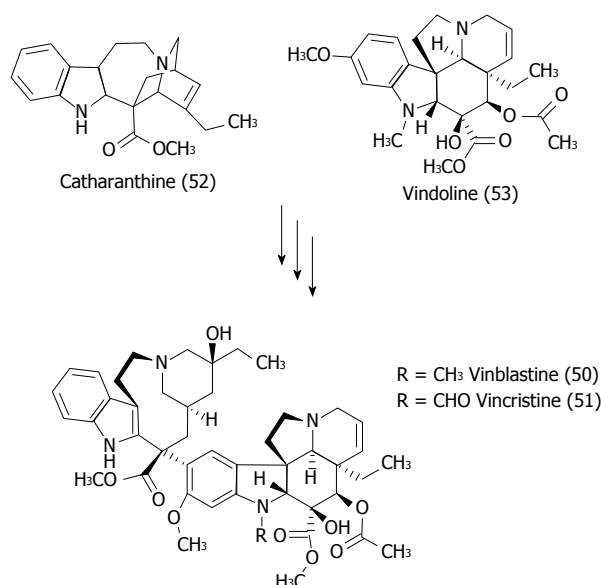


Figure 26 Structures of the vinca alkaloids (50-51) and its biochemical precursors (52-53).

(54) in a less costly way. However, Taxol® remains a very expensive drug^[111].

In the β-terminal subunit of tubulin [region (+)], the nature of the anchored nucleotide determines whether there will be polymerization or depolymerization. The presence of GTP provides for polymerization, and the presence of GDP promotes microtubule depolymerization instead. These processes take place because GDP hydrolysis alters the conformation of the β-tubulin, which causes a cascade of events that changes the structure of protofilaments, making them more curved and causing them to protrude out of the microtubules (structure previously shown in Figure 22, item 3). The presence of paclitaxel (54) anchored in the region adjacent to the GDP-binding site (the so-called "taxol site") stabilizes the polymer structure, preventing the depolymerization needed to maintain the microtubule dynamic equilibrium. The microtubule stabilization compromises different processes that depend on the microtubules, such as mitosis, disabling cell duplication^[93]. These three tubulin

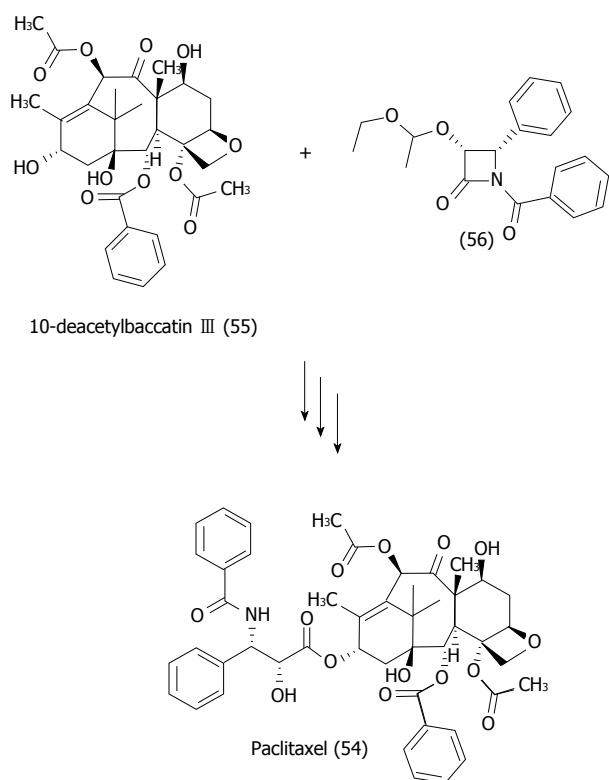


Figure 27 Synthetic strategy developed by Ojima *et al.*^[110] on the synthesis of paclitaxel (54) from the natural precursor 10-deacetylbaccatin III (55).

binding sites are the primary paradigms in the research and development of bioactive compounds, with the aim of modulating cellular phenomena through involvement with microtubules, and they are shown in Figure 28. Each of these sites has a number of known ligands, the structures of which are depicted in Figure 29.

The first work reporting the capacity of taxol (54) to act against *T. cruzi* was published by Baum *et al.*^[112] in 1981, in which the authors used transmission and scanning electron microscopy experiments to find several parasites containing multiple flagella and multiple intracellular organelles such as the nucleus and kinetoplasts. However, cell division by binary fission does not occur, which corroborates the hypothesis of (54), showing that it acts on a specific structure during cytokinesis^[112]. After that, other authors studied the effects of different compounds such as the natural amide piperine (36), as reported by Freire-de-Lima *et al.*^[113]. Natural piperine (36) acts in the blockage of cytokinesis of *T. cruzi* epimastigotes, and it leads to cellular ultra-structural alterations similar to those observed in taxol-treated parasites^[113].

Some of the well-known tubulin inhibitors can interact with other sites on the tubulin heterodimer. Curcumin (67, Figure 30), for example, is a natural diarylheptanoid with a recognized involvement in cell cycle modulation. It acts by binding to tubulin in HeLa and MCF-7 cells, reducing the GTPase activity and partially inhibiting the activity of colchicine (49) in these cells^[114]. Banerjee *et al.*^[115] also reported that curcumin acts by suppressing the dynamic instability of microtubules in MCF-7 cells, maintaining

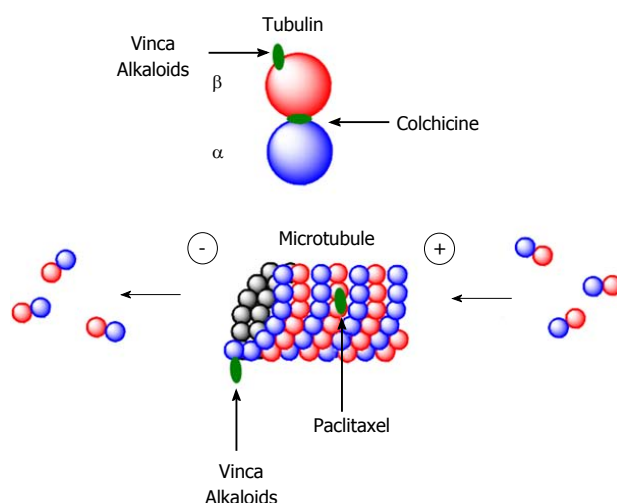


Figure 28 Representation of the binding site locations for the main tubulin ligands.

the microtubules in a metastable state, similar to paclitaxel (54). However, Banerjee's work suggested that curcumin did not interact with any of the three most popular binding sites of tubulin (taxol, vinca alkaloids and colchicine sites), which led Chakraborti *et al.*^[116] to perform experiments that allowed them to elucidate the binding site of curcumin (67) to tubulin. Using Fluorescence Resonance Energy Transfer, Chakraborti *et al.*^[116] determined that curcumin (67) interacts between two α , β -tubulins, which are heterodimers that are 32 Å away from the colchicine binding site. This interaction features a new binding site that is potentially useful for planning new antitubulin agents. Aiming to justify the trypanocidal properties of curcumin (67) and other natural diarylheptanoids, Sueth-Santiago *et al.*^[117] build a theoretical model of *T. cruzi* tubulin. Molecular docking studies have shown a good correlation between the binding scores and the trypanocidal activities of the four natural diarylheptanoids. The results obtained from the cell cycle studies corroborated this hypothesis, showing alterations on parasites cell cycle in the same way of the positive control with accumulation of parasite cells in the G2 phase^[117].

CONCLUSION

Despite the fact that Chagas disease was described more than 100 years ago, it remains a death sentence to millions of people who are in the chronic phase of the illness. Due to the lack of interest of the pharmaceutical industry in developing new drugs to treat neglected illnesses such as Chagas disease, there is no effective treatment available at present. However, given that, we have found a huge and growing amount of information that has been published about the parasite and its complex relationship with the host, the discovery of an effective drug comes closer each day. In this sense, detailed knowledge of both the differences and similarities

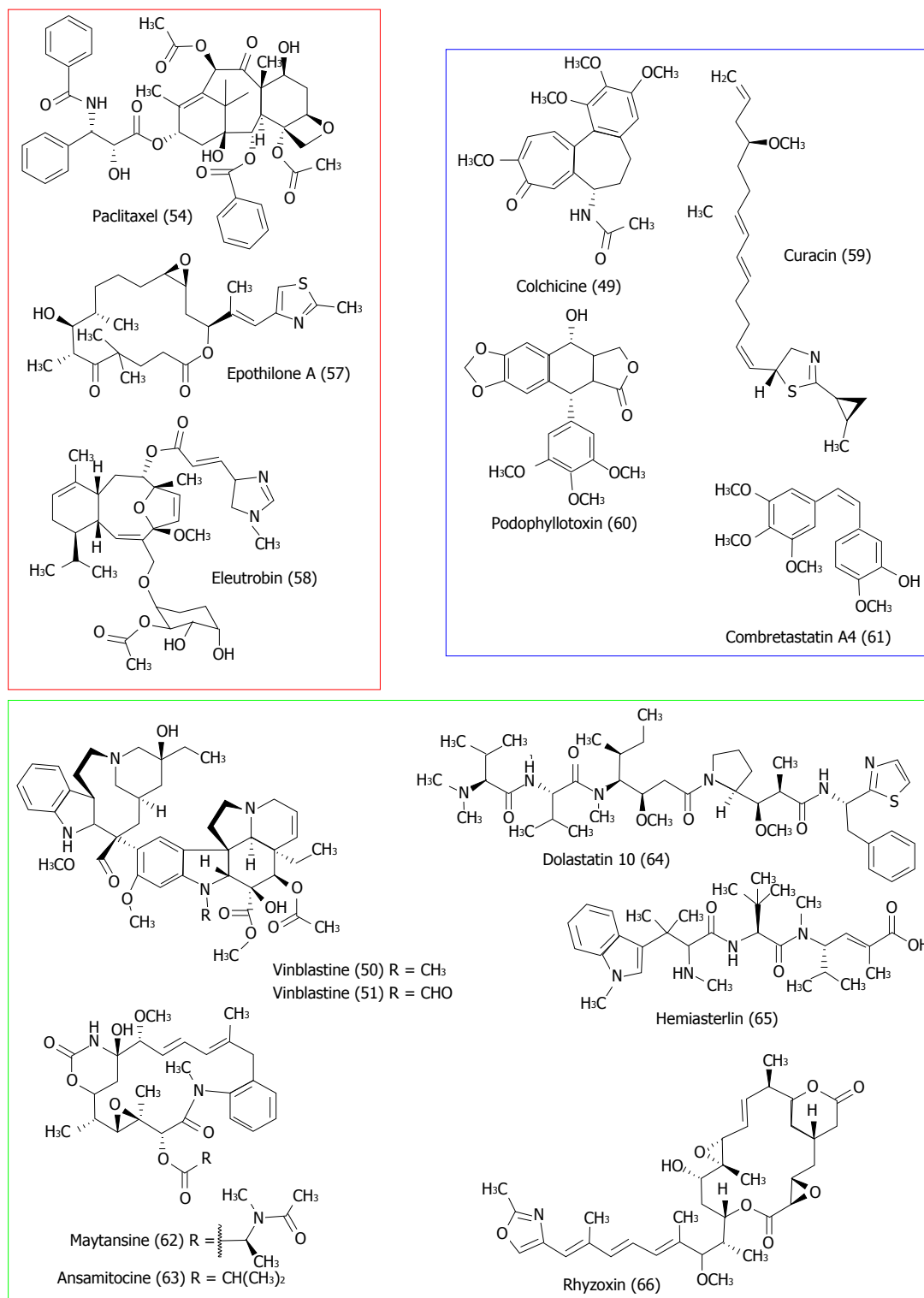


Figure 29 Structures of some ligands of the three main binding sites of tubulin: Taxol site (red), vinca alkaloids site (green) and colchicine site (blue).

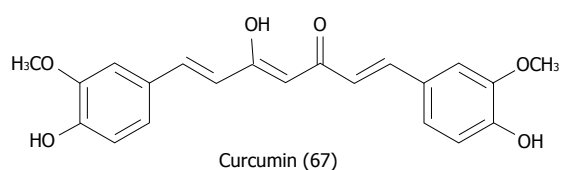


Figure 30 Chemical structure of curcumin (67), a natural product that stabilizes microtubules by binding in a unique binding site of tubulin.

between *T. cruzi* and its human host's biochemical targets may be the key for curing this severe and debilitating sickness.

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Use of thyroglobulin as a tumour marker

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it identifies the patients who are unlikely to show rising TG levels in the presence of thyroid cancer. Those who have high serum TG before surgery will show up recurrence as rising serum TG during the postoperative period. Those who do not have high serum TG before surgery will not show up rising serum TG in the presence of recurrent disease. In the latter situation, normal TG level gives only a false reassurance regarding recurrence of disease. Nevertheless, rising serum TG during the postoperative period must be interpreted cautiously because this could be due to the enlargement of non-cancerous residual thyroid tissue inadvertently left behind during surgery.

Key words: Thyroglobulin; Thyroid cancer; Recurrent thyroid cancer; Anti-thyroglobulin antibodies; Tumour marker

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Core tip: Although serum thyroglobulin (TG) is widely used as a tool to detect recurrence of thyroid cancer, it is widely held that preoperative TG measurement is unnecessary. It is true that preoperative TG level is hardly of much diagnostic value, but without a preoperative TG report, it is not possible to safely utilize serial serum TG subsequently as a monitoring tool. Routine measurement of serum TG before surgery is, therefore, recommended.

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Abstract

It is worthwhile to measure serum thyroglobulin (TG) level in thyroid cancer before subjecting patients to surgery for two reasons. Firstly, if the level is high, it may give a clue to the local and metastatic tumour burden at presentation; secondly, if the level is normal,

INTRODUCTION

Serial measurement of thyroglobulin (TG) is being practised by many clinicians to detect recurrence of thyroid cancer after surgery^[1-3]. Apart from that, there is hardly

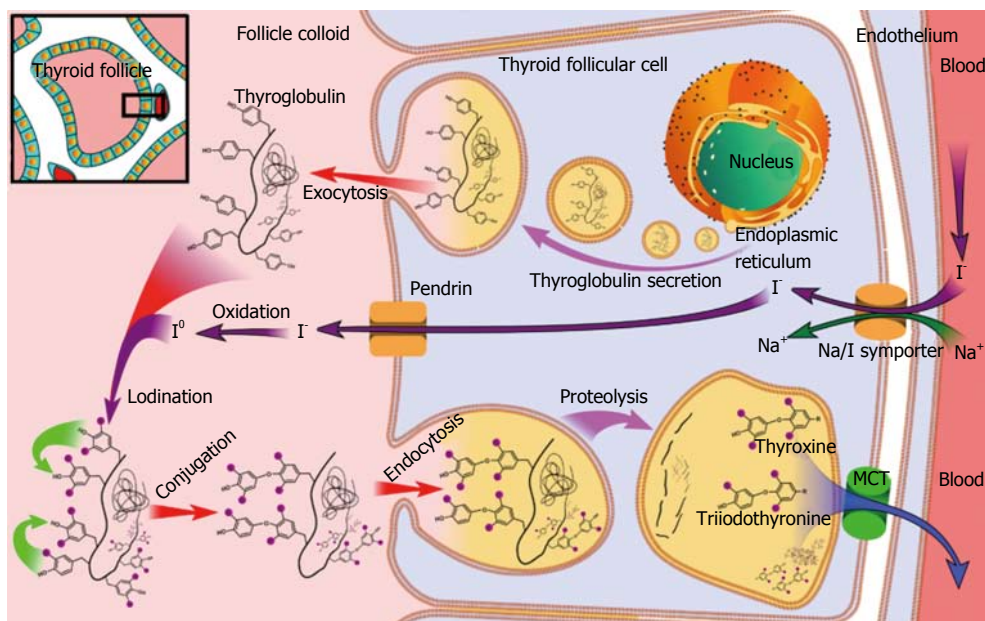


Figure 1 Metabolism of thyroglobulin^[20].

any use of TG as a diagnostic modality of thyroid cancer because it is not tumour specific^[4]. The serum level of TG reflects the thyroid tissue burden in the body regardless of underlying pathology whether benign or malignant. Since the serum levels are proportional to the volume of thyroid tissue, it can be used successfully to evaluate the "cancer burden" at presentation as well as to assess the adequacy of surgery, monitor the recurrences and progression of disease before and after cancer treatment. Its use as a monitoring tool is only valid provided certain precautions are seriously taken into account. Otherwise, false reassurance is inevitable in the presence of on going disease.

BIOCHEMISTRY

TG is a glycoprotein molecule synthesised exclusively by the thyroid follicular cells. These are stored as colloid within the thyroid follicles, iodinated and degraded to thyroxine and tri-iodothyronine. The whole process is regulated by thyroid stimulating hormone (TSH) (Figure 1).

TG in serum has a half-life of 65 h^[5]. The serum level TG is proportional to the volume of thyroid tissue in the body at a rate 1 ng/mL per 1 g of thyroid mass. Since the size of the normal thyroid gland is 20-25 g, the reference range has to be generally about 20 to 25 ng/mL. However, the normal serum TG level depends on the gender and the level of iodine intake of the patient. The gender specific reference range has been given as 1.40-29.2 ng/mL for males and 1.50-38.5 ng/mL for females^[6]. In countries with iodine deficiency the reference range may be higher^[7,8]. This is because of elevated TSH in serum in the presence of iodine deficiency; TSH stimulates the thyroid follicular cells to synthesise more TG. Apart from gender and iodine

intake, the measured TG level is also influenced by autoantibodies against TG (anti-TGAb), cigarette smoking and TSH status unrelated to iodine intake. The measured serum TG levels are masked by the presence of anti-TGAb in blood. Anti-TGAb bind to the TG molecules masking the epitopes on the TG molecule to which the antibodies of the radio immuno assay (RIA) test bind. As a result, the measured TG level by RIA in such patients, will be falsely low. Anti-TGAb can be seen in cases of Hashimoto's disease, Graves' disease, 20% of patients with thyroid carcinoma^[9], and 10% of normal population without any thyroid disease^[10].

CLINICAL USES OF TG

Evaluation of completeness of surgery

TG can be elevated in any thyroid pathology. Serum TG level is proportional to the thyroid mass rather than the type of pathology^[8]. As a guide, one gram of normal thyroid tissue is equivalent to 1 ng/mL of TG in serum in euthyroid state; in a TSH suppressed state, this is equivalent to 0.5 ng/mL. After total thyroidectomy, theoretically, the level has to drop to zero because no thyroid tissue is expected to leave behind after total thyroidectomy. Persistent presence of TG after total thyroidectomy, even at low concentrations, points to the possibility of inadequate surgery leading to residual thyroid tissue at the thyroid bed (in the absence of local or distant metastases). After hemithyroidectomy, the preoperative level should theoretically drop to 50% of preoperative value. After near total thyroidectomy, *i.e.*, total lobectomy on one side plus isthmusectomy plus subtotal lobectomy on the other side (leaving behind overall 1/8th of the size of a normal sized gland weighing 25 g), a TG level of 3-4 ng/mL can be expected to

present in serum when TSH is normal, and 1.5-2 ng/mL when TSH has been suppressed with high doses of thyroxine.

The serum TG level post surgery reflects the amount of residual thyroid mass. In the absence of metastatic disease, this reflects the size of the thyroid remnant left behind during surgery. Since TG has a half-life close to 3 d, it can be shown that the TG level has to be less than 0.5 ng/mL at the end of three weeks after total or near total thyroidectomy for a goitre of a size of three times normal. This can be used as a guide to initiate radio active iodine treatment for remnant ablation, the residual thyroid mass being as a result of inadequate surgery or metastatic disease. However, although such a hypothesis carries high positive predictive value, a negative result does not reliably exclude the possibility of residual disease. The TG serum level at 3 wk post surgery not only depends on the residual thyroid mass but also the size of the gland at the time of surgery as well as TG producing capacity of the tumour.

As a monitoring tool

TG can be secreted by differentiated cancer cells of the thyroid. This includes nearly two thirds of differentiated thyroid cancers (follicular neoplasms, all papillary carcinomas, Hurthle cell tumours of the thyroid), up to 50% of poorly differentiated and anaplastic carcinomas of thyroid and some medullary carcinomas. The cancerous nodules can be stained with immunohistochemical methods to specifically demonstrate TG. TG has been demonstrated in cancerous follicles both in the thyroid as well as in metastatic deposits in lymph nodes and elsewhere.

The use of TG as a screening and diagnostic tool of differentiated thyroid cancer is poor (Sensitivity and specificity is 70% and 80% respectively for follicular carcinoma) because both benign and malignant thyroid glands secrete TG^[11]. Elevated TG levels can be seen in benign conditions like Graves' disease and thyroiditis, as well as in thyroid cancer. Further, there is a subset of thyroid cancer patients (12%) whose TG level is low or low-normal as opposed to a minority whose blood level is very high (> 1000 ng/mL). A level > 5000 ng/mL is indicative of metastatic disease^[12]. Patients who develop cancer as a result of mutations of TG gene may not express TG even though they have a hypothyroid goitre (high TSH) as well as cancer^[13].

Those who have high serum TG one month after thyroidectomy are more likely to develop cancer recurrence, the value being proportional to the burden of remaining metastatic disease^[14]. However, high serum TG could be due to distant metastatic disease or incomplete excision of the gland during thyroidectomy. Holsinger *et al*^[15] reported that 57% of patients who were supposed to have had total thyroidectomy for thyroid cancer had residual thyroid tissue at the thyroid bed when tested by postoperative radioactive iodine uptake test. Therefore, the surgeon must be sure that the whole thyroid gland has been removed before utilizing TG as a prognostic indicator. Or else the patient must have undergone

remnant ablation with radioactive iodine before TG testing.

TG has been used frequently to monitor recurrent disease after thyroidectomy. Not only the first recurrence but also subsequent recurrences can be detected by monitoring serum TG level. The TG level has to be done every 6 to 12 mo. However, certain points need to be kept in mind when utilizing TG alone as a tumour marker to detect recurrence. TG can rise after thyroidectomy when the residual thyroid gland enlarges even though it does not harbour malignant cells. This could happen after near total thyroidectomy (total lobectomy on one side plus isthmusectomy plus subtotal lobectomy on the other side) when about 4 g of the gland is left behind purposely on one side, or glandular tissue left behind inadvertently in the neck because of difficult thyroidectomy. In such instances, serum TG could give false positive result. Therefore, more often than not, surgeons prefer to subject all patients to remnant ablation therapy after thyroidectomy for cancer. Such patients are more suitable for monitoring by TG than otherwise.

It may not be able to see high serum TG after thyroidectomy if the TG level before surgery had been low or normal. Therefore, it is necessary to recognise patients who would not have rising TG in the presence of recurrence before using TG as a tumour marker. This can only be done if the serum TG level before the surgery is known. Those who have normal or low serum TG before surgery will not show rising serum TG when there is recurrence of disease^[16]. This happens irrespective of the anti-TGAb status of the patient^[17]. Such patients must be offered iodine scintigraphy to detect recurrence. Furthermore, there is a possibility of transforming a differentiated thyroid cancer to anaplastic variety over time^[18]. In such a case, TG will not rise in the presence of recurrence.

PRECAUTIONS

It may take nearly one month (7 to 10 $t_{1/2}$) for the TG to disappear from the circulation after total thyroidectomy in the absence of metastases^[5].

Since there is a theoretical possibility of releasing TG in to the circulation following trauma, it would be a valid precaution to measure TG before FNAC test or 2 wk after FNAC, although there are no references to strongly recommend this approach.

In postoperative patients, thyroxine must preferably be stopped at least six weeks before the measurement of serum TG if the patient has been on thyroxine or two weeks before if the patient has been on liothyronine (tri-iodothyronine). The idea is to allow TSH level to rise from the suppressive levels to higher levels making the residual cancer tissue to synthesise and secrete TG stimulated by TSH. This is also the standard practice in postoperative use of radioactive iodine for diagnostic and therapeutic purposes. Alternatively, to minimize the risks associated with stopping thyroxine for six weeks, thyroxine can be stopped only for three weeks before the TG test,

Table 1 Interpretation and management of different serum thyroglobulin values

Stage of disease	Serum TG level			Uses and further action
	Low	Normal	Elevated	
Before surgery	Anti-TGAb or TG non-secreting tumour			TG can not be used subsequently to monitor disease activity
3-4 wk after surgery	Anti-TGAb, successful local surgery or no metastatic disease	Incomplete local surgery, metastatic disease or recurrence	Benign or malignant thyroid disease	TG can be used subsequently to monitor disease activity
				Do 125-Iodine scan to screen for residual local disease and distant metastases
Long term follow-up	Anti-TGAb Cure or stable disease		Recurrence or progression	Remnant ablation therapy (131-Iodine or further surgery) is indicated
				Do other tests to monitor disease activity Disease activity can be monitored by periodic TG measurements Further investigations and treatment

TG: Thyroglobulin; anti-TGAb: Autoantibodies against TG.

but the test may have to be repeated after six weeks of cessation of thyroxine if TG and TSH are normal^[19]. Another alternative is to stop thyroxine six weeks before and to put the patient on liothyronine for four weeks and do the test two weeks later after stopping liothyronine. Liothyronine has a shorter half-life than thyroxine. Recombinant human TSH can also be used, instead, to stimulate residual thyroid tissue without stopping thyroid suppression treatment.

CONCLUSION

Interpretation of serum TG measurements could be a quite complex task. It may be elevated in both benign and malignant disease of the thyroid gland. Therefore, its use as a diagnostic tool of cancer is negligible. TG level 3-4 wk post surgery can be used to assess the size of the residual thyroid mass either locally or distally, and guide remnant ablation therapy. Rising levels in the postoperative period could happen due to progression of cancer or growth of residual thyroid tissue at the thyroid bed following inadequate surgery. It may remain normal in malignant thyroid disease both pre and post operatively due to non-secreting varieties of thyroid cancer or due to the presence of anti-TGAb in serum, and such cases could only be detected by checking TG levels before surgery.

The changes of TG that can be expected at different stages of the disease and how different TG levels assist in decision making and the management of thyroid cancer has been summarised in Table 1.

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Basic Study

L-carnitine protects C2C12 cells against mitochondrial superoxide overproduction and cell death

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Abstract

AIM

To identify and characterize the protective effect that L-carnitine exerted against an oxidative stress in C2C12 cells.

METHODS

Myoblastic C2C12 cells were treated with menadione, a vitamin K analog that engenders oxidative stress, and the protective effect of L-carnitine (a nutrient involved in fatty acid metabolism and the control of the oxidative process), was assessed by monitoring various parameters related to the oxidative stress, autophagy and cell death.

RESULTS

Associated with its physiological function, a muscle cell metabolism is highly dependent on oxygen and may produce reactive oxygen species (ROS), especially under pathological conditions. High levels of ROS are known to induce injuries in cell structure as they interact at many levels in cell function. In C2C12 cells, a treatment with menadione induced a loss of transmembrane mitochondrial potential, an increase in mitochondrial production of ROS; it also induces autophagy and was able to provoke cell death. Pre-treatment of the cells with L-carnitine reduced ROS production, diminished autophagy and protected C2C12 cells against menadione-induced deleterious effects.

CONCLUSION

In conclusion, L-carnitine limits the oxidative stress in these cells and prevents cell death.

Key words: Superoxide anions; Mitochondria; Reactive Autophagy; Muscle; Carnitine; Oxygen species; Cell death

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Core tip: The overall goal of this study was to identify and characterize the protective effect of L-carnitine on oxidative stress in muscle cells. We, first, induced an oxidative stress in cultured muscle cells and parameters associated with the stress were measured. In another set of experiments, cells were treated with L-carnitine and the same parameters measured. The comparison among the data allowed showing that L-carnitine was able to fight against oxidative stress and dramatically limit cell death. In conclusion, our results show that, at least in muscle cells, L-carnitine can be considered as a non-conventional antioxidant.

Le Borgne F, Ravaut G, Bernard A, Demarquoy J. L-carnitine protects C2C12 cells against mitochondrial superoxide overproduction and cell death. *World J Biol Chem* 2017; 8(1): 86-94 Available from: URL: <http://www.wjgnet.com/1949-8454/full/v8/i1/86.htm> DOI: <http://dx.doi.org/10.4331/wjbc.v8.i1.86>

INTRODUCTION

During oxidative stress, a massive production of reactive oxygen species (ROS) induces alterations in cell structure. Oxidative stress may play a role in the onset of a wide variety of disorders^[1] and participate at the ageing process^[2]. ROS are able to interact with proteins, lipids and DNA leading to the formation of adducts accompanied with a loss of function^[3]. These deleterious effects are normally kept under control by antioxidants. Most antioxidant systems are composed of an enzyme (e.g., superoxide dismutase, catalase, glutathione peroxidase^[4]) usually under the control of a nutrient (vitamins or minerals); but besides these "classical" systems, several compounds have been shown to regulate metabolic activity inside the cells preventing the overproduction of ROS. These molecules are not strictly antioxidants but are able to reduce ROS production. Melatonin^[5], creatine^[6], coenzyme Q10^[7] and carnitine^[8] belong to this family.

An adult body contains around 25 g of L-carnitine^[9]. L-Carnitine found in a human body is either derived from food stuff, especially from meat and dairy products^[10], or derived from an endogenous synthesis. In mammals, L-carnitine is mainly synthesized in the liver, the testis and the kidney, this biosynthesis requires lysine and methionine as ultimate precursors and five enzymatic reactions^[11,12].

Dietary or biosynthetic carnitine is excreted into the blood stream (at a concentration of 50-100 $\mu\text{mol/L}$) and distributed to organs and tissues depending on L-carnitine. Among these, muscle concentrates most of the L-carnitine: In a human body, around 98% of all the L-carnitine is found in skeletal and heart muscles and the ratio between plasma and muscle carnitine concentration is around 1:50^[13]. None of these organs is able to synthesize

L-carnitine and this molecule has to be imported from the blood stream using specific transporters^[14].

The primary role of carnitine is to permit the transport of long chain fatty acids into mitochondria where they can enter the β -oxidation pathway. L-carnitine is also a cofactor for peroxisomal enzymes^[15] and recognized as an inhibitor for HDAC^[16]. L-carnitine has also been used as a protective agent against neurotoxic agents. The precise mechanisms involved are still to be clearly identified and several hypotheses remain. L-carnitine may exert its protective effects as a regulatory element in energy production and/or by interacting with the production of free radicals^[17].

ROS production is associated with several muscle diseases^[18] and it is clear that improving free radical metabolism could be beneficial to cells, animals or patients with muscle disorders^[19]. The goal of this study was to evaluate the beneficial effect that L-carnitine treatment may exert on muscle cells undergoing an oxidative stress.

MATERIALS AND METHODS

Chemicals

Culture media were purchased from Lonza (Levallois-Perret, France). L-Carnitine (carnipure™) was a generous gift from Lonza. Probes used for cytometry analyses were purchased from Molecular probes (Cergy-Pontoise, France). All others chemicals were obtained from Sigma Aldrich (St Quentin Fallavier, France).

Cell culture

Murine myoblastic C2C12 cells were obtained from the European Collection of Cell Cultures (ECACC, Salisbury United Kingdom) and cultured according to their recommendations. Cells were grown in DMEM (Gibco, Cergy-Pontoise, France) with 10% heat-inactivated fetal calf serum (FCS; Gibco), 4 mmol/L L-glutamine (Gibco) at 37 °C in a humidified atmosphere with 5% CO₂. Cells were trypsinized when they reached semi confluence. Dishes were seeded at a density of 2000 cells per square centimetre.

Treatments

Menadione was dissolved in 70% ethanol and added to culture medium (without phenol red) at the desired concentration (from 0 to 12 $\mu\text{mol/L}$); these varying concentrations were used as they may induce from moderate to severe oxidative stress^[20]. Cells were treated for 1 to 24 h for all measured parameters. L-carnitine treated cells were cultured with L-carnitine at the final concentration of 500 $\mu\text{mol/L}$, at all the stages of the experiment. L-carnitine was dissolved in DPBS, filtrated on a 22 μm filter and added to the culture medium. This concentration was chosen as it remains in the physiological range (the extracellular concentration in carnitine is estimated to be in the 50-100 $\mu\text{mol/L}$ range and in the muscle cells this concentration is at least 20 times higher) and as preliminary data showed that this concentration was

efficient for limiting oxidative stress.

Flow cytometric analyses

All experiments were carried out with a Becton Dickinson Facsan equipped with a 488 nm Laser, and fluorescence signals were measured on three channels: FL1 (530/30 nm), FL2 (585/42 nm) and FL3 (670 nm LP). Data were acquired with the Cellquest software (BD Biosciences, Le Pont De Claix, France) and analyzed with the Cyflogic Software (<http://www.cyflogic.com>).

Propidium iodide assay

After treatment with menadione and/or L-carnitine, the culture medium was removed and stored in a 15 mL tube; cells were rinsed with DPBS and trypsinized. The culture medium, the DPBS used for washing and the trypsinized cells were collected and centrifuged at 600 g for 3 min. The supernatant was discarded and the cells resuspended in DPBS. Propidium iodide was added to the cell suspension at a final concentration of 5 µg/mL. After a 5 min incubation, cells were analyzed with a flow cytometer^[21]. FL3 fluorescence was recorded for 10000 cells. Lethal dose 50 was determined with the BioStat 2009 software (AnalystSoft) as described in^[22].

Identification of LC3-II

LC3 has been used as a marker for autophagy. Cells treated with either L-carnitine, menadione (as before) and 3methyladenine (final concentration of 5 mmol/L) were harvested, washed twice in ice-cold PBS and resuspended in a RIPA solution (Tris-HCl, 20 mmol/L pH 8.0; 150 mmol/L NaCl, 2 mmol/L Na₂EDTA; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS, 5 mmol/L sodium fluoride) containing protease inhibitors (0.1 mmol/L phenylmethylsulfonyl fluoride, 5 µg/mL aprotinin, 5 µg/mL pepstatin A and 1 µg/mL chymostatin). The lysate was spun at 13000 g for 20 min at 4 °C. Protein concentration was determined using the Bradford assay. Proteins (20 µg) were separated on a 15% SDS-PAGE and transferred onto a PVDF membrane. The membrane was first incubated with antibodies directed against LC3 (Sigma L8918; 1:2000) and then with secondary antibodies (Santa Cruz, France) coupled with HRP (1:5000). Immunoreactive proteins were visualized on a Biorad Imager and bands size and density were measured using the Image Lab Software. The data were normalized against actin as an internal control.

Tbars production

C2C12 cells (10⁶ cells) were harvested and homogenized in a Tris-HCl buffer (150 mmol/L, pH 7.1) and Tbars production determined as in^[23]. The results were expressed as nmoles of MDA produced per mg of proteins. Protein amount was determined using the Bicinchoninic Acid Protein Assay (Pierce, France) with BSA as a standard.

Flow cytometric measurement of the superoxide production in the mitochondria with the MitoSox Red dye
MitoSoxRed labeling was carried out according as re-

commended by the manufacturer (Molecular Probes). Cells were harvested as above and resuspended in fresh medium. The reaction was carried out for 15 min at 37 °C in the dark with a final concentration of 5 µmol/L of MitoSox Red^[24]. The cells were then centrifuged at 600 × g for 3 min and resuspended in DPBS. Analysis was performed using a Facsan cytometer (BD - Bioscience), using 10000 cells and fluorescence was recorded with the FL3 channel.

Flow cytometric measurement of the mitochondrial transmembrane potential ($\Delta\psi_m$) using the 3,3'-dihexyloxacarbocyanine iodide dye

After treatment, the cells were harvested, the culture medium was removed and kept in a 15 mL tube; the cells were washed with DPBS and trypsinized. The culture medium, washing DPBS and trypsinized cells were pooled and centrifuged at 600 g during 3 min. The cell pellet was resuspended in fresh medium. dihexyloxacarbocyanine iodide [DiOC₆(3)] labeling was carried out according to Molecular Probes recommendations; briefly cells were incubated 20 min at 37 °C with 20 nmol/L of DiOC₆(3), cells were then centrifuged at 600 × g for 3 min then resuspended in DPBS. The analysis was performed using a Facsan cytometer, counting 10000 cells and recording fluorescence with the FL1 channel.

Cellular organization

Cells were grown on Labtek slides and treated as described above. After treatment, cells were labeled with MitoSox Red^[25] and Hoechst 33342 (final concentration of 10 µg/mL). After staining, cells were fixed with 4% formaldehyde in DPBS during 1 h. Cells were mounted in Dakocytomation fluorescence medium (Dako, Copenhagen, Denmark) and after solidification of the mounting medium, cells were observed with a LSM confocal microscope (Zeiss) in plane mode with an EC Plan-Neofluar objective (40 ×/1.30 Oil DIC M27).

Statistical analysis

Results are expressed as the mean ± SD. The statistical significance of differences between treatments was determined with KaleidaGraph (Synergy software) using the ANOVA test with Dunnett Post Hoc test.

RESULTS

Menadione alters mitochondrial membrane potential in C2C12 cells, L-carnitine restores mitochondrial membrane potential

The effect of menadione on mitochondrial integrity was estimated by monitoring potential variations using the potential sensitive fluorochrome DiOC₆(3). In the absence of menadione, no effect of L-carnitine was observed. In the presence of menadione, a significant decrease in the mitochondrial transmembrane potential was observed (Figure 1). This decrease appeared to be dose-dependent: While 77% of the cells were able to accumulate the fluorochrome in the absence of menadione, only 51%

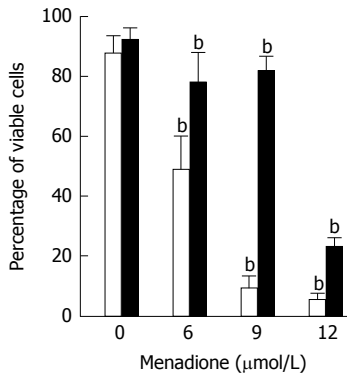


Figure 1 Protection against menadione-induced mitochondrial depolarization with L-carnitine. C2C12 cells were either untreated (white histogram) or pretreated with 500 $\mu\text{mol/L}$ of L-carnitine (dark histogram) and incubated for 24 h with desired concentration of menadione (from 0 to 12 $\mu\text{mol/L}$). Mitochondria integrity was evaluated using DiOC6(3) probe staining; fluorescent cells were counted using a cytometer. Results are presented as the mean value $\pm$ SD. Two different values ($P < 0.005$) are indicated by a star above the two histograms.

were concentrating the Dioc in the presence of 6 $\mu\text{mol/L}$ of menadione and the percentage decreased to respectively 8% and 6% at concentrations of menadione of 9 and 12 $\mu\text{mol/L}$. L-carnitine treatment appeared to prevent menadione-induced loss of mitochondrial transmembrane potential. As shown on Figure 1, the number of cells able to preserve their mitochondrial transmembrane potential was much higher among the L-carnitine treated cells than among untreated cells. At 9 $\mu\text{mol/L}$ of menadione and in the presence of L-carnitine, 70% of the cells exhibited fluorescence. At 12 $\mu\text{mol/L}$ of menadione and in the presence of carnitine, the percentage of mitochondrial integrity was much lower than in control cells, but was still higher than in menadione-only treated cells.

Mitochondrial morphology is altered by menadione

To evaluate intracellular organization of the nucleus and the mitochondria, cells were stained with Hoechst 33342 and MitosoxRed. Cells mounted in fluorescence medium were observed with a LSM confocal microscope in plane mode (Figure 2). Control cells exhibited well identified mitochondria, with a homogeneous repartition in the cytoplasm; the same organization was observed in cells treated with L-carnitine. After treatment with 9 $\mu\text{mol/L}$ of menadione during 24 h, the mitochondrial network appeared damaged with most of the mitochondria located around the nucleus. Such modifications were not observed on cells simultaneously treated with 500 $\mu\text{mol/L}$ L-carnitine and with 9 $\mu\text{mol/L}$ menadione for 24 h. In these conditions, mitochondria and cell structure were similar to those of control cells.

L-carnitine prevents menadione-induced free radical generation in the mitochondria

The global ROS production was evaluated by the measure of thiobarbituric reactive species. Tbars production in C2C12 cells was determined at intervals from 0 to 24 h

after treatment with four different concentrations of menadione (0 $\mu\text{mol/L}$: Figure 3A; 6 $\mu\text{mol/L}$: Figure 3B; 9 $\mu\text{mol/L}$: Figure 3C and 12 $\mu\text{mol/L}$: Figure 3D). In the absence of menadione, no effect on Tbars production was observed and L-carnitine supplementation remained without effect. In the presence of 6 $\mu\text{mol/L}$ of menadione, Tbars production increased after 6 h of treatment, and was found to be maximal after 8 h of treatment. L-carnitine supplementation fully inhibited this increase and no differences were found among L-carnitine treated cells. With a treatment of 9 $\mu\text{mol/L}$ of menadione, Tbars production was increased earlier than before and a significant difference was observed after 2 h of treatment. The effect of menadione was maximal after 4 h of treatment. Again, L-carnitine addition fully abolished the effect of menadione and in the presence of L-carnitine, no increase in Tbars production was observed. In the presence of 12 $\mu\text{mol/L}$ of menadione, the increase in Tbars production was rapid and appeared to be maximal after 2 h of treatment. L-carnitine supplementation was able to prevent this increase, even if one can observe a slight increase after 24 h of treatment (Figure 3A).

The mitochondrial generation of ROS was evaluated by analyzing C2C12 cells stained with MitosoxRed by flow cytometry. Carnitine treatment did not alter the basal production of mitochondrial ROS. Menadione treatment was found to induce an increase in the production of ROS in the mitochondria in a dose dependent manner. At a concentration of 6 $\mu\text{mol/L}$ of menadione, a significant increase in the number of cells producing ROS was observed (Figure 3B) and when the concentration of menadione reached 9 $\mu\text{mol/L}$, more than 90% of the cells produced ROS. L-carnitine treatment was able to decrease the mitochondrial production of ROS for concentrations of menadione less than 12 $\mu\text{mol/L}$.

L-carnitine limits the autophagy process induced by menadione in C2C12 cells

Microtubule-associated protein light chain 3 (LC3) is considered as one of the more accurate markers for autophagy^[26]. During the initiation of autophagy, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine leading to the formation of LC3-II^[27]. Due to its hydrophobic property, the PE group increases the migration of the LC3 protein in a SDS-PAGE gel. We examined the changes in endogenous LC3 after menadione and carnitine treatments in C2C12 cells.

Menadione significantly increased the level of LC3-II proteins in a dose-dependent manner (Figure 4). L-carnitine treatment did not modify autophagy in control cells (*i.e.*, untreated with menadione). At the concentrations of 6 and 9 $\mu\text{mol/L}$ of menadione, adding carnitine decreases the level of LC3-II significantly suggesting a protective effect of carnitine on autophagy. At the concentration of 12 $\mu\text{mol/L}$ of menadione, L-carnitine supplementation remained ineffective in reducing autophagy. At this concentration, the level of LC3-II was found to be the same between L-carnitine treated and untreated cells.

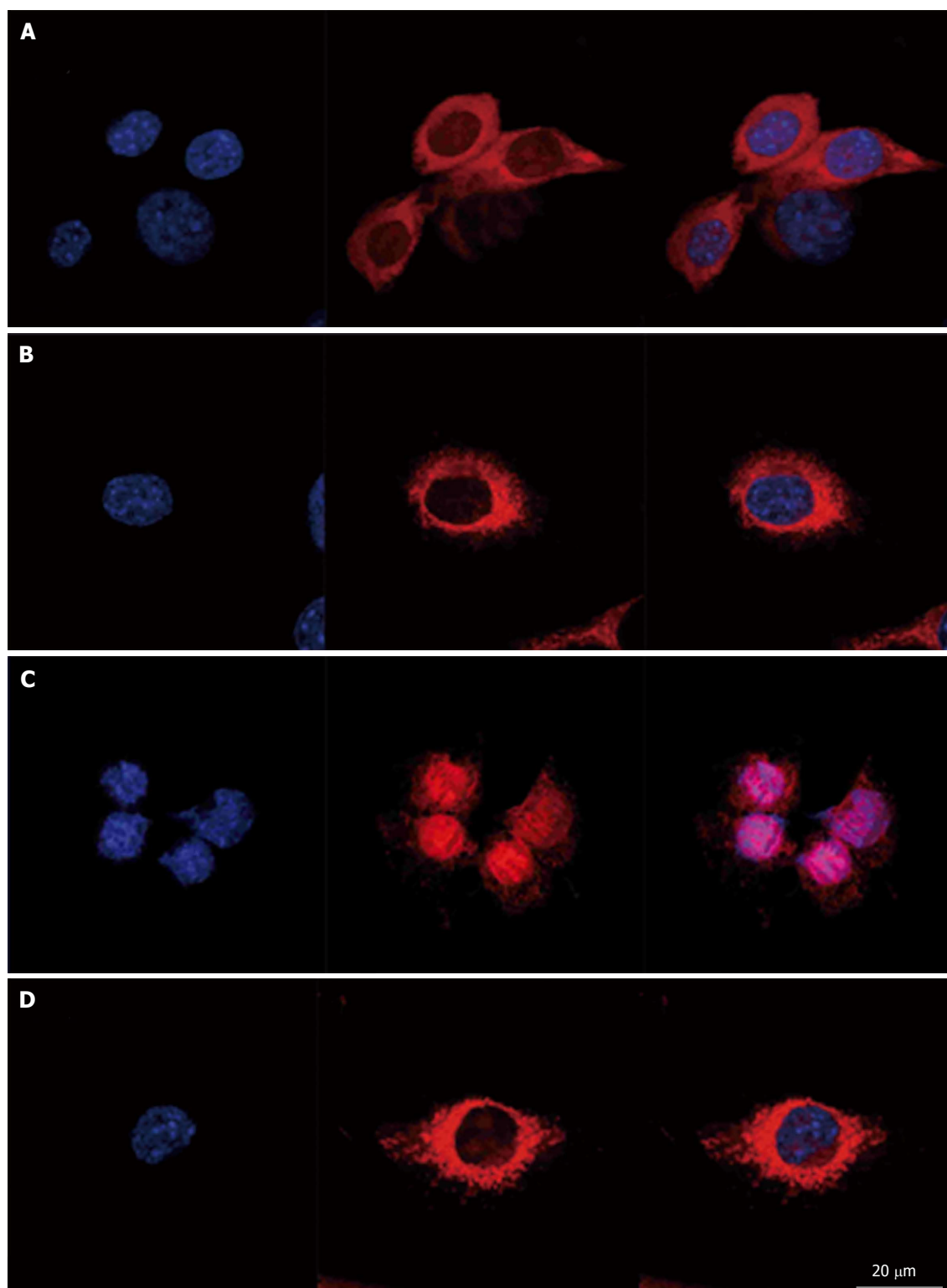


Figure 2 Prevention of menadione-induced mitochondrial distribution with L-carnitine. C2C12 cells were either untreated or pretreated with 500 $\mu\text{mol/L}$ of L-carnitine and incubated for 24 h with menadione (0 and 9 $\mu\text{mol/L}$). Nucleus and mitochondria morphology was evaluated after staining with Hoechst 33342 and MitoSoxRed, respectively. From left to right, staining with nuclei, mitochondria and both. Cells mounted in fluorescence medium were observed with a LSM confocal microscope. A: C2C12 untreated with menadione and untreated with L-carnitine; B: C2C12 pretreated with L-carnitine and untreated with menadione; C: C2C12 untreated with L-carnitine and treated with 9 $\mu\text{mol/L}$ of menadione; D: C2C12 pretreated with L-carnitine and treated with 9 $\mu\text{mol/L}$ of menadione.

Menadione induces cell death in C2C12 cells, L-carnitine has a protective effect

Cell death was evaluated by flow cytometry by measuring membrane permeability to propidium iodide (PI)

(Figure 5). Treatment with menadione induced a significant decrease in cell survival. At a concentration of 6 $\mu\text{mol/L}$ of menadione, only 51% of the cells remain unstained by PI. The cell survival decreased as the menadione con-

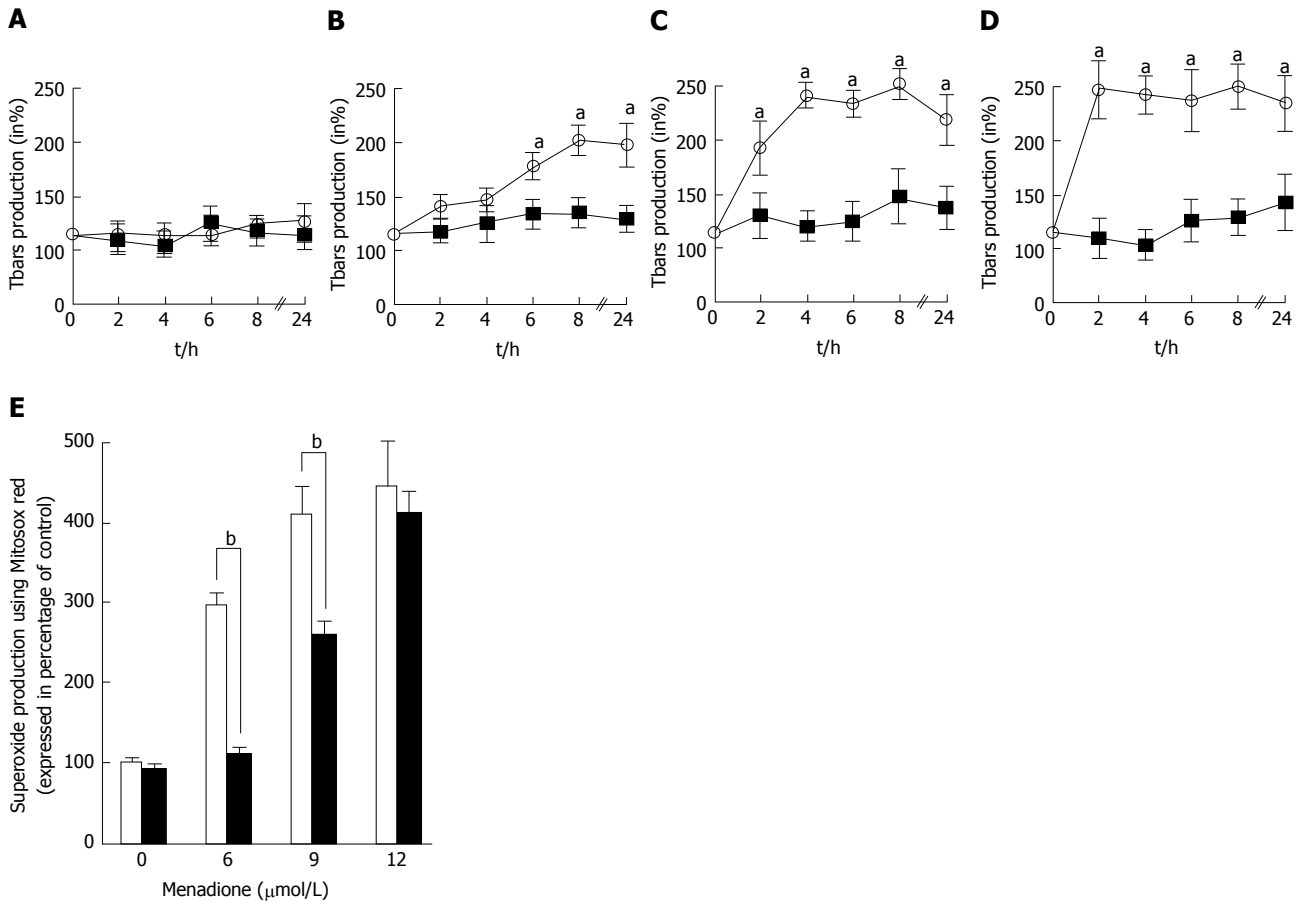


Figure 3 Characterization of reactive oxygen species production. A-D: Tbars production was determined in C2C12 cells in the presence of various amounts of menadione from 1 to 24 h. Results were expressed in percentage of the control cell Tbars production. Tbars production was analyzed in the presence of 0 (A), 6 (B), 9 (C) and 12 $\mu\text{mol/L}$ (D) of menadione in control cells (empty circles and dashed line) and in cells pre-treated with 500 $\mu\text{mol/L}$ of carnitine (black squares and full line). An asterisk on top of a symbol indicates a significant difference ($P < 0.05$); E: Superoxide anion production at the mitochondrial level on menadione-treated C2C12 cells with MitoSoxRed. C2C12 cells were either untreated (white histogram) or pretreated with 500 $\mu\text{mol/L}$ of L-carnitine (black histogram) and incubated for 24 h with desired concentration of menadione (from 0 to 12 $\mu\text{mol/L}$). MitoSoxRed (MSR) is a cell permeable dye that is targeted to the mitochondria, it is oxidized by superoxide and exhibit red fluorescence. Superoxide production was measured by cytometry after staining with MSR. Results are presented as the mean value $\pm$ SD. A star on the top of two histograms indicates statistical difference ($P < 0.005$) between these two values (Untreated vs L-carnitine treated cells).

centration increased.

Noteworthy, cells treated with L-carnitine were less sensitive to menadione. Whatever the concentration of menadione was, the number of viable cells was always higher with L-carnitine treatment. At a concentration of 9 $\mu\text{mol/L}$ of menadione, only 5% of C2C12 cells cultured in the absence of L-carnitine remained unstained by PI and in the presence of L-carnitine, 79% were unstained. In the presence of 12 $\mu\text{mol/L}$ of menadione, 23% of cells treated with L-carnitine were viable and this percentage was only of 5 % in cells untreated with L-carnitine.

DISCUSSION

Due to the high rate of oxidative processes and the abundance of myoglobin, muscle cells are very susceptible to ROS damage which are responsible of various types of damages that can lead to cell death or to cell ineffectiveness^[28]. It has been shown that ROS are implicated in the normal aging process but also in pathological processes. Muscle diseases like Duchenne

muscular dystrophy make muscle cells more susceptible to ROS damages^[29,30], leading to premature death or malfunction. Menadione (2-methyl-1, 4-naphthoquinone, vitamin K₃) is a quinone-containing compound. Reductive enzymes such as microsomal NADPH-cytochrome P450 reductase and mitochondrial NADH-ubiquinone oxidoreductase are able to metabolize menadione with the subsequent formation of unstable semiquinones. These molecules can enter into a redox cycle with molecular oxygen leading to the formation of quinone and the generation of reactive oxygen species, including H_2O_2 , $\text{O}_2^{\bullet-}$ and, in the presence of metal ions, to $\text{OH}^{\bullet}$ ^[31]. Menadione is widely used to study the oxidative stress on various types of cells^[29,31,32].

In this study, we showed that menadione induced an oxidative stress in C2C12 cells leading to cell death. The lethal dose 50 was calculated and found to be 6 $\mu\text{mol/L}$. In addition, menadione induced a significant increase in ROS generation in the mitochondria; at a dose of 6 $\mu\text{mol/L}$ of menadione, more than 67% of the cells were over producing ROS. Menadione was also able to

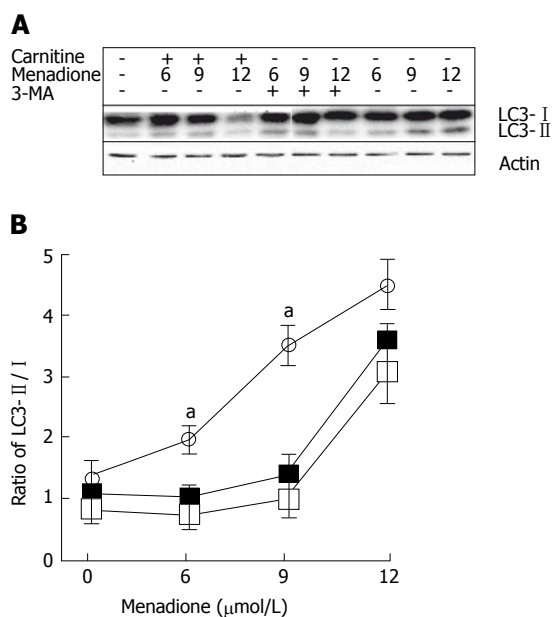


Figure 4 Menadione induces autophagy in C2C12 cells. A: Western blot for the determination of LC3 I and II forms. Initial cells were either treated with L-carnitine, menadione or 3-MA; B: LC3- I and II isoforms were detected by western blotting and quantified. The ratio between I and II forms was calculated and plotted in this figure. The black squares represent L-carnitine treated cells whereas empty circles represent C2C12 cells that were not pretreated with L-carnitine. Empty squares represent cells treated with 5 mmol/L of 3-MA. Each point is the mean of 4 independent experiments ($\pm$ standard values). For each concentration of menadione, a star indicates a significant difference between untreated and L-carnitine treated cells ($P < 0.05$).

induce autophagy in C2C12 cells. The oxidative stress is also associated with a rearrangement in mitochondrial distribution inside the muscle cell. This aspect is also encountered in several diseases associated with a loss of myofibrillar organization (e.g., cells lacking desmin^[33] or plectin^[34]). It is very likely that the change in mitochondrial distribution impacts cell function.

The data presented in this paper are partly in agreement with those of Chiou *et al.*^[32] who described that menadione (used on C2C12 in a culture medium without FCS) was able to induce massive cell death. Privation of serum for C2C12 cells is known to initiate differentiation and deeply changes the phenotype of the cells^[35]. In our experimental conditions, L-carnitine was added before the induction of the oxidative stress by menadione and appeared to protect C2C12 cells against ROS damages. For any concentration of menadione, L-carnitine treatment protected cells against menadione toxicity. This phenomenon is observed at all tested menadione concentrations and while the LD50 of untreated C2C12 was 6 μmol/L, in the presence of L-carnitine, the LD50 was calculated at 11 μmol/L. In C2C12 cells, L-carnitine supplementation was able to diminish cell death but also to reduce ROS production and to protect against mitochondrial transmembrane depolarization.

Currently, it is known that L-carnitine is a cofactor in the channeling of fatty acids inside the cell. It is involved in fatty acid oxidation by playing a role of cofactor in

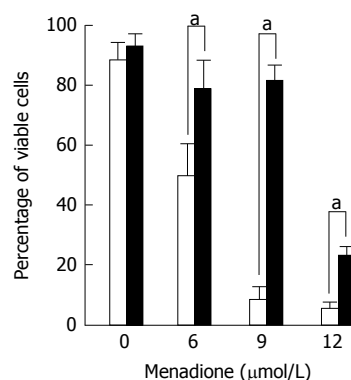


Figure 5 Protection of menadione-induced C2C12 cell death by L-carnitine. C2C12 cells were either untreated (white histogram) or pretreated with 500 μmol/L of L-carnitine (dark histogram) and incubated for 24 h with desired concentration of menadione (from 0 to 12 μmol/L). Cell survival was evaluated by the analysis of membrane permeability to PI measured by flow cytometry. Each value represents the average percentage of viable cells remaining in the total population $\pm$ standard values. Black histograms indicate the proportion (%) of C2C12 that was not PI positive. White histograms indicate the proportion (%) of C2C12 pretreated with L-carnitine that was not PI positive. A star on top of two histograms indicates a significant difference between untreated and L-carnitine treated cells ($P < 0.05$).

the transport of acyl groups across the mitochondrial membrane^[36]. Beside its role in fatty acid oxidation, L-carnitine, added to the food, has been shown to counteract some effects associated with aging on mitochondria and on muscle^[37-39]. Moreover, L-carnitine has been shown to prevent oxidative stress, to regulate nitric oxide production and to control the activity of enzymes involved in the defense against oxidative damage^[40] and mitochondrial dysfunction^[41]. Among the enzymes whose activity is protected by L-carnitine are found the catalase and the superoxide dismutase^[42], two of three major enzymes involved in ROS detoxification. In this study we were able to prove that L-carnitine may act on the intracellular ROS status either by decreasing mitochondrial ROS production, either by increasing defense against these reactive molecules produced in mitochondria, or either by scavenging ROS at the mitochondrial level.

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COMMENTS

Background

By controlling metabolism, L-carnitine seems to be able to limit reactive oxygen species (ROS) production under certain circumstances. In this paper, the protective effect of L-carnitine was assessed on muscle cells. L-carnitine appeared to limit ROS production in muscle cells and protecting them against oxidative stress.

Research frontiers

Muscle is a tissue with a high concentration of mitochondria and likely to produce high level of ROS. ROS may then induce oxidative stress and damage to muscle cells. Protecting muscle cells against oxidative stress was the authors' objective.

Applications

Limiting oxidative stress may find many applications, especially in limiting damage to cells, organs and tissues.

Terminology

Oxidative stress theory: In this theory, highly reactive molecules, related to oxygen, interact and finally alter some vital molecules, leading to disease and sometimes to death. **Carnitine:** A small molecule that is involved in the metabolism of fat. **Autophagy:** Autophagy is a cellular mechanism consisting in the partial degradation of the cell cytoplasm by its own lysosomes. Depending on the circumstances, autophagy may lead to a large spectrum of effects: From cell repair to cell death.

Peer-review

The manuscript is interesting. The main objective study was to evaluate the beneficial effect that L-carnitine treatment may exert on muscle cells undergoing an oxidative stress.

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Case Control Study

Device-associated infection rates, mortality, length of stay and bacterial resistance in intensive care units in Ecuador: International Nosocomial Infection Control Consortium's findings

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Author contributions: All authors were involved in study conception and design, drafting of the manuscript, provision of study patients, collection of data, critical revision of the manuscript for important intellectual content, and final approval of the manuscript; Rosenthal VD was responsible for software development, data assembly, analysis, and interpretation, epidemiologic analysis, statistical analysis and technical support.

Institutional review board statement: Every hospital's Institutional Review Board agreed to the study protocol, and patient confidentiality was protected by codifying the recorded information, making it only identifiable to the infection control team.

Informed consent statement: All involved persons (subjects or legally authorized representative) gave their informed consent prior to study inclusion.

Conflict-of-interest statement: All authors report no conflicts of interest related to this article.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at victor_rosenthal@inicc.org.

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Abstract**AIM**

To report the results of the International Nosocomial Infection Control Consortium (INICC) study conducted in Quito, Ecuador.

METHODS

A device-associated healthcare-acquired infection (DA-

HAI) prospective surveillance study conducted from October 2013 to January 2015 in 2 adult intensive care units (ICUs) from 2 hospitals using the United States Centers for Disease Control/National Healthcare Safety Network (CDC/NHSN) definitions and INICC methods.

RESULTS

We followed 776 ICU patients for 4818 bed-days. The central line-associated bloodstream infection (CLABSI) rate was 6.5 per 1000 central line (CL)-days, the ventilator-associated pneumonia (VAP) rate was 44.3 per 1000 mechanical ventilator (MV)-days, and the catheter-associated urinary tract infection (CAUTI) rate was 5.7 per 1000 urinary catheter (UC)-days. CLABSI and CAUTI rates in our ICUs were similar to INICC rates [4.9 (CLABSI) and 5.3 (CAUTI)] and higher than NHSN rates [0.8 (CLABSI) and 1.3 (CAUTI)] - although device use ratios for CL and UC were higher than INICC and CDC/NHSN's ratios. By contrast, despite the VAP rate was higher than INICC (16.5) and NHSN's rates (1.1), MV DUR was lower in our ICUs. Resistance of *A. baumannii* to imipenem and meropenem was 75.0%, and of *Pseudomonas aeruginosa* to ciprofloxacin and piperacillin-tazobactam was higher than 72.7%, all them higher than CDC/NHSN rates. Excess length of stay was 7.4 d for patients with CLABSI, 4.8 for patients with VAP and 9.2 for patients CAUTI. Excess crude mortality in ICUs was 30.9% for CLABSI, 14.5% for VAP and 17.6% for CAUTI.

CONCLUSION

DA-HAI rates in our ICUs from Ecuador are higher than United States CDC/NHSN rates and similar to INICC international rates.

Key words: Ventilator-associated pneumonia; Catheter-associated urinary tract infection; Healthcare-associated infection; Antibiotic resistance; Developing countries; Intensive care unit; Surveillance; Central line-associated bloodstream infections; Hospital infection

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Core tip: This is a prospective, cohort, surveillance study on device-associated infection rates, mortality, length of stay and bacterial resistance conducted in intensive care units (ICUs) in Ecuador from October 2013 to January 2015. Device-associated healthcare-acquired infection rates in our ICUs from Ecuador are significantly higher than United States Centers for Disease Control and Prevention's National Healthcare Safety Network's rates and similar to International Nosocomial Infection Control Consortium's international rates.

Salgado Yopez E, Bovera MM, Rosenthal VD, González Flores HA, Pazmiño L, Valencia F, Alquinga N, Ramirez V, Jara E, Lascano M, Delgado V, Cevallos C, Santacruz G, Pelaéz C, Zaruma C, Barahona Pinto D. Device-associated infection rates, mortality, length of stay and bacterial resistance in intensive care

units in Ecuador: International Nosocomial Infection Control Consortium's findings. *World J Biol Chem* 2017; 8(1): 95-101 Available from: URL: <http://www.wjgnet.com/1949-8454/full/v8/i1/95.htm> DOI: <http://dx.doi.org/10.4331/wjbc.v8.i1.95>

INTRODUCTION

Device-associated healthcare-acquired infections (DA-HAIs) are one of the main threats to the safety of patients, causing patient morbidity, mortality, excess costs and prolonged length of hospital stay (LOS), particularly in intensive care settings of limited-resource countries^[1-3].

Multifaceted infection prevention programs integrating target DA-HAI surveillance methods were proved effective in several United States studies, which showed the occurrence of DA-HAI could be reduced by more than 30%, along with an analogous reduction in DA-HAI-related hospital costs^[4].

Antimicrobial-resistant infections is another primary issue that needs to be addressed in infection control programs in order to prevent the spread of resistant strains through the report of DA-HAI-associated pathogens and their susceptibility to antibiotics^[5].

During the last four decades, the United States Centers for Disease Control and Prevention (CDC)'s National Healthcare Safety Network (NHSN)^[6] has developed benchmarking data on DA-HAIs in intensive care unit (ICU) patients, which has afforded the International Nosocomial Infection Control Consortium (INICC) an essential insight^[5].

The INICC is an open, international, non-profit, collaborative healthcare-associated infection (HAI) control network comprised of 2000 hospitals in 500 different cities in 66 countries in Africa, Asia, Eastern Europe, Latin America and the Middle East transcontinental region^[5,7]. Since it was established in 1998 in Argentina, it has been the first multi centre surveillance and research network centered on the reduction of the rates of DA-HAI in the ICU and of hospital-wide surgical site infections through tools and resources provided for free to healthcare centers to assist them in with the prevention and control this public health burden through the implementation of infection prevention programs^[6-9]. The INICC network operates by means of an online surveillance system - the INICC Surveillance Online System (ISOS) - and a systematic multidimensional approach - the INICC Multidimensional Approach (IMA) - whose effectiveness for the decrease of DA-HAI rates was shown in the scientific literature^[8-23]. The ISOS applies the definitions of HAIs developed by the CDC/NHSN and standardized methodologies, thereby promoting applied research and evidence-based infection prevention practices.

This is the first study to report an analysis of data on DA-HAI rates from Ecuador using ISOS between October 2013 and January 2015 in 2 ICUs from 2 hospitals of the INICC network^[5].

MATERIALS AND METHODS

Background on INICC

The INICC is focused on the surveillance and prevention of HAI in adult, pediatric ICUs and neonatal ICUs, step down units, inpatient wards, and of surgical site infections in surgical procedures hospital wide^[5]. Through the ISOS, INICC provides free training and surveillance tools to hospitals worldwide, which allows them to measure HAI consequences, and to evaluate the impact of infection control and prevention practices^[24].

Study design and setting

This is a prospective surveillance, cohort study made on all the patients admitted, between October 2013 and January 2015, to 2 adult medical/surgical ICUs from 2 medium-sized hospitals (1 private and 1 public hospital) in Quito, Ecuador, through the implementation of the IMA. The IMA uses 6 components for HAI control to be applied simultaneously, as follows: (1) bundles of infection control interventions; (2) educational and training sessions; (3) outcome surveillance on HAI-related rates; (4) process surveillance; (5) feedback on HAI rates and their related adverse effects; and (6) feedback on health care workers' performance^[5].

In accordance with the INICC protocol, hospitals' identities are kept under confidentiality and patient data was anonymized. Due to the fact that this was an epidemiological surveillance study, which did not include tests of experimental drugs, biomedical devices or products, and that patient data were anonymized, an informed consent was not necessary according to the ethics committees that evaluated and approved the study.

ISOS

The ISOS applied CDC/NSHN's methods and definitions published in January 2015^[25], and also included INICC methodology. The methods developed by the CDC/NSHN to determine HAI rates indicate that the numerators are the total number of each type of HAIs, and the denominators are the device days recorded from all ICU patients, in the form of pooled data; that is, the number of device days and the characteristics of a particular patient are not calculated^[25]. By contrast, the INICC methodology, through the implementation of the ISOS, included the collection of data per specific patient, from all ICU patients (with and without HAI), including surrogates of HAI (low blood pressure, high temperature, antibiotic therapy, results of cultures, LOS and mortality), and data on risk factors, including invasive devices. To have data on all ICU patients enabled a match of patients with and without HAI by various patient features necessary to calculate excess mortality, LOS and HAI-related hospital cost^[5].

Analysis and collection of data

Infection control professionals (ICPs) uploaded their daily-collected data on DA-HAIs using ISOS. Data on

central line-associated bloodstream infection (CLABSI), ventilator-associated pneumonia (VAP), and catheter-associated urinary tract infection (CAUTI), denominator data, patient-days and specific device-days in the ICUs were used to determine the rates of each DA-HAI per 1000 device days, LOS and mortality, as follows: (1) device days equaled the number of central line (CL)-days, mechanical ventilator (MV)-days and urinary catheter (UC)-days; (2) DA-HAI crude excess mortality equaled crude mortality of patients with DA-HAI hospitalized in the ICU minus crude mortality of ICU patients who had not acquired a DA-HAI; (3) DA-HAI crude excess LOS equaled crude LOS of patients with DA-HAI hospitalized in the ICU minus crude LOS of patients ICU patients who had not acquired a DA-HAI; and (4) Device utilization ratio (DUR) equaled the number of device days divided by the number of bed days^[5].

Training

ICPs were trained at hospitals by the INICC team. Instruction manuals, training tools and tutorial movies describing in detail how to conduct surveillance and upload data to ISOS were also provided to ICPs. Finally, ICPs received technical and methodological support from the INICC team *via* email, telephone calls and webinars.

Definitions

The ISOS applied the definitions and criteria published in 2015 by CDC's NHSN for HAI surveillance^[25].

Statistical analysis

Data analysis and the calculation of rates of DA-HAI, LOS, mortality, device utilization were done using ISOS version 2.0 (City of Buenos Aires, Argentina). Relative risk ratios, *P*-values and 95% confidence intervals were calculated using SPSS 16.0 (SPSS Inc. an IBM company, Chicago, Illinois, United States) and EpiInfo® version 6.04b (CDC, Atlanta, GA, United States). The statistical review of the study was done by a biomedical statistician.

RESULTS

From October 1st 2013 to January 30th 2015, 776 patients were admitted to the 2 participating medical/surgical ICUs, for a total of 4818 bed days. During the study period, the mean length of participation of each ICU was as follows: + SD 14.5 + 2.1 mo, range from 13 to 16 mo.

Table 1 provides data on crude excess LOS and mortality in all patients (with and without DA-HAI) admitted to the ICUs over the period of study. CLABSI was associated with the highest pooled excess mortality in the ICUs. The excess LOS of patients with CAUTI was the greatest among the analyzed DA-HAIs.

Table 2 shows the DA-HAI rates and related results of this report from Ecuador benchmarked against the INICC report of data from 43 countries for the period 2007-2012, against the United States CDC/NHSN report of 2013 and against the United States NHSN report of 2009-2010^[6,26,27].

Table 1 Pooled means of the distribution of crude mortality, crude excess mortality, length of stay, and crude excess length of stay, of adult intensive care unit patients with and without device-associated healthcare-acquired infection

Patients	Patients, <i>n</i>	Deaths, <i>n</i>	Pooled crude mortality, %	Pooled crude excess mortality, % (95%CI)	LOS, total days	Pooled average, LOS (d)	Pooled average, excess LOS (d) (95%CI)
Without DA-HAI	678	107	15.80	-	3579	5.3	
With CLABSI	15	7	46.70	30.9 (8.1-54.7)	190	12.7	7.4 (5.8-9.2)
With CAUTI	12	4	33.30	17.6 (-3.2-46.4)	174	14.5	9.2 (7.3-11.4)
With VAP	43	13	30.20	14.5 (4.1-27.4)	434	10.1	4.8 (4.1-5.7)

DA-HAI: Device-associated healthcare-acquired infection; CLABSI: Central line-associated bloodstream infection; VAP: Ventilator-associated pneumonia; CAUTI: Catheter-associated urinary tract infection; LOS: Length of stay.

DA-HAI rates pooled means were as follows: 6.5 (*n*, 39) CLABSIs per 1000 CL-days, with a DUR of 1.24 for 5998 CL-days; 44.3 (*n*, 69) VAPs per 1000 MV-days, with a DUR of 0.32 for 1559 MV-days, and 5.7 (*n*, 21) CAUTIs per 1000 UC-days, with a DUR of 0.77 for 3699 UC-days.

Overall, our CLABSI and CAUTI rates were similar to the INICC report data, but our VAP rate was substantially higher than INICC's. On the other hand, the incidence rates of DA-HAI were higher compared with United States NHSN report data. Our DURs for CL and UC were higher compared both to United States NHSN's and INICC's; however, our DUR for MV was lower than INICC's. Most of the resistance rates found in our ICUs were significantly higher than those found in the US ICUs as reported by the CDC's NHSN.

DISCUSSION

DA-HAIs in Ecuador have not been systematically analyzed in the scientific literature to date. The incidence of DA-HAIs in this study is significantly higher than other recent analogous studies carried out in Latin America. In Colombia, it was recently shown that DA-HAI rates per 1000 device days were higher than ours: The CLABSI rate was 47.4, the VAP rate was 32.3, and the CAUTI rate was 20.3^[28]. By contrast, pooled crude mortality was higher in our study than in a study conducted in Colombia, whose findings showed the crude unadjusted mortality attributable to DA-HAI was 18.5% for patients with CLABSI (95%CI: 1.42-2.87); 16.9% for patients with VAP (95%CI: 1.24-3.00); and 10.5% for patients with CAUTI (95%CI: 0.78-3.18)^[28]. In Peru, Cuellar *et al.*^[29] found a CAUTI rate of 5.1 per 1000 UC days, a VAP rate of 31.3 per 1000 MV days and a CLABSI rate of 7.7 per 1000 CL-days. In a comparable study conducted in Brazil, Salomao *et al.*^[30] found a rate of 20.9 VAPs per 1000 MV days, a CAUTI rate of 9.6 per 1000 UC days and a CLABSI rate of 9.1 per 1000 CL days.

The statistically significantly higher rates of DA-HAI rates and DURs found in the analyzed ICUs of Ecuador compared with the rates reported by the US CDC's NHSN represent the current burden of HAIs in high-income countries^[6]. On the other hand, CLAB and CAUTI rates found in the international INICC Report (2007-2012) for 43 countries^[26], which would represent middle and low-income economies, were similar to our rates, although

our pooled DURs were higher for CL and UC^[6,26]. By contrast, although our VAP rate was remarkably higher than INICC's, our DUR for MV was lower, which means there are other risk factors different from DURs influencing DA-HAI rates. Regarding antimicrobial resistance, the resistance percentages found in this study were also higher than those found in United States CDC's NHSN^[27] and INICC^[26] reports' for *Pseudomonas aeruginosa* as resistant to piperacillin-tazobactam, ciprofloxacin, amikacin, and imipenem or meropenem, as well as the resistance percentages determined for *Acinetobacter baumannii* to imipenem or meropenem.

Different factors can elucidate the possible reasons for these higher DA-HAI rates compared with the United States CDC's NHSN and INICC's reports. As also occurs in other developing countries, we consider that adherence to infection control bundles in Ecuador is variable, there is frequently a low nurse-patient staffing ratio (with a nurse-patient ratio higher than 4:1) and the number of experienced nurses or trained healthcare workers is deficient - which has been demonstrated as significantly associated with considerably high DA-HAI incidence rates in the ICU patient^[31]. In addition, there is hospital over-crowding. According to World Health Organization standards^[32], there should be between 8 and 10 hospital beds available per 1000 persons, but in 2011, in Ecuador, there were only 1.5 per 1000, with many hospitals remaining at full capacity^[33].

The risk of infection of patients hospitalized in ICUs can be reduced though the implementation of surveillance targeted on DA-HAI, because it is successful to focus on characteristics of the burden of DA-HAIs. These surveillance data is necessary to increase ICPS's sensitivity and aids them to detecting HAIs and avoiding underreporting^[5].

In addition, surveillance should be complemented with the performance of other practices for DA-HAI control and prevention^[34,35]. Therefore, INICC has played a crucial by facilitating free infection prevention tools and resources through the use of ISOS, as well as by fostering increasing awareness about the risks posed by DA-HAIs amongst health care professionals^[5,36].

Limitations

The difference in time periods for the diverse data sources was not considered for the benchmarking of our findings against the United States CDC's NHSN and

Table 2 Benchmarking of device-associated healthcare-acquired infection rates, device utilization and antimicrobial resistance in this report against the report of the International Nosocomial Infection Control Consortium (2007-2012) and the reports of the United States Centers for Disease Control and Prevention's National Healthcare Safety Network data (2013 and 2009-2010)

	This report 95%CI	INICC report (2007-2012) ^[26] 95%CI	United States CDC/NHSN report (2013) ^[6] /2009-2010 ^[27]
Medical/surgical ICU			
Central line, DUR	1.24 (1.21-1.27)	0.54 (0.54-0.54)	0.37
CLABSI rate	6.5 (4.6-8.9)	4.9 (4.8-5.1)	0.8
Mechanical ventilator, DUR	0.32 (0.31-0.34)	0.36 (0.36-0.36)	0.24
VAP rate	44.3 (34.4-56.0)	16.5 (16.1-16.8)	1.1
Urinary catheter, DUR	0.77 (0.74-0.79)	0.62 (0.62-0.62)	0.54
CAUTI rate	5.7 (3.5-8.7)	5.3 (5.2-5.8)	1.3
Antimicrobial resistance % (n)			
Pathogen, antimicrobial	CLABSI (n)	CLABSI	CLABSI
<i>Staphylococcus aureus</i>			
Oxacillin	60% (5)	61.20%	54.60%
<i>Pseudomonas aeruginosa</i>			
Ciprofloxacin	71.4% (7)	37.50%	30.50%
Piperacillin or piperacillin-tazobactam	100% (5)	33.50%	17.40%
Amikacin	71.4% (7)	42.80%	10.00%
Imipenem or meropenem	71.4% (7)	42.40%	26.10%
<i>Klebsiella pneumoniae</i>			
Ceftriaxone or ceftazidime	60% (5)	71.20%	28.80%
Imipenem or meropenem	20% (5)	19.60%	12.80%
<i>Acinetobacter baumannii</i>			
Imipenem or meropenem	100% (2)	66.30%	62.60%

ICU: Intensive care unit; CLABSI: Central line-associated bloodstream infection; VAP: Ventilator-associated pneumonia; CAUTI: Catheter-associated urinary tract infection; DUR: Device use ratio; CI: Confidence interval; INICC: International Nosocomial Infection Control Consortium; United States CDC/NHSN: Centers for Disease Control and Prevention's National Healthcare Safety Network of the United States.

INICC reports. Due to the low economic resources of our ICUs, very few cultures were taken, which could have influenced the rates of CLABSI and CAUTI, as they could not be documented because they did not fulfill all the United States CDC/NHSN criteria. In addition, the number of patients to whom blood and/or urine cultures should have been taken, but were actually not due to lack of economic resources, is unknown as this data was not registered.

Conclusions

The findings of this study highlight that DA-HAIs pose major challenges for public health and the wellbeing of patients in Ecuador. One of INICC's primary goals is to provide health care facilities worldwide with free tools and resources to support the introduction of systematic infection prevention practices in order to address this burden effectively by accomplishing a reduction in DA-HAI rates and their adverse effects.

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COMMENTS

Background

The International Nosocomial Infection Control Consortium (INICC) program is focused on surveillance of device-associated healthcare-acquired infections (DA-HAIs) in the intensive care units (ICUs), step down units and general wards, and surveillance of SSIs hospital wide. This particular study was focused on ICUs, because they are the healthcare settings that represent the highest HAI rates, due to patients' critical condition and exposure to invasive devices. Through the last 12 years, INICC has undertaken a global effort in Africa, Eastern Mediterranean, Europe, Latin America, South East Asia and Western Pacific to prevent and control DA-HAIs, and has achieved extremely successful results, by increasing hand hygiene compliance, improving compliance with other infection control bundles and interventions as described in several INICC publications, and consequently reducing the rates of DA-HAI and mortality. To compare a hospital's DA-HAI rates with the rates identified in this report, it is required that the hospital team concerned collect their data by applying the methods and methodology described for United States NHSN and INICC, and then calculate infection rates and DU ratios for the DA-HAI Module.

Applications

The particular and primary application of these data is to serve as a guide for the implementation of prevention strategies and other quality improvement efforts in Ecuador for the reduction of DA-HAI rates to the minimum possible level.

Peer-review

This is a nice prospective multi-center trial showing similar nosocomial infection

rates in Ecuadorian hospitals as compared with international hospitals.

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Common therapeutic target for both cancer and obesity

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Abstract

Obesity and cancer are two interrelated conditions of high epidemiological need, with studies showing that obesity is responsible for nearly 25% of the relative contribution to cancer incidence. Given the connection between these conditions, a drug that can operate on both obesity and cancer is highly desirable. Such a drug is accomplishable

through the development of potent anti-angiogenesis agents due to the shared underlying role of angiogenesis in the development of both diseases. Prior research has demonstrated a key role of type-2 methionine aminopeptidase (MetAP2) for angiogenesis, which has led to the development of numerous of novel inhibitors. Several irreversible MetAP2 inhibitors have entered clinical trials without great success. Though this lack of success could be attributed to off-target adverse effects, the underlying causes remain unclear. More promising reversible inhibitors have been recently developed with excellent pre-clinical results. However, due to insufficient knowledge of the biological functions of N-terminal protein processing, it is hard to predict whether these novel inhibitors would successfully pass clinical trials and thereby benefit cancer and obesity patients. Significantly more efforts are needed to advance our understanding of the regulation of methionine aminopeptidases and the processes by which they govern the function of proteins.

Key words: Methionine aminopeptidase; Angiogenesis; Cancer; Obesity; Diabetes; Protein processing; Protein stability; Protein maturation; Protein modification

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Core tip: There were approximately 14 million new cancer cases worldwide each year and more than half of the population in the developed countries are overweight or obese. Obesity is responsible for nearly 25% of the relative contribution to cancer incidence, which ranks second only to tobacco use. It would be, therefore, highly desirable to have drugs that work for both cancer and obesity. In this article, the biological function of a common therapeutic target, methionine aminopeptidase-2, and the status of some of its inhibitors in pre-clinical and clinical trials for cancer and/or obesity were discussed.

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METHIONINE AMINOPEPTIDASE-2 IS A COMMON THERAPEUTIC TARGET FOR BOTH CANCER AND OBESITY

According to the most recent data from the World Health Organization, there are approximately 14 million new cancer cases and 8.2 million cancer-related deaths worldwide each year. The number of new cancer cases is expected to rise by about 70% over the next 20 years^[1]. The financial costs of cancer in the United States per year are approximately \$263 billion. Moreover, more than half of the population in the developed countries are overweight or obese^[2]. The prevalence in children in all developed countries is about 15%. It has been predicted that, if current trends continue, the number of children under 5 who are overweight or obese would rise to 70 million worldwide by 2025. Obesity is commonly associated with numerous diseases, including type 2 diabetes mellitus, hypertension, stroke, gallbladder disease, dyslipidemia, sleep apnea, hepatic steatosis endometrial disorder, and cancer^[3-5]. Obesity is responsible for nearly 25% of the relative contribution to cancer incidence, which ranks second only to tobacco use^[1]. Significance evidence shows that both neoplastic and non-neoplastic tissue growth are dependent on angiogenesis. Furthermore, it is well established that cancer mortality is mainly due to metastatic tumors, and that angiogenesis is required for tumor metastasis^[6]. This suggests that anti-angiogenesis agents could provide a novel therapeutic option for the prevention and treatment of both human obesity and cancer. One major molecular target for developing anti-angiogenesis agents is type-2 methionine aminopeptidase (MetAP2)^[7-13]. This molecular target provides a crucial link of the functions of methionine aminopeptidases (MetAPs) to both cancer and obesity.

TWO DISTINCT TYPES OF EUKARYOTIC METAPS ARE RIBOSOME-ASSOCIATED METALLOPROTEASES AND EMETAP2 IS BI-FUNCTIONAL

MetAPs are responsible for the removal of the initiator methionine (iMet) during protein synthesis. The iMet is removed when the second residue is small and uncharged (G, A, P, S, C, T, V), a function that has been evolutionarily conserved across microbes and humans^[14-23]. There are two types of MetAPs in eukaryotic cells^[20-26]. These two types of MetAPs share low sequence similarity. However, they are both metalloproteases and belong to the "pita-bread" (or M24) protease family with a pseudo two-fold axis of symmetry in the catalytic site and a metal ion binding site located at the interfaces between domains^[27]. In addition, each eukaryotic MetAP (eMetAP) contains a unique N-terminal domain linking each MetAP to a distinct site near the exit of the nascent polypeptide

chains^[23,28-31]. The eukaryotic type 1 MetAP (eMetAP1) contains two zinc finger-like motifs that are involved in ribosomal association^[28-31]. It has been demonstrated recently that nascent polypeptide-associated complex (NAC) and eMetAP1, like signal recognition particle (SRP), contacts the ribosome *via* Rpl25/35, the universal adaptor site of the ribosome. It has also been shown that NAC prevents antagonism between SRP and eMetAP1 binding^[32]. The eukaryotic type 2 MetAP2 (eMetAP2), on the other hand, contains polycharged Lys-rich block(s) that play a role in its ribosome association^[31]. This unique N-terminal motif is also associated with POEP (protection of eIF2 α phosphorylation) activity^[33] which can prevent the phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2 α). This function of eMetAP2 connects the processing of iMet with the regulation of the initiation of protein synthesis. However, the interplay between these two functions is yet to be discovered.

METAPS ARE ESSENTIAL FOR CELL GROWTH, WHICH GOVERN THE FUNCTION AND STABILITY OF CERTAIN PROTEINS

MetAP activity is essential for cell growth, as demonstrated by several deletion experiments, including the deletion of a single MetAP in *Escherichia coli* and *Salmonella typhimurium* and the deletion of both MetAP1 and MetAP2 in *Saccharomyces cerevisiae*^[25], all of which result in lethality. Failure to remove initiator methionine may therefore have several detrimental effects on protein function. This could be due to the importance of methionine removal for subsequent N-terminal modifications, including N-myristoylation and N-acetylation, the lack of which may alter protein stability, localization, or functional interactions. Multiple examples have demonstrated that inappropriate retention of initiator methionine can, in some circumstances, result directly in decreased protein stability^[34,35]. Another possible cause is that the removal of initiator methionine may be required to expose a mature N-terminal residue involved in catalysis. An example is the case of some N-terminal nucleophile aminotransferases, which have a nucleophilic N-terminal cysteine^[36]. It would be very interesting to find out whether there are other classes of enzymes whose functions are controlled by the removal of iMet.

THE METAP2 IS A COMMON THERAPEUTIC TARGET FOR CANCER VIA ANTI-ANGIOGENESIS

Angiogenesis, the process of the formation of new blood vessels, is known to be important for the pathogenesis of several major human diseases. These include rheumatoid arthritis, diabetic retinopathy, and cancer^[37-39]. It has been firmly demonstrated that angiogenesis is

essential for tumor growth and metastasis^[40,41]. Fumagillin has been demonstrated as a potent angiogenesis inhibitor *in vivo*^[42-46]. One of the fumagillin analogs, TNP-470, was shown to be a much more potent inhibitor of tumor progression than fumagillin in multiple animal models, and entered clinical practice in 1992^[42,47]. Despite encouraging preliminary findings against several cancers^[48-50], further progress of this compound in clinical trials was hampered in part by its toxicity to central nervous system (CNS) and rapid clearance.

To understand the molecular basis of angiogenesis inhibition by fumagillin, ovalin, and TNP-470, eMetAP2 was identified as a common molecular target for this class of inhibitors^[7-13]. These findings indicate that eMetAP2 plays a role in human endothelial cell proliferation by blocking the G1 phase entry of the cell cycle^[51], thereby becoming a potential therapeutic target for cancer. In addition, it was found that the His-231 residue in the active site was irreversibly modified by fumagillin spiroepoxide^[9-12]. In the fumagillin/MetAP2 complex, the hexenyl chain of fumagillin functionally mimics Met thioether. Following this discovery, PPI-2458, a novel fumagillin derivative was developed. This compound differs from fumagillin in that the polyolefinic chain, which extends out of the active site, is replaced by a carbamoyl-linked D-valinamide moiety^[52,53]. PPI-2458 has shown efficacy in rodent models for melanoma^[54], non-Hodgkin lymphoma^[55], and arthritis^[49,56-58]. The safety and tolerance of PPI-2458 in patients with non-Hodgkin's lymphoma and solid tumors was examined in phase I clinical trials between 2004 and 2007^[49]. All metabolites retained significant MetAP2 inhibitory and antiproliferative activities. It has also been demonstrated that there is a good correlation between highly efficient inhibition of circulating MetAP2 and rapid metabolite formation, confirming the importance of active metabolites in the efficacy of PPI-2458. Unfortunately, no further reports could be identified after this Phase I study report in 2009. To increase the oral bioavailability and specificity of TNP-470, as well as decrease its toxicity, TNP-470 was conjugated into a polymeric delivery system N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer with a Gly-Phe-Leu-Gly linker. This conjugate is believed to selectively accumulate in tumor vessels due to an enhanced permeability and retention (EPR) effect^[59]. However, though a multi-institutional phase II study of TNP-470 in patients with metastatic renal carcinoma revealed its manageable toxicity, it does not lead to any significant objective responses^[60].

A more potent fumagillin derivative, CKD-732 (also known as ZGN-433 or beloranib), exhibits a 2-fold increase in sensitivity than TNP-470 and a 1000-fold increase in selectivity than fumagillin for vascular endothelial cells^[61]. Based on the anti-angiogenic activity of CKD-732, a Phase I study plus XELOX was conducted in metastatic colorectal cancer (mCRC) patients who have been previously treated with irinotecan-based chemotherapy. In this study, a very promising clinical benefit rate of 100% was observed with a small number of participants. Unfortunately, there have been no further reports after

the publication in 2012. It would be highly beneficial to have Phase II data that may further elucidate the value of eMetAP2 as a target for cancer therapy.

THE METAP2 IS ALSO A THERAPEUTIC TARGET FOR OBESITY VIA ANTI-ANGIOGENESIS

Worldwide prevalence of obesity has nearly doubled since 1980. Recently, inhibition of pathological angiogenesis in adipose tissue has attracted the attention of researchers in the anti-obesity field. Although MetAP2 inhibitors were originally developed as anti-cancer therapies, as described above, MetAP2 inhibitors target angiogenesis, which would prevent further development of adipose tissue and thus obesity. Unlike anti-angiogenic therapy for cancer, MetAP2 inhibition for obesity treatment might not lead to drug resistance due to the genomic stability of obesity-related adipocytes and endothelial cells^[62]. In addition, the timescale of current obesity treatment are not designed for longitudinal study and the treatment can be stopped when target body weight is achieved^[62]. Since angiogenesis is important for wound healing, it is predicted that patients who are obese and have already developed cardiometabolic complications, such as hypertension, might not be appropriate for this type of therapy^[63].

ZGN-433 (beloranib, CKD-732) described above, was found to be an effective MetAP-2 inhibitor for anti-diabetes. In the phase II study, beloranib produced statistically significant and clinically meaningful weight loss in obese participants for up to 12 wk in the absence of any dietary or exercise intervention^[63]. Statistically significant improvements in cardiometabolic risk factors, including waist circumference, lipids and blood pressure, were observed when compared to a placebo. Overall, adverse events were mild to moderate, and they are resolved over the course of the study. Robust Phase II clinical data of ZGN-433 (beloranib) indicated that a high potential for weight reduction in moderate to severe obese patients without serious adverse effects. Beloranib has moved into Phase III clinical trials^[64]. Unfortunately, in December 2015, there was a second patient death. In order to determine whether the deaths were treatment related, Zafgen halted the Phase III clinical trial of beloranib for Prader-Willi Syndrome. After discussions with the Food and Drug Administration, they found that there are insurmountable obstacles to gaining approval and thus, product development for beloranib was ended.

REVERSIBLE VS IRREVERSIBLE METHIONINE AMINOPEPTIDASE INHIBITORS

All the MetAP2 inhibitors that have entered clinical trials so far are irreversible inhibitors containing a highly reactive spiroepoxide (Table 1). It remains unclear whether the

Table 1 Current status of selected type-2 methionine aminopeptidase inhibitors

Name	Type of inhibitor	Preclinical/clinical trials
Fumagillin ^[7-10]	Irreversible	N/A
TNP-470 ^[12,13]	Irreversible	Phase I
PPI-2458 ^[49]	Irreversible	Phase I
CKD-732 ^[61-64] (ZGN-433, beloranib)	Irreversible	Phase II / III
SDX-7320 ^[59,60]	Irreversible	Phase II
Bengamides ^[65] (LAF389)	Reversible	Phase I
2-Hydroxy-3-aminoamides ^[66]	Reversible	Preclinical
Anthranilic acid sulfonamides ^[67]	Reversible	Preclinical
Triazoles ^[68]	Reversible	Preclinical
Indazoles ^[69]	Reversible	Preclinical
Pyrazolo[4,3-b]indoles ^[70]	Reversible	Preclinical

N/A: Not applicable.

major adverse effects are caused by the interaction of the spiroepoxide with non-specific targets or simply the nature of the biological function of eMetAP2. Many reversible MetAP2 inhibitors have been developed (Table 1). They include bengamides, 2-hydroxy-3-aminoamides, anthranilic acid sulfonamides and triazole analogs^[65-68]. Most of the reversible MetAP2 inhibitors, except the bengamides, have not entered clinical trials because they are not as potent as irreversible^[66]. Recently, using the fragment-based drug discovery approach (FBDD), a 6-substituted indazole core was identified as an orally efficacious potent reversible MetAP2 inhibitor^[69]. Based on those findings, a pyrazolo[4,3-b]indole core was designed using the structure-based drug discovery (SBDD) approach^[70]. One pharmacokinetically acceptable compound was further evaluated in a DIO-mouse model for obesity and a 4% reduction in body weight was observed. In addition, this compound was high specific based on the data evaluated in a Ricerca Comprehensive Pharmacological Profile panel including 100 biological targets and a panel of proteases^[69,70]. These findings showed great promise of developing potent reversible MetAP2 inhibitors for obesity and hopefully for anti-cancer drugs in the foreseeable future.

CONCLUSION

Despite initial promising pre-clinical and clinical studies using MetAP2 as the target for anti-cancer and anti-obesity drug development, many important biological questions remain unsolved. Insights into the basic biology of eMetAPs have only recently emerged. These include a recent large-scale N-terminus profiling in cells responsive and unresponsive to fumagillin treatment^[71]. Changes in glutathione status were observed in fumagillin-sensitive cells, but not in unresponsive cells. Proteo-transcriptomic analyses revealed that both eMetAPs accumulated in a cell-specific manner and that cell sensitivity to fumagillin was related to the expression levels of eMetAPs, particularly eMetAP1. It is also worth noting that the authors suggested that MetAP1 levels could be routinely checked in several

types of tumors and used as a prognostic marker for predicting responses to treatments inhibiting MetAP2^[71]. Moreover, additional publications have allowed a growing understanding of the regulation of the MetAPs and the interplay between MetAPs, ribosomes, and other factors involved in protein synthesis and protein processing, as well as the regulation of MetAP functions^[72-74]. These significant advances in our understandings of these basic biological functions will help advance the development of potent anti-cancer and anti-obesity MetAP inhibitors to significantly reduce the risk of unexpected adverse effects occur during clinical trials.

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Endocrine disrupting chemicals in mixture and obesity, diabetes and related metabolic disorders

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Abstract

Obesity and associated metabolic disorders represent a major societal challenge in health and quality of life with large psychological consequences in addition to physical disabilities. They are also one of the leading causes of morbidity and mortality. Although, different etiologic factors including excessive food intake and reduced physical activity have been well identified, they cannot explain the kinetics of epidemic evolution of obesity and diabetes with prevalence rates reaching pandemic proportions. Interestingly, convincing data have shown that environmental pollutants, specifically those endowed with endocrine disrupting activities, could contribute to the etiology of these multifactorial metabolic disorders. Within this review, we will recapitulate characteristics of endocrine disruption. We will demonstrate that metabolic disorders could originate from endocrine disruption with a particular focus on convincing data from the literature. Eventually, we will present how handling an original mouse model of chronic exposition to a mixture of pollutants allowed demonstrating that a mixture of pollutants each at doses beyond their active dose could induce substantial deleterious effects on several metabolic end-points. This proof-of-concept study, as well as other studies on mixtures of pollutants, stresses the needs for revisiting the current threshold model used in risk assessment which does not take into account potential effects of mixtures containing pollutants at environmental doses, *e.g.*, the real life exposure. Certainly, more studies are necessary to better determine the nature of the chemicals to which humans are exposed and at which level, and their health impact. As well, research studies on substitute products are essential to identify harmless molecules.

Key words: Endocrine disrupting chemicals; Persistent organic pollutants; Phthalates; Bisphenol A; Metabolic

disorders; Insulin resistance

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Core tip: Evidences are accumulating showing that some pollutants endowed with endocrine disrupting activities, the so-called endocrine disrupting chemicals, may contribute to the pandemic evolution of obesity and related metabolic disorders including diabetes. Within this review, we present the concept of endocrine and metabolic disruption and give an overview of the current knowledge of the field, including data from our laboratory and others, specifically focusing on the cocktail effect of pollutants which is one of the biggest concern caused by pollutants nowadays.

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INTRODUCTION

Obesity is a major public health problem because it is a risk factor for the development of metabolic disorders such as type 2 diabetes, cardiovascular diseases and some cancers whose evolution is pandemic. These diseases represent a major societal challenge in health and quality of life with large psychological consequences in addition to physical disabilities linked to overweight and diabetes. Metabolic disorders are also one of the leading causes of morbidity and mortality. By 2030, it is predicted that the number of overweight people will reach 3.3 billion while diabetes will affect more than 400 million people worldwide. In 2010, 10% of overweight children were between the ages of 5 and 17^[1]. Furthermore, a conservative estimate of the cost of pollutants on health impact, in the field of obesity and diabetes, exceeds the annual 18 billion in Europe^[2]. Several causative factors have been identified, especially excessive food intake and decreased physical activity. Yet, neither these well-recognized risk factors nor the genetic predispositions and the observed reductions in sleep length can explain the kinetics of the epidemic. Thus, it has been put forward that pollutants, which exponential manufacturing coincides with obesity trends and prevalence of diabetes^[3,4], may well constitute new actors of these multifactorial diseases, specifically chemicals endowed with endocrine disrupting activities, *i.e.*, the endocrine disrupting chemicals. Herein, we summarized the current knowledge about endocrine and metabolic disruptions, to illustrate that these pollutants are indeed causative factors in the obesity and diabetes pandemic. Some studies analyzing the cocktail effects were also described, as we are exposed to thousands of chemicals.

THE CONCEPT AND HYSTORY OF THE ENDOCRINE DISRUPTION

If industrialization fostered societal progress improving life expectancy, it also led to the presence, in the different compartments of the environment, of thousands of anthropic molecules sometimes transported over very long distances globalizing pollution. Some of these chemicals (an estimate of 900 molecules classified after their characteristics including their half-lives, Table 1) can affect the hormonal system, thereby interfering with the development of the organism and representing the endocrine disrupting chemicals (EDCs). Historically, the first warnings came from researchers and physicians invested in reproductive biology. They outlined the detrimental effects of some pesticides in the environment particularly on the birds^[5] or alerting on the diethylstilbestrol (DES) tragedy with DES given to millions of women between 1941 and 1971 to prevent miscarriages. An estimated 2 to 5 million children were exposed *in utero*, a large number developed genital malformations and cancers^[6-8].

At the Wingspread conference of 1991, the concept of endocrine disruptor was proposed to account for new scientific discoveries on chemicals such as pesticides, plasticizers or persistent organic pollutants (POP) capable of mimicking a hormonal action or, conversely, able to antagonize the hormone action, or to interfere with the mechanisms of hormonal production, transport or metabolism. Today, the concept of endocrine disruption is still debated, but there is a consensus on the definition given by the World Health Organization (WHO) stating that "an endocrine disruptor is an exogenous substance or mixture that alter(s) function of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations"^[9].

Thus, endocrine disruption is characterized by a modification of the endocrine system which may result in a toxic effect when the homeostatic regulations are disrupted. Specifically, the endocrine system is constituted by a set of so-called endocrine glands which secrete chemical messengers or hormones, carried by the bloodstream to target distant organs expressing the corresponding high-affinity receptors. The endocrine system includes well defined glands such as the pituitaries, gonads, adrenals or thyroid glands but also organs such as liver, pancreas, gut and adipose tissues. This system is highly sensitive, able to react to very low doses (pM) of hormones in a non-linear relationship between hormone levels and receptor occupation, with opposite effects observed when hormone levels exceed the physiological range (*e.g.*, hyper and hypothyroidism lead to opposite metabolic defects with regards to body weight and energy expenditure). Importantly, hormonal effects varied with the stage of development and the targeted organ. In adults, they are characterized by negative regulatory feedback loops with loss of biological effects at the highest hormonal doses to maintain physiological homeostasis. Besides, fetal and neonatal

Table 1 Chemicals, sources and routes of exposure, examples, and some demonstrated metabolic effects

Chemicals	Sources	Examples	Some demonstrated metabolic effects
Alkylphenols	Lubricating oil additives; detergents; emulsifiers, pesticides; NP plastics Exposure occurs <i>via</i> water drinking and food consumption ^[67]		Estrogenic activities ^[68]
Dioxins	Byproducts of industries from incomplete combustion; release during natural events such as wood burning and volcanic eruption Diet is the main route of exposure ^[69]	TCDD	Hepatic steatosis ^[70] and fibrosis ^[71] ; increased adipocyte differentiation (<i>in vitro</i>) ^[72]
Flame retardants	Used in electronic equipment, furniture, plastics...and then, present in dust, air and soil Dermal exposure is a significant route of exposure ^[73]	Penta-BDE	Decrease in glucose oxidation ^[74]
Organotin compound	Used as biocide in anti-fouling paint, heat stabilizer in Poly Vinyl Chloride Exposure mainly by consumption of seafood ^[75]	TBT	Induction of adipocyte differentiation ^[76] , increase of body weight and hepatic steatosis ^[77] , transgenerational effects on fat depots and hepatic steatosis ^[39]
Phenolic derivatives	Plastic components, cosmetics, disinfectants, thermal paper receipts Food and water drinking are the major routes of exposure ^[78]	BPA, BPS	Estrogenic activities ^[79] ; alteration of pancreatic β cell functions and hepatic insulin signaling (BPA) ^[47] ; induction of lipid accumulation and differentiation (<i>in vitro</i> , BPS) ^[80]
Pesticides	Due to their persistence, accumulation in soils and sediments; bioaccumulation throughout the food chain	DDT and its metabolite	Alteration of systemic glucose homeostasis and hepatic lipid metabolism ^[83] ; Glucose intolerance, hyperinsulinemia, dyslipidemia and altered bile acid metabolism ^[84]
Phthalates	Processing of agriculture products (banned in Europe); Dietary sources ^[81] as well as inhalation and dermal routes of exposure ^[82] Plastic components, cosmetics, medical equipment; Exposure mainly derives from dietary sources for high molecular weight phthalates (<i>e.g.</i> , DEHP) and non-dietary sources for low molecular weight phthalates (<i>e.g.</i> , DBP) ^[86]	Atrazine (C ₈ H ₁₄ ClN ₅) DBP, DEHP	Increased body weight, intra-abdominal fat and insulin resistance ^[85] Anti-androgenic effects ^[87] ; Transgenerational inheritance of obesity ^[88] ; Increased adipocyte differentiation ^[89]
PCBs	Synthetic compounds now banned but previously used, in particular, in electrical capacitors; still release in environment due to their persistence Food consumption contributes over 90% of total exposure ^[90]	PCB153 (C ₁₂ H ₅ Cl ₆), PCB170 (C ₁₂ H ₅ Cl ₇), PCB187 (C ₁₂ H ₅ Cl ₇) (non dioxin-like); PCB126 (C ₁₂ H ₅ Cl ₃), PCB77 (C ₁₂ H ₆ Cl ₄) (dioxin-like)	Increased adipocyte differentiation (<i>in vitro</i>); increased body weight, adipocyte hypertrophy ^[72] , increased hepatic steatosis and visceral adiposity in the context of a lipid-enriched diet ^[91]
PAH	Byproducts of incomplete combustion of organic compounds (cigarette smoke, wood burning, overcooked meat...) Contamination primarily through inhalation and consumption of certain foods ^[92]	B[a]P	Carcinogenic Alteration of estrogen metabolism in human mammary carcinoma-derived cell lines ^[93] Inhibition of lipolysis, increased fat accumulation and weight gain ^[94]
PFAA	Water and oil repellent; used for treatments of clothing, insulation and fire-fighting foams Oral and dermal exposure ^[95]	PFOA	Elevated serum leptin and insulin; overweight after <i>in utero</i> exposure ^[96]

PAH: Polycyclic aromatic hydrocarbon; PFAA: Perfluoroalkyl acids; PCBs: Polychlorobiphenyls; Np: Nonylphenols, C₁₅H₂₄O; DBP: Dibutyl phthalates, C₁₆H₂₂O; BPA: Bisphenol A, C₁₅H₁₆O₂; BPS: Bisphenol S, C₁₂H₁₀O₄S; TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin, C₁₂H₄Cl₄O₂; Penta-BDE: Pentabrominated diphenyl ethers, C₁₂H₅Br₅O; TBT: Tributyltin, (C₄H₉)₃Sn; DDT: Dichlorodiphenyltrichloroethane, C₁₄H₉Cl₅; DDE: p,p'-dichlorodiphenyldichloroethylene, C₁₄H₈Cl₄; DEHP: Diethyl hexyl phthalate, C₂₄H₃₈O₄; B[a]P: Benzo[a]pyrene, C₂₀H₁₂; PFOA: Perfluorooctanoic acid, C₈HF₁₅O₂.

development are periods of high sensitivity to hormonal signals, with hormones shaping sexual differentiation and behavior (testosterone, estradiol), cognitive development (thyroid hormone) but also feeding behavior (testosterone, estradiol, glucocorticoids, leptin).

While endocrine disruption is localized at the interface between endocrinology and toxicology, risk assessment relies on toxicology principles and the linearity of the harmful effects of chemicals beyond a threshold value. This is the Paracelse's statement that "all substances are poisons: There is none which is not a poison. The right dose differentiates a poison from a remedy". Hence,

health risks assessment by international agencies such as the United States Environmental Protection Agency (EPA) or the European Food Safety Agency (EFSA) relies on the setting of toxicological reference values, *e.g.*, the tolerable daily intake (TDI) doses. The TDI is an estimate of the amount of a given chemical in food or drinking water that can be ingested daily over a lifetime without a significant health risk. Its calculation is empiric in that it assumes a dose-linear response curve from the no- or the lowest-observed-adverse-effect-levels (NOAELs or LOAELs, respectively) in animal studies performed in the most sensitive species and examining the most

sensitive endpoint; the value determined is then divided by an uncertainty factor (from 100 to 1000) to take into account interspecies as well as inter-individuals variation^[10].

While such calculations result in reference doses which may appear sufficiently protective from a toxicological point of view, the discovery of adverse effects in rodents at doses lower than the TDI outlines the necessity of integrating the principles of Endocrinology for higher protection. Specifically, care should be taken at the non-monotonicity dose-response curves^[11] challenging the Paracelse' paradigm. The developmental origin of human health diseases should as well be considered. It integrates that EDCs may exert their adverse effects long after the individuals have been exposed (for instance during fetal and neonatal development)^[12,13]. It is also important to consider that humans are exposed to a plethora of chemicals, not a single chemical, some being highly persistent in the environment (e.g., the persistent organic pollutants, POPs) which outlines the necessity in risk assessment to consider the possible additive, antagonistic or synergistic activities of the resulting mixture to which humans are exposed. This is recognized as the "cocktail effect" that will be more detailed in the last part of this review.

ENERGY METABOLISM AND METABOLIC DISRUPTION

Regulation of energy metabolism relies on the integrated action of a large number of hormones operating centrally to control food behavior and peripherally to maintain glycaemia at a physiological range whilst covering energy demands. It both involves insulin secretion from pancreas and responsiveness to insulin by the metabolically active tissues (liver, muscle and adipose tissues) in response to food intake that elevates blood glucose. In addition to insulin, hormones (and their corresponding high-affinity receptors) are involved in energy metabolism. They include, but are not limited to, glucocorticoids, thyroid hormone, leptin and adiponectin, gut hormones (such as ghrelin and Glucagon-like peptide 1 or GLP1), the growth hormone and the sexual hormones estrogen/androgen, energy metabolism being highly sexually marked with sex-dimorphic insulin sensitivity, eating behavior, distribution of fat, etc. The protective role of estrogens against metabolic disturbances has been well demonstrated conferring positive metabolic adaptations to women^[14].

Obesity results in energy imbalance between energy intakes determined by food consumption and energy expenditure comprising basal metabolism, thermoregulation and physical exercise in obese patients. Along with energy supplies exceeding energy requirement, glycaemia will remain at values exceeding physiological range between meals whilst pancreas will secrete higher insulin levels in an attempt to lower glycaemia. First signs of insulin resistance will arise in the metabolic tissues

liver, muscle and adipose tissues. Hepatic production of glucose namely gluconeogenesis will be no longer well controlled by insulin resulting in higher levels of glucose in blood. Glucose uptake will be less effective in muscles and lipolysis will be enhanced leading to elevated levels of free fatty acids in circulation (lipotoxicity). Eventually, type 2 diabetes develops with persistent and progressive deterioration of glucose tolerance. Gradually, the body's ineffective use of insulin evolves as a tryptic of hyperglycemia, hyperinsulinemia and hypertriglyceridemia, in a vicious circle where hyperglycemia aggravates hyperinsulinemia and hyperinsulinemia aggravates hyperglycemia and hypertriglyceridemia^[15,16].

Importantly, lipid and glucose metabolisms are under a tight regulation not only by the hormones mentioned above and their associated hormone receptors but also by several nuclear receptors such as peroxisome proliferator-activated receptors (PPARs), liver X receptors (LXR) and farnesoid X receptor (FXR) as well as the xenosensors Pregnane X receptor (PXR), Constitutive androstane receptor (CAR) and aryl hydrocarbon receptor (AhR). These transcription factors integrate the changes in environmental or hormonal signals through direct gene regulation or through cross-talk with other transcriptional regulators to maintain the vital function of nutrient homeostasis between the fed and the fasting states^[17]. Interestingly, PXR, CAR and AhR were first identified as controlling xenobiotic and drug metabolism and promoting their clearance^[18,19]. However, analysis of the phenotypes of mice deficient in one particular nuclear receptor or in which a xenosensor has been activated by a strong agonist showed their important roles in the metabolism of fatty acids, lipids and glucose^[20,21] and on obesity and/or insulin resistance (Table 2). Besides, like the receptors for estrogens, androgens, glucocorticoids or thyroid hormones, the PPARs, LXR, and FXR can also be targeted by specific environmental chemicals identified as endocrine disruptors (Table 3). Therefore, whilst homeostasis of the energy metabolism highly depends on the integrated and beneficial contribution of nuclear receptor activation, these receptors can as well be the sites of endocrine disruption. This underlines the concept that insulin resistance, resulting from the inappropriate activation of one of the nuclear receptor mentioned above, well constitutes an endocrine disruption. This concept does not exclude that pollutants may exert toxic effects by mechanisms distinct from endocrine disruption including oxidative stress and mitochondrial alteration^[22] as well as inflammation^[23,24].

It has been well illustrated with the studies on anti-diabetic medications that obesity does not necessary lead to insulin resistance. Indeed, the insulin sensitizer properties of the thiazolidinedione class of drugs were based on their abilities to activate PPAR γ which is a master transcription factor involved in adipogenesis^[25]. However, while enhancing insulin sensitivity, PPAR γ activation also leads to enhanced body weight. Thanks to molecular biology research, the dissection of estrogen receptor beta (ER β)-deficient mouse phenotype led to the

Table 2 Metabolic characteristics of mice deficient in some nuclear receptors¹

Insulin status	Obesity	No body weight change
Insulin resistance	ER α (-/-) in both males and females ^[38]	
No difference in insulin sensitivity	AR (-/-) in males only ^[97]	
Improved insulin sensitivity	ER β (-/-) (study on males only) ^[26]	CAR activation (study on males only in HFD context, activation by TOBOBOP) ^[99]
	ERR β (deletion in neurons; study on males only) ^[98]	AhR (-/-) (studies on males only) ^[100]
		AhR (-/-) (studies on males only, in HFD context) ^[101]
		PPAR α (-/-) (studies on males only, in HFD context) ^[102]
		PXR (-/-) (studies on males only, in HFD context) ^[103]

¹Mice were fed standard diet or high-fat diet when mentioned. HFD: High-fat diet; AhR: Aryl hydrocarbon receptor; CAR: Constitutive androstane receptor; PPAR α : Peroxisome proliferator-activated receptor α ; PXR: Pregnane X receptor.

Table 3 Interactions of some nuclear receptors with endocrine disruptors

Nuclear receptors	Interactions with chemicals
Steroid receptors	
ER	BPA (Er α ^[38] , GPR30 ^[104])
AR	BPA ^[105]
GR	BPA; phthalates ^[106]
PR	BPA ^[107]
TR	BPA ^[108] ; brominated flame retardants, BFR ^[109]
RXR heterodimers	
PPAR α	Phthalates ^[110] ; polyfluoroalkyl compounds ^[111] ; pyrethrins ^[112]
PPAR γ	Phthalates ^[110,113] ; organotin ^[76] , BPA ^[114]
FXR	Pyrethroids ^[115]
CAR	Phthalates ^[116,117]
LXR α	Phthalates; BPA ^[118]
PXR	Phthalates; BPA ^[119,120]
Other receptors	
AhR	Dioxines; PCB dioxin-like ^[72,121,122]

BPA: Bisphenol A; PCB: Polychlorobiphenyl; ER: Estrogen receptor; AR: Androgen receptor; GR: Glucocorticoid receptor; PR: Progesterone receptor; TR: Thyroid hormone receptor; PPAR: Peroxisome proliferator-activated receptor; FXR: Farnesoid X receptor; CAR: Constitutive androstane receptor; LXR: Liver X receptor; PXR: Pregnane X receptor; AhR: Aryl hydrocarbon receptor.

discovery that the metabolic actions of ER β are mediated by a negative cross-talk with PPAR γ acting as an insulin sensitizer^[26]. Several other cross-talks were evidenced and reviewed recently^[27]; for example, between the estrogens acting through estrogen receptors and the AhR allowing under some circumstances regulation of estrogen target genes by dioxins^[28]. Other examples of cross-talk include CAR-target genes regulating the metabolism of estrogens^[29]; the control of bile acid homeostasis by xenobiotics as a result of cross-talk between FXR, CAR and PXR^[27] and more recently, the evidence that liver ER α regulates female hepatic metabolism through interaction with LXR α ^[30].

Thus, the threat represented by xenobiotics is challenging an already complex and multilayer physiological mechanism that tightly regulates insulin secretion and sensitivity whilst living organisms oscillate between fed and fasting states to meet energy demands. On top of this, several EDCs can activate or interact with multiple transcription factors or hormone receptors as described above in a context of exposure to numerous pollutants

and possibly interacting with a high-fat nutritional context undermining appropriate adaptive responses. Diet is a primary route of exposure to pollutants, linking the amount of food ingested and the levels of exposure to pollutants. Other routes of exposure include dermal, inhalation as well as subcutaneous and intravenous infusions *via* medical equipment (Table 1)^[31,32].

EPIDEMIOLOGICAL AND EXPERIMENTAL EVIDENCES SUPPORTING INVOLVEMENT OF EDCs IN THE OBESITY AND DIABETES EPIDEMICS

First evidences were brought from occupational exposure to a class of toxic molecules or accidental setting as for dioxins. For example, veterans exposed to Agent Orange have an increased relative risk of developing diabetes^[33]. After the Seveso industrial explosion in Italy, the risk of developing diabetes increased in women who have been

exposed^[34]. Associations between polychlorobiphenyls (PCBs) and diabetes have also been evidenced in humans and obesity has been suggested as an aggravating factor^[35]. Recently, it has been shown that plasma POP profile could discriminate patients who are metabolically healthy or insulin resistant^[36]. In that study, menopausal women were obese based on their body mass index (BMI) and they were subdivided in 2 groups depending on their insulin sensitivity index. Interestingly, their metabolic health status was inversely correlated with plasma POP profile^[36]. Mechanisms of action have not been explored but it could be hypothesized that resistance to insulin was in part linked to the known pro-inflammatory effects of POPs.

Experimental studies mostly done in rodents with strong evidences of endocrine disrupting mechanisms and subsequent metabolic disorders advanced the understanding of the mechanisms involved in these effects. Historical examples have shown that neonatal administration of diethylstilbestrol (DES) to mice causes obesity in adult age. This effect involves at least the estrogen receptors as DES binds with very high affinity to these receptors^[37]. Strikingly enough, estrogens are protective against metabolic diseases, and male and female estrogen receptors ER α deficient mice are obese and insulin resistant^[38], illustrating the pleiotropic effects of this hormone. It could result from the various cross-talks of ERs with other receptors or transcription factors as mentioned above. Another historical chemical inducing metabolic disorders upon exposure is tributyltin (TBT). It belongs to organotin compounds and was widely used as an antifouling painting for ships (now banned). TBT provided a very clear example of endocrine disruption not only in the reproductive field area as for DES but also in metabolism. Indeed, TBT is the obesogen molecule *per se* in that it induces adipocyte differentiation targeting PPAR γ . Due to its strong and consistent effect on adipocytes, TBT has been used for multigenerational studies in mice (3 successive generations called F1, F2, F3)^[39]. In that study, mice were not tested for insulin sensitivity or tolerance to glucose. However, it was shown that early-life exposure to TBT causes hepatic accumulation of triglycerides (steatosis) and reprogramming of the adipocyte stem cells to favor the adipocyte lineage in the F1 and F2 exposed mice as well as in the non-exposed F3 generation. These experiments are an illustration of the DOHaD hypothesis. This hypothesis states that threat during the highly vulnerable period that constitutes the maternal period (e.g., food restriction or excess; adverse environmental milieu because of the presence of pollutants) will provoke diseases later in life including metabolic diseases (obesity, diabetes, cardiovascular diseases) and some cancers^[13]. Occurrence of adverse effects distant from the exposure period as well as in non-exposed generation, questioned the possible involvement of epigenetic mechanisms (e.g., acetylation or methylation of histones, DNA methylation) leading to alteration in the chromatin organization and alteration of transcriptional patterns that could explain the phenotypes described with TBT in early-life exposed TBT

mice^[39]. In addition, the TBT experiments have opened the way for addressing the question as to whether *in vitro* standardized model systems using the 3T3-L1 fibroblast cell line could be used to monitor the obesogenic properties of chemicals^[40,41]. It remains that PPAR γ is also known as an insulin sensitizer and these *in vitro* experiments will not answer to the question as to whether insulin sensitivity is altered upon exposure to the tested chemicals.

Solid evidences of insulin resistance triggered by exposure to POPs were originally reported using adult rats fed a high fat diet containing either crude salmon oil identified as a source of lipophilic compounds or refined oil (deprived of POPs) for 28 d. The authors^[42] demonstrated that rats fed the contaminated oil gained weight and developed abdominal obesity, insulin resistance and hepatic steatosis. Molecular analysis revealed an alteration of insulin signaling, indicative of an endocrine disruption. In another study, rats were exposed to ozone^[43], and the authors indicated that oxidative stress was the first hit causing later on impaired insulin signaling in muscle and whole-body insulin resistance.

An archetypal chemical of the endocrine disrupting field is Bisphenol A (BPA). BPA is widely used in the production of polycarbonates, epoxy resins and polyester resins and its global production exceeded 5 million tons in 2015. Although it does not bioaccumulate in the body, its exposure is universal and it is estimated that more than 95% of the general population is contaminated with diet as a primary route of exposure^[44]. BPA has been shown to act through several receptors including at least the estrogen receptors ER α , ER β and membrane receptor GPR30, the estrogen receptor-related ERR γ and possibly the PPAR γ ^[11,45,46] probably explaining the pleiotropic effect of this chemical and the difficulties at precisely defining its mechanism of action with effects and amplitude varying with age, sex, nutritional context, period of exposition and dosage. A selected list of publications will be presented to illustrate the large range of the reported BPA metabolic effects at doses in the range of the reference dose thus a thousand times lower than the supposed NOAEL dose of 5 mg/kg per day. To explore how exposure to environmental chemicals may affect metabolic health later in life, *i.e.*, the DOHaD hypothesis, a large number of experiments have been based on maternal exposure with a survey of metabolic traits in offspring at adulthood. Hence, maternal exposure to BPA led to the dysfunction of beta pancreatic cells and altered hepatic insulin signaling, resulting in impaired glucose tolerance and insulin sensitivity at adulthood, a phenotype that is exacerbated in offspring fed a high-fat diet^[47]. Mechanisms may involve changes in pancreatic cell mass through enhanced proliferation and diminished apoptosis of the beta-cells, partly acting *via* ER β activation which will result in excess insulin signaling during early life followed by a tendency to reduced pancreatic mass later in adulthood possibly contributing to the observed glucose intolerance^[48]. Interestingly, BPA exposure during gestation reproduced part of the effects of a high-fat diet with hyperinsulinemia and

impaired glucose tolerance in the adult male offspring^[49]. Worthy of note, BPA has been found not only to target insulin secretion through its action on pancreas but to target insulin-sensitive tissues as well with evidences of impaired insulin signaling in both muscles and liver^[50,51]. Others demonstrated that alterations in glucose tolerance and in serum levels of leptin, insulin and adiponectin with some of the effects mimicked by DES, thus indicative of an estrogen dependency^[52]. In addition to maternal effects, BPA also impacts the metabolic health of adults exposed during their adulthood. For example, exposure to low doses of BPA resulted in altered hepatic expression of genes involved in lipid synthesis and in accumulation of cholesteryl esters and triglycerides contributing to hepatic steatosis. Importantly, Marmugi *et al.*^[53] used a large range of doses and they could demonstrate non-monotonic dose-response curves for the expression of several genes related to lipid synthesis. In addition, analysis of the hepatic transcriptome of rats exposed to either TDI or NOAEL doses of BPA showed distinct sets of responsive genes depending on dosage indicating that different doses lead to different responses^[53]. The adipose tissue is also targeted by BPA and Hugo *et al.*^[54] demonstrated, using human adipose explants and adipocytes, that BPA at environmentally relevant doses could inhibit adiponectin release, a marker of insulin sensitivity. A down-regulation of adiponectin release was also demonstrated by Menale *et al.*^[55] using adipocytes from subcutaneous explants recovered from children undergoing orchidopexy surgery. In this study, the authors evidenced a strong and inverse association between BPA and adiponectin within a population of 141 obese children. A reduced glucose utilization coupled to alterations in insulin signaling was also demonstrated in human subcutaneous adipocytes^[56]. Questions remain on the possible activation of PPAR γ by BPA^[41,46], potentially explaining part of its obesogenic effect. Collectively, several *in vitro* and *in vivo* data evoke the possibility for BPA to be a metabolic disruptor with many of its adverse effects linked to endocrine disruption. Notwithstanding, some discrepancies remain in epidemiological studies due to limitations at defining causality between exposures to BPA and the risk to develop diseases. However, these limitations may be linked to difficulties at estimating the usual dietary BPA intake in a context of multiexposure^[44,51,57].

EVIDENCES OF A COCKTAIL EFFECT

In the laboratory, we aim to approach the question of the multi-exposure to environmental pollutants through setting an original mouse model of chronic and lifelong exposure starting in the prepubertal period of the dams-to-be until the adulthood of the offspring, dissecting the metabolic traits in both males and females. Intending to approach a realistic scenario, the mixture of pollutants is made of both persistent and short-lived low-dose chemicals in the range of the TDI for each chemical, all of great concern for human health and specifically

metabolic health^[18,35,58]. The mixture comprises 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), PCB153, di-[2-ethylhexyl]phthalate [DEHP] and BPA. These chemicals have been categorized as endocrine disruptors with either estrogeno-mimetic activities or anti-androgen activities. Moreover, these chemicals activate a broad range of signaling pathways as recapitulated in Table 3. In addition, to mimic the environmental main route of exposure, the mixture of pollutants was incorporated into the diet. Initially, we used a high-fat high-sucrose diet to explore the hypothesis that obese individuals may be more sensitive to exposure to pollutants because they are at risk of developing metabolic disorders. Data yielded brought solid evidences for a cocktail effect linked to endocrine disruption and resulting in metabolic disorders. Indeed, in a preliminary study, we used the NOAEL dose that is the no-observed-adverse-effect level dose in animal studies or 10 times the NOAEL doses for each pollutant of the mixture. It resulted in maternal toxicity with decreased pup survival. At the TDI dose range, there was no maternal toxicity which was compulsory for studying the metabolic health of the offspring but we evidenced sex- and age-dependent metabolic effects in the absence of weight modification^[59]. Specifically, in male offspring, although pollutants did not aggravate glucose intolerance, insulin resistance, plasma levels of triglycerides or cholesterol resulting from the high-fat high-sucrose consumption, we observed changes in cholesterol metabolism with a decrease in hepatic cholesterol levels and an increase in the expression of genes encoding proteins related to cholesterol biosynthesis. A different phenotype was observed in females which exhibited an aggravation of glucose intolerance. Although no change in insulin sensitivity was observed, we interestingly measured decreased levels of the major hepatic estrogen receptor (ER α) together with enhanced expression of the estrogen sulfotransferase (EST/SULT1E1) which metabolizes estrogens. To reconcile these data, we put forward the hypothesis that enhanced estrogen metabolism in the liver of pollutant-exposed females lowered the physiological protection of estrogens against metabolic disorders which could explain the worsening of their glucose intolerance^[59]. Furthermore females with lower plasma estrogens (*i.e.*, young adults) responded differently when exposed to the same mixture of pollutants. Specifically, females (not males) exhibited an alleviated-glucose intolerance with no change in gluconeogenesis and hepatic steatosis, an enhanced lean/fat mass ratio, an enhanced insulin sensitivity in skeletal muscle and a reduced expression of genes encoding inflammatory markers in the adipose tissue^[60]. We suggested that these opposite effects according to the age of the females may result from the hormonal environment. The pollutant mixture could exert an additional and positive estrogenic effect on metabolic traits in the young females but a negative effect in adult females when estrogens are high, with the induction of EST/SULT1E1 as a means to lower estrogen effects within physiological dose range^[32,59,60].

More experiments are underway to better characterize the effects of the mixture and to define whether they represent adverse or adaptive events, and what the contribution of the nutritional context is. For example, using a pollutant-mixed standard diet instead of a high-fat high-sucrose diet, we observed the activation of common metabolic pathways in the liver of challenged females with partial overlapping between the set of dysregulated genes induced by exposure to the mixture of pollutants in a standard diet and by a high-fat high-sucrose diet not containing the pollutant mixture. This study is highly relevant for understanding the synergistic effects between pollutants and the obesogenic diet (Labaronne *et al.*, Submitted). Collectively, these studies constitute a proof-of-concept that low doses of pollutants at supposedly ineffective doses for humans, are not harmless when in mixture.

Importantly, several laboratories have also developed studies to help answering to the today's context of exposure characterized by contamination with a plethora of chemicals at rather low levels. Combined effects of estrogens or anti-androgens chemicals have first been used to demonstrate the "something from nothing" phenomenon with mixtures of endocrine disrupters^[61]. For example, a mixture of 8 estrogenic chemicals produced strong estrogenic effects at doses too low to mediate any measurable effect when tested alone^[62]. Another study reported the same additivity when using mixtures of up to 30 anti-androgen chemicals^[63]. The toxic equivalence factor (TEF) was also formulated for dioxins, PCBs and polyaromatic hydrocarbons resulting in the summation of the doses of each chemical of the mixture multiplied by its respective TEF^[64]. Worthy of note, it was shown recently that the synergistic effect of the mixture containing a pharmaceutical estrogen and a persistent pesticide was due to their cooperative binding to the PXR receptor leading to its synergistic activation, when each chemical alone exhibited low efficacy^[65]. It illustrates how much pollutant interactions in a context of multi-exposure represent a *bona fide* challenge for policy makers^[66].

CONCLUSION

The pandemic evolution of obesity and its associated metabolic disorders that are considered as one of the major health burdens worldwide stress the need for extensive research towards the identification of new etiologic factors with the hope to prevent further augmentation and even more to reduce the kinetics of expansion. These past 20 years, evidences that endocrine disrupting compounds constitute etiologic factors have largely progressed. Certainly, more studies are to be undertaken to better determine the nature of the chemicals to which humans are exposed and at which level. In parallel, substitution research should be encouraged for identifying harmless molecules. Eventually, scientists may think on interventional strategies based on the use of benefit compounds with the aim at counteracting the deleterious metabolic effects of

pollutants. However, it should as well be considered that evidences are more than convincing and that regulatory decision makers should take into account the accumulated and solid scientific results and enjoin to considerably limit the use and spread of chemicals to better protect human health, as recently achieved for BPA in baby bottles.

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Role of pro- and anti-inflammatory phenomena in the physiopathology of type 2 diabetes and obesity

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Abstract

In obesity, persistent low-grade inflammation is considered

as a major contributor towards the progression to insulin resistance and type 2 diabetes while in lean subjects the immune environment is non-inflammatory. Massive adipose tissue (AT) infiltration by pro-inflammatory M1 macrophages and several T cell subsets as obesity develops leads to the accumulation - both in the AT and systemically - of numerous pro-inflammatory cytokines, including interleukin-1 β (IL-1 β), tumor necrosis factor α , IL-17 and IL-6 which are strongly associated with the progression of the obese phenotype towards the metabolic syndrome. At the same time, anti-inflammatory M2 macrophages and Th subsets producing the anti-inflammatory cytokines IL-10, IL-5 and interferon- γ , including Th2 and T-reg cells are correlated to the maintenance of AT homeostasis in lean individuals. Here, we discuss the basic principles in the control of the interaction between the AT and infiltrating immune cells both in the lean and the obese condition with a special emphasis on the contribution of pro- and anti-inflammatory cytokines to the establishment of the insulin-resistant state. In this context, we will discuss the current knowledge about alterations in the levels on pro- and anti-inflammatory cytokines in obesity, insulin resistance and type 2 diabetes mellitus, in humans and animal models. Finally, we also briefly survey the recent novel therapeutic strategies that attempt to alleviate or reverse insulin resistance and type 2 diabetes *via* the administration of recombinant inhibitory antibodies directed towards some pro-inflammatory cytokines.

Key words: Type 2 diabetes; Crown-like structures; Adipose tissue inflammation; Macrophages; Eosinophils; Obesity

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Core tip: Low-grade inflammation of adipose tissue (AT) contributes to insulin resistance and type 2 diabetes in obese patients. On the contrary, in lean individuals, the immune environment of AT is non-inflammatory. In obesity,

AT is infiltrated by pro-inflammatory macrophages and T cells leading to the accumulation of interleukin-1 β (IL-1 β), tumor necrosis factor α , IL-17 and IL-6. On the contrary, M2 macrophages, Th2 and T-regs cells producing anti-inflammatory IL-10, IL-5 and interferon- γ , are present in AT of lean individuals. Here, we discuss the interaction between AT and infiltrating immune cells in the lean *vs* the obese condition, with emphasis on the contribution of pro- and anti-inflammatory cytokines to the establishment of insulin resistance.

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INTRODUCTION

The steadily increasing incidence of obesity and associated morbidities is recognized as a major public health problem, reaching epidemics proportions both in industrialized and developing countries.

In obesity, adipose tissue (AT) depots are subjected to extensive hypertrophy, with expansion of the visceral AT compartments being a strong predictor of the development of insulin resistance^[1]. The AT of obese individuals is in a persistent condition of low-grade inflammation, which is dictated by the infiltration within the AT of several classes of pro-inflammatory immune cells^[2], including monocytes, macrophages, natural killer cells, and lymphocytes, resulting in secretion of adipokines and proinflammatory cytokines by both adipocytes and the population of infiltrating immune cells^[3]. Here, we discuss the multifaceted interplay existing between the AT and the immune system with an emphasis on the alterations occurring during the transition from the homeostatic state of adipose depots in the lean condition to the AT accumulation experienced throughout the development of obesity.

ANTI-INFLAMMATORY STATE OF THE AT: PEACEFUL TIMES DURING HOMEOSTASIS

The immune environment in the lean AT is predominantly non-inflammatory. In this tissue, eosinophils and innate lymphoid cells drive a bias towards a type 2 immune response, secreting cytokines such as interleukin-4 (IL-4), IL-5 and IL-13, which maintain AT macrophages in an anti-inflammatory, M2-like state. However, this picture is not so simplified. Indeed, IL-10 and IL-33 are also secreted; invariant natural killer (NK)-T cells are involved, as well as newly identified populations of T and B regulatory cells (T-regs and B-regs), some of which appear to be exclusive of AT. Adipocytes are also active regulators of immune

responses by means of their own secreted hormones. At the end, research has recently made an intense effort to fully comprehend the nature of the healthy AT, in pursuance of new pathways to successfully treat and win the battle against the expanding epidemic of obesity and type 2 diabetes mellitus (T2DM).

Despite a growing body of evidence linking inflammation and metabolism, the cellular sources of inflammatory mediators in the AT were still unknown at the very beginning of 2000's. Only in 2003, AT macrophages were pointed out as the culprits, increasing significantly in number and producing a range of inflammatory mediators during obesity^[4,5]. In fact, Weisberg *et al.*^[4] estimated the percentage of macrophages ranging from 10% in lean AT to almost 50% in obese mice and humans. These infiltrated phagocytes augmented the inflammatory environment in the AT and were demonstrated to be responsible for the increase in local and systemic insulin resistance and metabolic abnormalities associated with obesity^[5,6]. It is well known that visceral adipose tissue (VAT) expansion present higher risk for the development of metabolic syndrome and insulin resistance than subcutaneous adipose tissue (SAT) growth^[7]. Unsurprisingly, macrophage accumulation in obese VAT tissue is greater than in SAT, as are the levels of the cytokines/chemokines MCP-1, CCR2 and of CD8⁺ T lymphocytes: These molecules and T cell subsets are essentially pro-inflammatory mediators^[8].

ROLE OF INFILTRATING MACROPHAGES IN LEAN AND OBESE AT

The functional relevance of macrophages and their phenotypic changes was established through loss- and gain-of-function experiments^[9,10]. Since the discovery of the increased infiltration of macrophages in the obese AT, the attention of researchers has been focused on the inflammatory type of macrophage easily visualized in the so-called "crown-like structures" (CLS) present around adipocytes and their contribution to metabolic disease. These recently recruited, inflammatory macrophages, were mostly of the "classically activated", M1-type^[11]. However, the role of macrophages in the homeostatic, lean AT, has been left mostly unexplored. In lean AT, macrophages seem to be the major population of immune cells, with most of them belonging to the "alternatively activated" class, often classified as the M2-type, with a ratio of M2:M1 reported to be approximately 4:1^[12,13].

M2 macrophages are immunosuppressive cells with a high phagocytic capacity, capable to perform antigen presentation and having the ability to secrete extracellular matrix compounds, angiogenic and chemotactic factors, and anti-inflammatory cytokines. Therefore, they contribute to the resolution of inflammation, tissue repair and remodelling^[14]. Despite being adopted here, and within the literature at large, one must bear in mind that the M1/M2 dichotomy seems to be an oversimplification, as macrophages with intermediate or different phenotypes

may also be found in the AT^[15]. Although a definitive standard set of markers for the identification of M2 cells is not available yet, a group of molecules often reported in the literature to be associated with this type of cell has been used, since adopting a single marker would be unrealistic^[16]. Arginase-1 (Arg-1) and CD206 are the two most frequently cited markers in AT macrophages classified as M2 cells^[13]. Arg-1 participates in amino acid metabolism, being strongly expressed in macrophages exposed to IL-4^[16]. Arg-1 metabolizes arginine to ornithine and polyamines, thereby inhibiting the production of nitric oxide (NO)^[17,18]. The mannose C-type 1 lectin receptor, CD206, is involved in pathogen recognition by the innate immune system^[19]. Other regularly cited markers of M2 macrophages are resistin-like β (Fizz1), CD301, Retnla, Dectin-1, MGL-1, peroxisome proliferator activated receptor (PPAR) γ and pSTAT6^[16]. Also, several cytokines, mostly with immunosuppressive characteristics, produced by M2 cells, include IL-10, transforming growth factor β (TGF- β) and some chemokines (CCL17, 18, 22 and 24)^[16,20,21].

Most, if not all, of the evidence found so far points out that resident, M2 macrophages, are the primary cells responsible for the homeostatic, anti-inflammatory state in lean AT, ultimately avoiding local and systemic insulin resistance^[13,22]. These cells are frequently found in the interstitial spaces between adipocytes in the lean AT. In obese AT, M2 cells can expand but not as much as the M1-type; some M2 cells are even localized in CLS, where their suggested role may involve the phagocytosis of dead adipocytes, angiogenesis and tissue remodeling^[11,18]. Macrophages recruited into the tip of the gonadal AT promote vascular development during tissue outgrowth^[23]. Other functions include a possible role in adipogenesis, suggested by the finding that lectin-binding CD68⁺ F4/80⁺ CD34⁺ macrophage-like cells are present in the adipogenic aggregates in the developing fat pads of young mice^[24]. M2 macrophages, expressing high levels of MGL-1 and IL-10, have been demonstrated to participate in iron metabolism and perhaps, iron homeostasis in AT, since up to 25% of the macrophages from lean AT have a twofold increase in iron content, making them, basically, ferromagnetic^[25]. Finally, cold exposure can induce alternative activation in macrophages from white AT, promoting tyrosine hydroxylase expression and catecholamine production, factors required for browning of WAT, with expression of uncoupling protein 1 (UCP1) by adipocytes and induction of thermogenic metabolism^[26].

MOLECULAR MEDIATORS INFLUENCING THE M1/M2 BALANCE

Because of their importance to insulin sensitivity and AT homeostasis, it is interesting to know about the mediators of M2 polarization in AT. Adipokines are substances secreted locally by adipocytes. One of them, adiponectin, appears to work mainly *via* enhancing insulin

sensitivity, particularly by impairing liver gluconeogenesis, increasing fatty acid oxidation and promoting glucose uptake^[27]. Adiponectin can also drive M2 polarization in both human and mouse macrophages by increasing the expression of Arg-1, IL-10 and macrophage galactose N-acetyl-galactosamine specific lectin-1 (Mgl-1) molecules^[28], although its effect on already differentiated M1 macrophages is mostly pro-inflammatory^[29]. Notwithstanding, there is ample evidence for the role of adiponectin as an anti-inflammatory molecule. Adiponectin production is higher in the lean AT and inversely correlated with obesity and levels of inflammatory markers such as C-reactive protein and IL-6 and can make macrophages secrete more IL-10^[29]. Recently, Shimizu *et al.*^[30] have found that adiponectin inhibits the production of high mobility group box 1 (HGMb1) proteins, an innate pro-inflammatory, damage-associated molecular pattern (DAMP) molecule, in tumor necrosis factor (TNF) α stimulated 3T3 adipocytes^[30]. Adiponectin decreases the expression of NF- κ B, inflammatory factors on endothelial cells and diminish monocyte migration to tissues. Through its activated receptors AdipoR1 and AdipoR2, adiponectin can down-regulate TNF α and MCP-1 gene expression and upregulate interleukin-1 receptor antagonist (IL-1Ra), respectively^[31].

Fatty acids are another class of molecules acting on macrophages to switch between the M1/M2 program. In general, saturated fatty acids fuel the development of M1 cells, while the unsaturated types aid the rise of alternatively activated phagocytes. For instance, supplementation of mice with dietary fish oils containing eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) can reduce pro-inflammatory gene expression and increase anti-inflammatory gene activity and adiponectin expression^[32]. Long chain omega-3 polyunsaturated fatty acids (PUFAs) may induce M2 polarization associated with down-regulation of pro-inflammatory mediators in inflamed AT from obese mice^[33]. In addition, omega-3 PUFAs can be metabolized into bioactive molecules: Resolvins, protectins and maresins. Titos *et al.*^[33] have also shown that resolvin D1 can decrease IFN γ production, while increasing the expression of Arg-1 in macrophages from AT^[33]. Incubation of macrophages in culture with the lipid mediator maresin R1 (MaR1) diminishes ROS and pro-inflammatory cytokine [IL-1, TNF α , IL-6, interferon- γ (IFN γ)] production and induces upregulation of the type 1 mannose receptor mRNA expression, a M2 marker^[34]. The role of PUFAs as beneficial nutrients or therapeutic agents is being actively investigated in the prevention/treatment of obesity, T2DM and several inflammation-related diseases and is discussed elsewhere^[32].

IL-4 AND IL-13: KEY CYTOKINES IN THE CONTROL OF M2 POLARIZATION

The differentiation and survival of M2 cells are dependent upon exposure to IL-4 and IL-13^[10]. An important

question arises, particularly on the context of lean AT surroundings: Where these two cytokines come from? Conditioned medium from 3T3 L1 adipocytes contains IL-13 but not much IL-4^[35]. It is well known that both IL-4 and IL-13 are produced by the Th2 lymphocyte, IL-4 being its hallmark^[12]. Nonetheless, the role of Th2 CD4⁺ lymphocytes exerting a protective function and control of AT inflammation is under debate^[10]. As explained below, Th2 cells are not the major producers of IL-4 or IL-10 in AT. The major populations expressing the Th2 marker GATA-binding protein 3 in VAT are FoxP3⁺CD4⁺ T-reg cells and group 2 innate lymphoid cells (ILC2's)^[10,36]. Thus, although a Th2-type of response is certainly present (see below), more studies must be made to ascertain the specific function of CD4⁺ Th2 cells in the lean, homeostatic AT.

Recently, it has been demonstrated that innate immune cells such as eosinophils are also a major source of IL-4 in VAT, with their cell numbers inversely correlated with the degree of adiposity^[37]. Using *Gata1*^{-/-} mice (deficient in eosinophils) fed a high-fat diet (HFD), these authors showed animals with increased visceral adiposity, high numbers of M1 macrophages and glucose intolerance/insulin resistance, while IL-4 transgenic mice (enriched in eosinophils) also fed HFD, presented a decrease in these parameters^[37]. Eosinophils are granulocytes involved in the combat to helminth infection and in immunopathological processes such as allergies. Not surprisingly, chronic infection of HFD mice with the helminth *Schistosoma mansoni* triggered strong increases in eosinophil and M2 macrophage numbers in white adipose tissue (WAT) together with improved insulin resistance, better glucose uptake and WAT insulin sensitivity. More importantly, the effects with injections of *S. mansoni*-soluble egg antigens extract (SEA), instead of the entire helminth, were similar^[38]. Eosinophils are not only helpful in decreasing the AT pro-inflammatory milieu and its adverse metabolic effects. Uncoupling protein-1 (UCP1) is critical for non-shivering thermogenesis since it interrupts the mitochondrial electrochemical gradient, creating a proton leak where the excess energy expenditure is dissipated in the form of heat. Precursor-adipocytes from WAT can adopt this cycle, expressing UCP1 and turning into so-called "beige" adipocytes; and this process can protect against obesity^[39]. Eosinophil-derived cytokines, signaling through STAT6, are required for the activation of adipocyte "beiging", since depletion of eosinophils or knockdown of *Il4ra* in macrophages both result in impaired AT beiging in response to a cold challenge^[26]. These authors also showed that treatment with recombinant IL-4 boosts UCP1 expression in both VAT and SAT, resulting in weight loss and improved glucose tolerance and insulin sensitivity.

Eosinophil differentiation and activation is dependent of GM-CSF, IL-3 and IL-5. Moreover, IL-5 is a signal for eosinophil migration and survival in ATs with IL-13 helping to enhance eosinophil's chemotaxis^[40,41]. To keep eosinophils fostering M2 macrophages and an anti-inflammatory milieu in the homeostatic AT, a major source of IL-5 and IL-13 was investigated and zeroed in on a recently discovered

subset of non-T cells named group 2 innate lymphoid cells or ILC2's^[41]. These cells resemble Th2 cells in their cytokine production but do not have T-cell receptors. The transcription factors retinoic acid receptor-related orphan receptor and GATA-binding protein 3 are important for ILC2's development. The role of IL-33 has been associated with tissue repair, parasite elimination, asthma and allergy^[42]. Molofsky *et al.*^[41] used mice where IL-5- and IL-13-producing cells were eliminated: In this model, they observed the disappearance of ILC2's, eosinophils and anti-inflammatory macrophages in VAT. Furthermore, ILC2's displayed a lower cell count in VAT from mice under HFD and IL-33 was identified as the cytokine able to rapidly promote the activation of ILC2's and the accumulation eosinophils and alternatively activated macrophages in VAT^[41].

ROLES OF ILC'S AND IL-33 IN THE MAINTENANCE OF LEAN AT

IL-33 is rapidly acquiring growing importance for the maintenance of an anti-inflammatory status in the AT and amelioration of obesity-related insulin resistance. Originally described as a member of the IL-1 cytokine family, IL-33 signals through its receptor, ST2 (suppression of tumorigenicity 2), present in several cell types such as Th2 lymphocytes, mast cells, CD8⁺ T cells, natural killer (NK) cells and, more importantly, ILC2's and T-reg cells. Both IL-33 and ST2 are strongly expressed in AT^[43-45]. Administration of IL-33 to obese mice improved both adipose-tissue inflammation and systemic insulin resistance, attributed by the authors to this cytokine's ability to promote polarization of macrophages to an M2-like phenotype and to foster the differentiation of Th2 cells^[43]. Furthermore, mice deficient in ST2 and fed HFD developed a high body weight and fat mass, glucose intolerance and impaired insulin sensitivity. Similarly, *Il-33*^{-/-} mice have aberrant metabolic parameters such as elevated AT mass and insulin/glucose disturbances even when fed a normal diet^[46]. Within the same study, Brestoff *et al.*^[46] also demonstrated the critical importance of IL-33 for the accumulation and maintenance of ILC2's in human WAT and went further to show mechanisms of IL-33/ILC2's metabolic regulation of homeostasis, such as *in vivo* beiging of WAT and production of methionine-enkephalin peptides by ILC2's that can act directly on adipocytes to upregulate the expression of *Ucp1*^[46].

Obesity inversely correlates with the amount of anti-inflammatory T-regs in the AT. In comparison with their lymphoid-tissue counterparts, a unique population of resident Foxp3⁺CD4⁺ T-reg cells accumulates in VAT of lean mice^[47-49], and they are highly overrepresented in lean individuals (40%-80% vs 5%-15% of the Foxp3⁺CD4⁺ T-cell compartment). These T-regs have a distinct transcriptome, particularly the profile of transcripts encoding transcription factors, cytokines/chemokines and their receptors as well as an atypical expression of molecules involved in lipid metabolism. They also

have an unusual, clonally expanded, repertoire of T-cell antigen receptors. Importantly, in rodents where these AT T-regs were experimentally deleted, an increase in AT inflammation (represented by high levels of TNF α , IL-6 and RANTES) and acutely reduced insulin sensitivity was observed^[47]. The unique phenotype of this AT T-regs population was emphasized as well by their expression of PPAR γ , a transcription factor usually associated with adipocyte differentiation and function^[48]. However, PPAR γ also drives T-regs cell accumulation, phenotype and function in visceral AT. The injection of pioglitazone, an agonist of PPAR γ , could increase the numbers of these AT T-regs in VAT and restore insulin sensitivity of mice under HFD^[48]. Interestingly, Han *et al.*^[49] have reported, in comparison with other T-regs populations, higher levels of the ST2 chain of the IL-33 receptor in most AT T-regs. The proportion of these ST2⁺ T-regs was reduced in obese VAT and their numbers could be restored by injections of recombinant IL-33, which was also able to reduce VAT inflammation and decrease insulin resistance in mice under HFD^[44,49,50]. Human omental AT T-regs cells also showed high ST2 expression, suggesting an evolutionarily conserved requirement for IL-33 in VAT-Tregs cell homeostasis^[50]. Thus, IL-33 promotes the accumulation and function of both ILC2 and T-regs cells. Interestingly, although AT T-regs can also respond directly to IL-33, *in vivo* ILC2-intrinsic activation by IL-33 is required before VAT T-regs cells accumulation^[51].

IL-10 is a classical immunosuppressive cytokine, which induces a general anti-inflammatory effect on monocytes/macrophages, T and B cells, mast cells and NK cells^[52]. If stimulated with IL-10, macrophages can turn into M2 cells, also secreting IL-10^[13,16]. VAT T-regs from 30-wk lean mice showed upregulated IL-10 expression as compared to conventional T-regs. Up to 13.9% of VAT T-regs express IL-10 (contrary to 1.8% of conventional T-regs) as detected by flow cytometry^[47]. The high expression of IL-10 by VAT T-regs is altered after HFD since VAT T-regs from obese mice display a significant reduction in IL-10 production^[53]. Because IL-10 is necessary for T-reg - mediated suppression of TNF α production from macrophages, this obesity-induced change in VAT T-reg function most likely contributes to inflammation and insulin resistance^[53].

THE ROLE OF NUCLEAR RECEPTORS IN THE INDUCTION OF M2 DIFFERENTIATION

M2 cells preferentially use fatty acids and oxidative metabolism, while M1 cells utilize glucose^[54], which is comprehensible since the latter needs increased levels of reactive-oxygen species (ROS) and NO to better perform their microbicidal activities. Interestingly, pushing oxidative metabolism into M1 macrophages seems to change their phenotype towards a M2-type^[55]. On the other hand, after IL-4 stimulation, STAT6 activation on macrophages can induce the co-activator protein PPAR γ -coactivator-1 β

(PGC1- β), which promotes mitochondrial respiration and biogenesis. PGC1- β is considered an important metabolic trigger for the switch towards the M2 profile^[55]. STAT6 activation also induces the transcriptional regulators PPAR γ and PPAR δ , both helping in the maintenance of the M2 phenotype: PPAR δ induces the expression of MGL-1, a marker often found on M2 cells^[13,55]. In addition, knock-down of PPAR δ can lead to insulin resistance^[56], demonstrating that its function is important for the expression of anti-inflammatory mediators by M2 macrophages. Another marker of M2 cells, arginase-1, is highly responsive to agonists of both PPAR γ and PPAR δ and the arginine metabolism is a relevant feature of M2 cells^[55,57]. Disruption of PPAR γ impairs the maturation of M2 macrophages and leads mice towards diet-induced obesity, glucose intolerance and insulin resistance^[58]. Treatment of macrophages from *ob/ob* mice with a thiazolidinedione (rosiglitazone), a pharmacological activator of PPAR γ , can induce anti-inflammatory M2 markers such as Arg1 and reduce the number of M1 macrophages even in *ob/ob* mice^[59]. Therefore, both PPAR γ and PPAR δ are important activators of M2 differentiation. Another regulator of the arginase-1 gene (*Arg1*), the hypoxia inducible factor-2 α (HIF-2 α), seems an important driver of M2 phenotype^[60]. However, in this context, the function of HIF-2 α still needs to be better elucidated since it also induces NF- κ B, a pro-inflammatory transcription factor^[61]. Macrophage metabolism seems to be important for insulin sensitivity and new investigations on this area will most certainly bring a better understanding of the role of macrophages in the AT on its homeostatic state.

LOW-GRADE CHRONIC INFLAMMATION OF THE AT: T CELLS AT PLAY DURING OBESITY

The establishment of a pro-inflammatory phenotype is viewed as the link between the development of obesity and the evolution of obesity towards insulin resistance and ultimately T2DM and its associated cardiovascular burden^[62].

As discussed above, during the development of obesity, hypertrophied AT experiences a stronger infiltration by macrophages and other immune cells and, critically, these infiltrating immune cells are mainly pro-inflammatory, as opposed to the milder infiltration in AT of the lean which is chiefly constituted by anti-inflammatory lineages^[3]. Infiltration of the AT by proinflammatory M1 macrophages occurs at an advanced stage of AT hypertrophy, and, while being necessary to promote inflammation, can be viewed as a secondary event^[4,5]. On the contrary, recent research promotes the idea that the initial events leading to the regulation of obesity-induced inflammation can be attributed to T cell lineages^[63]. The lean AT is populated by resident anti-inflammatory CD4⁺ Foxp3⁺ T-regs and Th2 cells. These T cells secrete IL-10, an anti-inflammatory cytokine known to improve adipocyte insulin sensitivity of adipocytes^[47], but also systemically as mice overexpressing

Table 1 Summary of the key adipose tissue-infiltrating immune cells and secreted cytokines contributing to the pro-inflammatory status of adipose tissue in obesity and the anti-inflammatory status in lean individuals

Immune cell lineage	Main secreted cytokines	Biological activity	Lineage-inducing stimulus	Ref.
Pro-inflammatory AT in the obese condition				
M1 macrophages	IL-1 β	Recruited at the advanced stage of AT hypertrophy during obesity	Induced by saturated fatty acids	[4,5]
Th1	TNF α	Induce the recruitment of M1 macrophages to the AT		[65]
Th17	IL-17/IL-22	Induce the recruitment of M1 macrophages to the AT	Induced by purinergic signalling	[65,71,73]
Anti-inflammatory AT in lean individuals				
M2 macrophages	IL-10, TGF- β Multiple chemokines (CCL17, 18, 22)	Secretion of multiple immunosuppressive cytokines and chemokines Phagocytosis of dead adipocytes	Induced by omega-3 polyunsaturated fatty acids Induced by IL-4 and IL-13	[16,20,21]
T-regs	IL-10	Promote polarization of M2 macrophages	Constitutively present in the AT of lean individuals	[13,16,47]
Th2	IL-4 and IL-13	Promote polarization of monocytes into M2 macrophages	IL-33	[43]
Eosinophils	IL-4	Promote "beiging" of adipose tissue. Promote UCP1 expression	Differentiation and activation dependent on GM-CSF, IL-3 and IL-5	[26]
ILC2's	IL-5 and IL-13	IL-5 and IL-13 secretion by ILC2's promotes eosinophils differentiation	IL-33 promotes the activation of ILC2's	[41]

AT: Adipose tissue; ILC2s: Group 2 innate lymphoid cells; UCP1: Uncoupling protein 1; IL: Interleukin; TNF: Tumor necrosis factor; TGF: Transforming growth factor.

IL-10 in skeletal muscle subjected to a high fat diet did not develop insulin resistance albeit becoming markedly obese^[64]. During the transition from a lean phenotype to obesity, infiltrating anti-inflammatory CD4⁺ decline, and pro-inflammatory T lineages Th1 and Th17 become predominant. Pro-inflammatory T cell infiltration precedes macrophage infiltration and, *via* the secretion of pro-inflammatory IL-17 and TNF α drive the expansion of the inflammatory state^[65]. In addition to this inflammatory amplification driven by Th1/Th17 cells and IL-1 β -producing M1 macrophages, the adipocytes are also secreting several pro- and anti-inflammatory adipokines that participate to the regulation of metabolic homeostasis. Notably, adipocytes of lean individuals mainly secrete anti-inflammatory adiponectin, while obese AT secretes pro-inflammatory IL-6^[66]. Therefore, a crosstalk between adipocytes and the immune cell populations infiltrating AT maintains an anti-inflammatory state in physiological conditions, but can switch to a state of sub-clinical inflammation characterized by an IL-1 β , IL-6 and IL-17- rich environment, a prerequisite for insulin resistance, during the development of obesity (Table 1).

IL-17: A NOVEL PLAYER IN OBESITY-INDUCED INSULIN RESISTANCE

The contribution of pro-inflammatory cytokines IL-1 β and TNF α in mediating insulin-resistance in the obese state is now widely accepted and has been comprehensively reviewed elsewhere^[67,68]. Similarly, IL-6, which is often increased in pro-inflammatory settings, is likewise viewed

as a pro-inflammatory cytokine, although such notion is now in part disputed and IL-6 might serve both in pro- and anti-inflammatory context depending on the global environment and the balance with other pro- or anti-inflammatory mediators^[69,70].

More recent data have also called into action Th17 cells - and their secreted cytokine IL-17 - in the establishment of inflammation associated to obesity^[71]. Studies in a mouse model indicated that T-cells derived from diet-induced obese mice accumulated the Th17 subset, thereby releasing IL-17 in an IL-6-dependent fashion^[72].

In a more clinically relevant setting, a 3 to 10-fold accumulation of IL-17 and IL-22 secreting Th17 cells was observed in AT from insulin-resistant obese subjects^[73], in VAT from morbidly obese women^[74] and in peripheral blood from obese children. Also, increased plasmatic levels of IL-17 have been observed in obese women^[75].

From a mechanistic point of view, several mechanisms, perhaps not mutually exclusive, have been proposed as participating into the polarization of T cells towards the Th17 lineage in obesity. Purinergic signaling resulting from the activation of the P2X7 receptor by ATP have been shown to promote Th17 polarization within the AT microenvironment^[76]. Also, co-culture of mature adipocytes derived from obese donors with peripheral blood mononuclear cells promoted increased release of IL-17 and IL-22 by the latter, and this cytokine production exacerbated inflammation by amplifying IL-1 β secretion by macrophages.

The two IL-17 isoforms, IL-17A and IL-17F, are central mediators of inflammation and contribute to the development of multiple autoimmune disorders and are thus

attractive therapeutic targets^[77]. The IL-17 receptors IL-17RA and IL-17RC are ubiquitously expressed, explaining the large spectrum of activities of these two cytokines^[78]. In addition to its pro-inflammatory action, IL-17 might affect metabolic homeostasis by inhibiting adipogenesis^[79]. The inhibition of adipogenesis, in the context of an hypercaloric diet, would hamper lipid storage in the AT and favor the increase in circulating levels of free fatty acids, contributing to the worsening of insulin resistance.

TREATING METABOLIC INFLAMMATION WITH TARGETED THERAPIES

The current understanding that inflammatory events in obesity and T2D are mediated by multiple cytokines, including IL-1 β , IL-6, TNF α and IL-17 produced by various cell types within the AT has lent support to the idea that inhibition of these cytokines by specifically designed inhibitory antibodies might curb the progression of the obese phenotype towards insulin resistance and diabetes^[80]. Several inhibitory antibodies acting on the IL-1 system, IL-6 and TNF α ^[81] have been tested in indications related to obesity and the metabolic syndrome^[82]. Canakinumab, an IL-1 β inhibitory antibody, originally used to treat pro-inflammatory diseases, has more recently been used in several clinical trials aiming at treating T2D and has been shown to induce mild improvements in glycated hemoglobin and beta cell functioning in patients with T2D^[83].

Undoubtedly, with the improved understanding of the anti- and pro-inflammatory phenomena playing a role in the development of obesity and T2D that we tried to summarize here, more efforts will be done in the next future to try to bring to clinical fruition targeted therapies aiming to treat metabolic inflammation *via* the inhibition of pro-inflammatory mediators or the activation of anti-inflammatory pathways.

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Immunological aspects of age-related diseases

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Abstract

The proportion of elderly people rises in the developed countries. The increased susceptibility of the elderly to infectious diseases is caused by immune dysfunction,

especially T cell functional decline. Age-related hematopoietic stem cells deviate from lymphoid lineage to myeloid lineage. Thymus shrinks early in life, which is followed by the decline of naïve T cells. T-cell receptor repertoire diversity declines by aging, which is caused by cytomegalovirus-driven T cell clonal expansion. Functional decline of B cell induces antibody affinity declines by aging. Many effector functions including phagocytosis of myeloid cells are down regulated by aging. The studies of aging of myeloid cells have some controversial results. Although M1 macrophages have been shown to be replaced by anti-inflammatory (M2) macrophages by advanced age, many human studies showed that pro-inflammatory cytokines are elevated in older human. To solve this discrepancy here we divide age-related pathological changes into two categories. One is an aging of immune cell itself. Second is involvement of immune cells to age-related pathological changes. Cellular senescence and damaged cells in aged tissue recruit pro-inflammatory M1 macrophages, which produce pro-inflammatory cytokines and proceed to age-related diseases. Underlying biochemical and metabolic studies will open nutritional treatment.

Key words: Elderly people; Damage associated molecular patterns; Immune dysfunction; Lymphoid lineage; Myeloid lineage; Shrinkage of thymus; Cytomegalovirus; Age-related tissue damage; Cellular senescence; Pro-inflammatory

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Core tip: The authors divide immune cells, which are involved in age-related pathological changes, into two categories. First category is aging of immune cell-itself, which include age-related myeloid lineage deviation of hematopoietic stem cells, the shrinkage of thymus followed by the decline of naïve T cells, the cytomegalovirus infection-mediated decline of T-cell receptor repertoire diversity and functional decline of myeloid cells. Second category is the involvement of immune cells to age-related pathological changes. Age-related tissue damage and cellular senescence activate pro-inflammatory (M1) macrophages, which induce many age-related diseases.

Biochemical works are needed to further elucidate age-related immune changes.

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INTRODUCTION

The proportion of elderly people rise world wide, especial in the developed countries. Aging-related changes in the immune system contribute to the increased susceptibility of the elderly to infectious diseases, cardiovascular disease and stroke caused by atherosclerosis, autoimmune disease such as rheumatoid arthritis, cancer and late onset anemia and degenerative diseases including Alzheimer diseases^[1,2]. Further metabolic syndrome, which is caused by obesity occurs from middle age and proceeds to tissue failure such as renal failure in advanced age, is tightly related to immune system. Chronic infections such as hepatitis virus and chronic tissue damages caused by tobacco inhalation induce tissue damage, which arouses immune responses and wound repair responses. Chronic inflammation follows tissue fibrosis in advanced age proceeding to tissue failure such as chronic obstructive pulmonary disease. Most prominent cause of age-related immune dysfunction is T cell immunosenescence. There are three causes of T cell immunosenescence. One is the age-related hematopoietic stem cells (HSCs) deviation from lymphoid lineage to myeloid lineage. Second is the shrinkage of thymus. Third is expansion of T cell clones to cytomegalovirus (CMV). Changes of HSCs also affect immunosenescence. HSCs deviate to myeloid lineage by aging. Both in mouse and human myeloid-lymphoid ratio elevates by aging^[3,4], which induces the decline of lymphoid cells (T and B cells) and erythrocytes and contribute age-related anemia and decline of adaptive immunity^[5,6]. The number of aged B cells decline and affinity and diversity of antibodies are low^[7]. Ageing related myeloid deviation increases the number of myeloid cells. However, the oxidative burst and phagocytosis of both neutrophils and macrophages are decreased^[8]. The antigen presentation of aged dendritic cells and the cytotoxicity of Natural killer cells are low^[9]. Pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor α (TNF- α) and IL-1 β are elevated in elderly people, which are called inflammaging and induce metabolic syndrome such as obesity, type II diabetes, atherosclerosis, cardiac diseases^[10]. However, other reports have shown that pro-inflammatory (M1) macrophages are replaced by anti-inflammatory (M2) macrophages by aging^[11]. Here to solve this discrepancy we propose to classify age-related immune changes as follows. Age-related pathological changes are classified as immune cell intrinsic changes by aging and involvement of immune cells to age-related pathological changes.

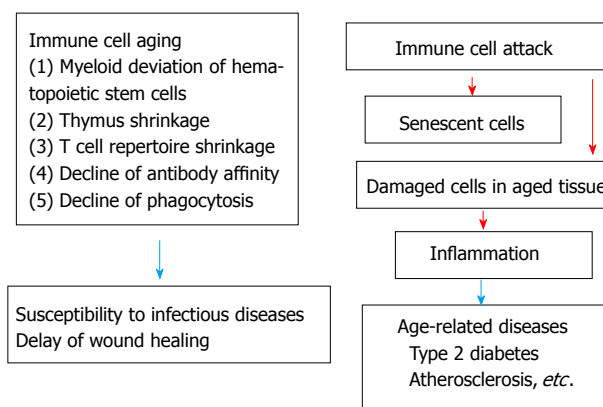


Figure 1 Two types of engagement of immune cells to aging.

Increased susceptibility of the elderly to infectious diseases is mainly caused by age-related immune cell intrinsic changes usually called immunosenescence. Metabolic syndrome and other related diseases, which occur at aged people, are mainly caused by immune cell attack to age-related tissue changes. Age-related tissue failure is caused by repeated immune cell attack. Here we first describe the recent findings from the points of immune cell intrinsic changes by aging and then go to age-related tissue changes, which induce immune reaction (Figure 1).

IMMUNE CELL INTRINSIC CHANGES BY AGING (IMMUNOSENESCENCE)

HSCs aging

Adult immune cells develop from HSCs that originate from the bone marrow (BM) (Figure 2). From HSCs common lymphoid progenitor cells (CLPs) and myeloid precursor cells develop. T cells emigrate from BM to enter thymus, where they develop to naïve CD4 and CD8 T cells. B cells develop in bone marrow from pre-pro-B cells, pro-B cells and emigrate from bone marrow as pre-B cells to circulation and secondary lymph nodes and spleen. Finally B cells further differentiate to plasma cells to produce antibodies (Figures 2 and 3).

Although the cellularity of BM decreases by aging^[12], the number of HSCs in aged mice differ between mouse strains. The number of HSCs of aged-C57BL/6 mice increases, whereas other strain of mice decreases^[13,14]. However, in HSCs of aged mice the self-renewal activity and homing activity to BM are two-fold less efficient compared with young HSCs^[2,6,15-17]. It has been found by transplantation experiments that old HSCs tend to have a myeloid-skewed blood cell production caused by a decreased ability to produce lymphoid cells^[3,18]. These age-related changes of HSCs induce age-related abnormalities such as myeloid leukemia^[19], late onset anemia^[20]. Biochemical mechanism underlining HSC aging has been elucidated recently. The small RhoGTPase Cdc42 in aged HSCs is elevated, which correlates with a loss of polarity in aged HSCs. It has been found that

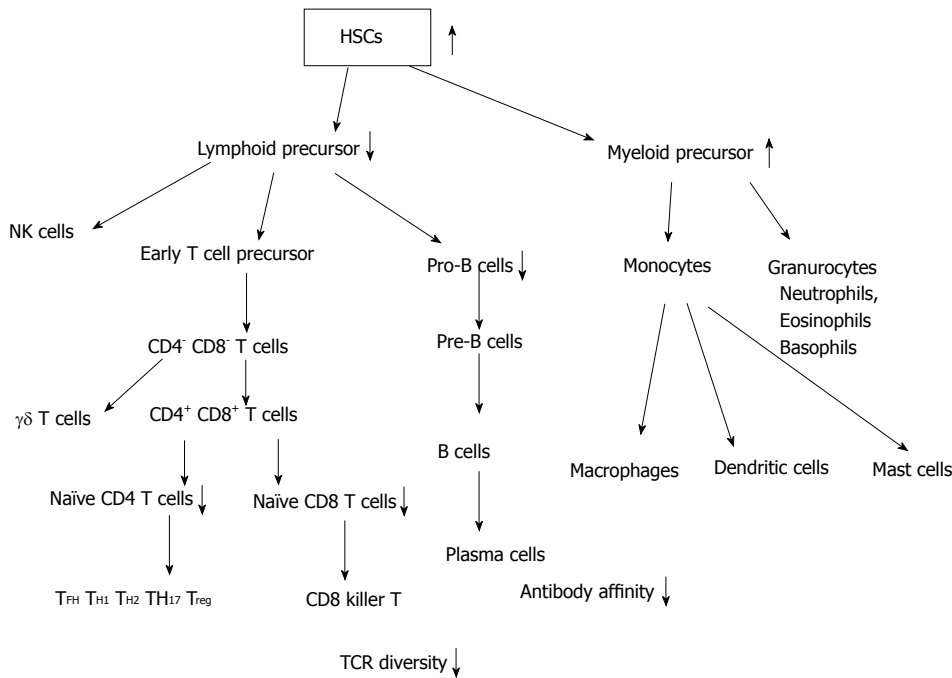


Figure 2 Hematopoietic stem cells to mature immune cells. HSCs: Hematopoietic stem cells; TCR: T-cell receptor.

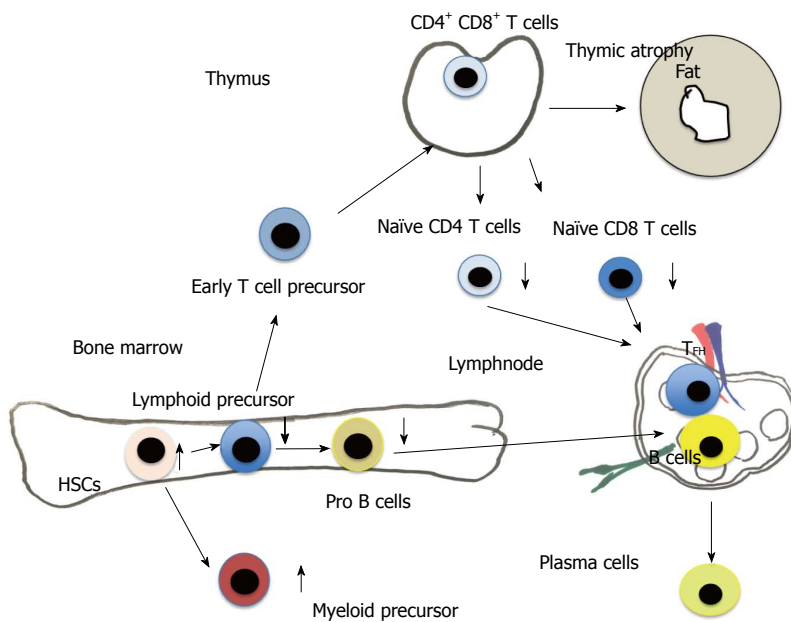


Figure 3 Differentiation of hematopoietic stem cells to mature immune cells.

in young HSCs Cdc42 and tubulin are asymmetrically distributed at the same location, whereas in aged HSCs Cdc42 and tubulin were distributed throughout the cell body in an unpolarized fashion^[21]. HSCs, which produce all mature blood cells, must counterbalance the deleterious effects of chronic stress by generating new cells to replace those that become damaged or destroyed. Cells adapt chronic stress by autophagy and apoptosis^[22]. Autophagy adapts starvation by using degraded own cell organelles for nutrient. One report has shown that autophagy is suggested to be decreased by aging^[23]. However, another report has shown that

by electron microscopic analysis autophagy is increased by aged mice, and old HSCs retain an intact FoxO3a-driven pro-autophagy gene program^[24]. *FOXO* genes are key regulators of longevity downstream of insulin and insulin like growth factor signaling^[25]. MicroRNAs, a class of small-noncoding RNAs, regulate normal function of HSCs, including cell cycling and engraftment potential^[26]. The microRNA-212/132 cluster (Mirc19) is enriched in HSCs and is up regulated during aging and plays a role in maintaining balanced hematopoietic output by buffering FOXO3 expression^[27]. There exist many other molecules, which relate HSCs aging. For instance recent work

done by Chang *et al.*^[28] showed that ABT263 (a specific inhibitor of the anti-apoptotic proteins BCL-2 and BCL-xL) rejuvenated aged HSCs.

T cell aging

One of the most early and dramatic changes in aging of immune system is the decrease of naïve T cells. Naïve T cells are derived from thymus and continuously recirculate through the peripheral lymphoid tissues. Naïve T cells differentiate into effector/memory T cells after primed with cognate antigen presented by dendritic cells. Naïve T cells originate from thymus. Naïve T cell production from thymus declines very early only after first year of life. These naïve T cell decline comes from the early thymic involution^[29,30] (Figures 2 and 3). This dramatic decline of human naïve T cell output is compensated by homeostatic proliferation^[31,32]. CD4⁺CD45RA⁺CCR7⁺ human naïve T cells are recently found to be divided by CD31. CD31⁺ naïve T cells are recent thymic emigrants (RTEs). By aging CD31⁺CD4⁺CD45RA⁺ decrease, while CD31⁺CD4⁺CD45RA⁺ increase during healthy aging by homeostatic proliferation^[33-35]. Recent work using neonatal thymectomy has shown that total number of CD3⁺, CD4⁺ and CD8⁺ cell numbers decrease in the first year after neonatal thymectomy. CD4⁺CD45RA⁺CCR7⁺ and CD31⁺CD4⁺CD45RA⁺ naïve T cells decrease, while effector memory CD4⁺ T cells (CD45RA⁺CCR7⁻) and central memory CD4⁺ T cells (CD45RA⁻CCR7⁺) increase^[36]. A human memory T cell subset with stem cell-like properties (CD4⁺CD45RA⁺CCR7⁺CD28⁺CD27⁺FAS⁺), which has been shown recently^[37] increase relatively. Wijk's group has shown recently that after more than 10 years of thymectomy most children have thymic regeneration and show almost the same number of CD31⁺ naïve T cells compared to healthy control. Only small numbers of children have no thymic regeneration with lower T cell count and low percentage of naïve T cells compared to thymectomized CD31⁺ children^[38]. Another report, which uses *in vivo* ²H₂O labeling, has shown that compensatory increase of peripheral T-cell division rates are not required to compensate the age-related decrease of thymic output^[34]. From these findings, Goronzy *et al.*^[39] summarized that even in early adulthood, thymic output contributes little to the maintenance of the size of the naïve T cell compartment. In order to build T cell repertoire, thymic output is necessary only in early life. To maintain T cell repertoire, thymic output is not necessary. Homeostatic proliferation of the existing T cell pool is enough to maintain CD4 T cell repertoire. Both IL-2 and IL-7 drive homeostatic proliferation and IL-2 receptor (CD25) expression is increased by aging. In contrast to CD4 naïve T cells, the CD8 naïve T cell compartment shrinks with age, which induces higher homeostatic proliferation in aged-CD8 naïve T cells than that in aged-CD4 naïve T cells^[39]. Another cause of T cell dysfunction of aged people instead of decreased production of naïve T cells is the decline in TCR repertoire diversity, caused by cytomegalovirus-driven T cell clonal

expansions^[40]. The naïve T cell repertoire are in the range of approximately 2.5×10^7 in humans. These repertoires are hijacked by CMV infection^[41]. Virus-specific clonally expanded CD8 T cells occupy more than 50% of memory pool and reached to 90% in aged mice^[42]. Naïve T cells are thought to be the precursor of memory T cells. By infection memory T cells respond quickly by producing cytokines, granzyme and perforin. Recently, new type of CD8 T cells have been discovered called "memory T cells with a naïve phenotype" (T_{MNP} cells), increased with age. T_{MNP} cells have Naïve T cell phenotypes, but quickly respond to infection by producing cytokines, granzyme and perforin. T_{MNP} cells have unique V 14 T cell receptor. T_{MNP} cells bound to multimers of CMV or EBV peptide and MHC class I^[43]. These results mean that some population of CD8 T cells, which are expanded by CMV, work as effective protection of other virus infection. Further works are needed to elucidate the effects on age-related T cell dysfunction caused by CMV infection. Here we do not describe B cell dysfunction of aged people. B cell function decreases directly by B cell aging itself and by the result of age-related T cell functional decline, because helper T cell affect B cells by direct binding and secreting cytokines.

Myeloid cell aging

Innate immune cells include granulocytes (neutrophils, eosinophils, basophils), macrophages, dendritic cells, natural killer cells and innate lymphoid cells and others^[44]. Among them "myeloid cell" here include granulocytes, macrophages and dendritic cells. We review recent findings, which relate to myeloid cell intrinsic aging. Phagocytosis is the most important effector function of myeloid cells especially neutrophils. Neutrophils play a crucial role in the immune defense against bacterial and fungal pathogens^[45]. Neutrophils survive for only 8-12 h in the circulation and up to 1-2 d in tissues^[46]. Neutrophils from aged human have impaired phagocytosis of bacteria^[46,47]. DHEA (Dehydroepiandrosterone sulfate), which increases the phosphorylation of p47phox *via* PKC- β and generate neutrophil superoxide to kill bacteria, decreases in old age^[48]. Neutrophils also work as an important player in wound healing. Neutrophils normally begin arriving at the wound site within minutes of injury, continuing for several days. Molecules called damage associated molecular patterns (DAMPs) are produced from wounded tissue cells, which activate neutrophils. Neutrophils engulf damaged cells and produce inflammatory cytokines and chemokines, which facilitate wound repair. We have shown that in aged C57BL/6 mice, wound healing is relatively inefficient. Anti-Gri-1 treatment delayed wound healing in aged mice, whereas G-CSF injection increased wound repair^[49]. In bacterial infection and wound healing, macrophages come to work after neutrophil and engulf dead cells including dead neutrophils.

Every tissue contains tissue macrophages, which are sometimes called tissue specific names such as microglia in brain, Langerhans cells in skin and kupper cells in liver.

Important functions of tissue macrophages include innate defense against infection and wound healing. Origin and turnover of tissue macrophages depend on tissue types. Adult's microglia derive from primitive myeloid progenitors of yolk sac that arise before embryonic day 8^[50]. Further non-parenchymal macrophages such as perivascular, meningeal and choroid plexus macrophages, which mediate immune responses at brain boundaries, also arise from yolk sac precursors during embryonic development and remain a stable population^[51]. Langerhans cells predominantly derived from embryonic fetal liver with a minor contribution of yolk sac^[52,53]. Tissue-resident macrophages with low turn over are self-maintain locally throughout adult life with minimal contribution from circulating monocytes^[54] and self renew after birth^[55]. In heart cardiac macrophages are primarily embryonic and are self-renewed, which reside throughout myocardium^[56]. Recent data have shown that by advanced age these embryo-derived tissue macrophages are replaced by blood monocytes derived from HSCs. In the heart embryo-derived cardiac macrophages decline self-renewal by age and are progressively substituted by monocyte-derived macrophages^[57]. Intestinal macrophages have a high turnover rate and are constantly replaced from bone marrow derived Ly6C⁺ blood monocytes from early lifetime^[58]. In peritoneal cavity, embryo-derived macrophages retain self-renewal at least until 4 mo in mice. By aging embryo-derived macrophages in peritoneal cavity are replaced by bone marrow-derived monocytes^[59].

There exist two types of macrophages namely M1 macrophages and M2 macrophages. M1 (inflammatory) macrophages produce NO radical from arginine with IFN γ or LPS stimulation, whereas M2 macrophages produce TGF β 1 and inhibit NO synthase and stimulate arginase. Arginase converts arginine to ornithine, which induces cell division^[60]. M2 macrophages are further subdivided into M2a and M2c. M2a is activated by IL-4/IL13, and relates to wound-healing process^[61]. M2c is activated by IL10, which has anti-inflammatory activity^[62]. It has been shown that macrophages of aged mice polarize to M2 macrophages^[11,63]. M2 macrophages promote angiogenesis in age-related eye disease^[11]. M2a macrophages have been shown to induce muscle aging by inducing fibrosis. Transplantation of young BM cells to aged mice shows fewer M2a macrophages and less accumulation of collagen^[64]. These findings of age-related M2 predominance contradicts so called "inflammaging", which predominates pro-inflammatory state.

AGE-RELATED TISSUE CHANGES, WHICH INDUCE IMMUNE CELL ATTACK

Although aged HSCs have reduced regenerative potential, aged HSCs are biased toward myeloid differentiation at the expense of lymphoid differentiation^[65]. Chronic, low-grade, systemic inflammation is the primary risk factor for major human chronic diseases, including

cardiovascular disorders, cancer, type 2 diabetes, and neurodegenerative disease. Increased production of inflammatory mediators that accompany this process, referred to as "inflammaging"^[66]. This inflammatory tendency in aged human has wide variability. The mechanism of inflammaging has still not reach complete understanding.

Damaged macromolecules and self-debris released as a consequence of cell/organelle injury work as DAMPs, which may activate pro-inflammatory macrophages. These DAMPs include free radicals from oxidative stress and advanced glycation end products, ATP, fatty acids and amyloid. Another sources, which are up regulated by aging, are leaked microbial products from gut working as pathogen associated molecular patterns (PAMPs)^[65]. These DAMPs and PAMPs may work as stimulator to M1 macrophages. These inflammaging and age-related M2 macrophage activation described previously seem to contradictive. How to consider this discrepancy? By acute infection or injury, neutrophils and M1 macrophages first predominate to the infected or injured site, then M2 macrophages resolve inflammation and induce tissue repair. In chronic inflammation, M1 macrophages continue to exist in inflamed tissue with late coming M2 macrophages. We speculate that both tissue fibrosis caused by M2 macrophages and pro-inflammatory status caused by M1 macrophages continue to exist in chronic inflammation. In acute infection or acute tissue damage, M2 macrophages compensate inflammation caused by M1 macrophages and induce tissue repair. However, in persistent infection such as hepatitis C or repeated tissue damage such as smoking, M1 macrophages, which work as inflammation and M2 macrophages, which resolve tissue damages by producing matrix materials, exist at the same time. Predominance of either M1 or M2 macrophages may dependent on disease process.

Metabolic syndrome and macrophages

Metabolic syndrome, which has the risk to proceed to Type 2 diabetes and atherosclerosis, is caused by obesity. Now it is established that visceral and subcutaneous white adipose tissues (WAT) are different concerning metabolic activity. Lipolytic effect of catecholamines is lower in visceral WAT than that in subcutaneous WAT^[67]. Two main causes of insulin resistance (which causes type 2 diabetes) are saturated fatty acids and pro-inflammatory cytokines. Saturated fatty acids can bind to the Toll-like receptors (TLR) 2 and 4, especially in adipocytes and macrophages, which induce c-Jun N-terminal kinase (JNK), phosphorylate IRS1 and decrease insulin receptor signaling. Saturated fatty acids also produce pro-inflammatory cytokines^[68,69]. The expansion of WAT leads to necrosis of hypertrophic adipocytes, which produce DAMPs. DAMPs recruit macrophages and produce pro-inflammatory cytokines, which aggravate insulin resistance^[70]. Atherosclerosis is mediated by the recruitment of monocyte-derived macrophages into the subendothelial space, where they ingest the lipoproteins^[71]. Type 2 diabetes proceeds to

many other age-related diseases such as renal failure. Atherosclerosis also proceeds to stroke, coronary heart disease. Thus macrophages especially M1 macrophages are engaged in early stage of age-related diseases.

Clearance of senescent cells by immune cells

Not only M1 macrophages but also aged-other tissue cells produce pro-inflammatory cytokines. Vascular aging enhanced secretion of inflammatory mediators from aging endothelial cells and vascular smooth muscle cells from disease free animals^[72]. One explanation of this age-related secretion of inflammatory mediators is senescence associated secretory phenotype (SASP). SASP is produced by senescent cells. From the studies using mice and aged human, aged innate immune system show elevation of basal inflammation^[73]. Especially older human have elevated level of pro-inflammatory cytokines^[74,75]. However, we should keep in mind that cellular senescence is an independent phenomenon to *in vivo* aging. Cellular senescence triggers tissue remodeling in embryonic tissue development and in tissue damage. Cellular senescence also engages in age associated loss of tissue functions^[76,77]. Cellular senescence has been first described by Hayflick at 1961 as limited replicative potential of human fibroblasts *in vitro*^[78]. Cellular senescence is cell cycle arrest with or without telomere shortening after cell division. There exist p53/ p21^{Waf1} pathways and p16^{INK4a}/pRB pathways in cellular senescence^[79]. Whether senescent cells contribute to age-related diseases remains unclear. Some reports have shown that senescent cells have a protective role in atherosclerosis. Mice lacking senescent markers such as p53, p21 or p19Arf show accelerated atherosclerosis^[76]. Senescent cells produce SASPs, which attract immune cells and eliminate senescent cells. Senescent cells attract not only granulocytes and macrophages but also NK cells, CD8 T cells and CD4⁺ T helper 1 (Th1 CD4 T) cells^[80,81]. However recent report showed that senescent foamy macrophages produced inflammatory cytokines (TNF α), monocyte recruiting chemokines (MCP1) and matrix proteases, which engaged in atherosclerosis^[82]. In this case, macrophages themselves became senescent.

CONCLUSION

From the demand to solve worldwide increase of age-related diseases, the future biochemical studies are needed, which elucidate the phenomena of immuno-senescence and immune cell attack to eliminate senescent cells or damaged cells by age-related stresses. In advanced age, diseases are caused by M1 or M2 macrophages as mentioned above. Which type of macrophages are involved depend on the situation. In order to prevent or treat age-related diseases we have to carefully evaluate the immune status. Because metabolic reprogramming of oxidative phosphorylation to glycolysis occurs in M1 pro-inflammatory macrophages and urea cycle intermediates such as arginine, ornithine, citrulline regulate M1/M2 polarization^[60], the measurement of

metabolic changes will elucidate the predominant type of macrophages. Studies of metabolic pathway of immune cells in aging will open the nutrient treatment of age-related diseases.

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Basic Study

Identification of neuron selective androgen receptor inhibitors

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Abstract**AIM**

To identify neuron-selective androgen receptor (AR) signaling inhibitors, which could be useful in the treatment of spinal and bulbar muscular atrophy (SBMA), or Kennedy's disease, a neuromuscular disorder in which deterioration of motor neurons leads to progressive muscle weakness.

METHODS

Cell lines representing prostate, kidney, neuron, adipose, and muscle tissue were developed that stably expressed the CFP-AR-YFP FRET reporter. We used these cells to screen a library of small molecules for cell type-selective AR inhibitors. Secondary screening in luciferase assays was used to identify the best cell-type specific AR inhibitors. The mechanism of action of a neuron-selective AR inhibitor was examined *in vitro* using luciferase reporter assays, immunofluorescence microscopy, and immunoprecipitations. Rats were treated with the most potent compound and tissue-selective AR inhibition was examined using RT-qPCR of AR-regulated genes and immunohistochemistry.

RESULTS

We identified the thiazole class of antibiotics as com-

pounds able to inhibit AR signaling in a neuronal cell line but not a muscle cell line. One of these antibiotics, thiostrepton is able to inhibit the activity of both wild type and polyglutamine expanded AR in neuronal GT1-7 cells with nanomolar potency. The thiazole antibiotics are known to inhibit FOXM1 activity and accordingly, a novel FOXM1 inhibitor FDI-6 also inhibited AR activity in a neuron-selective fashion. The selective inhibition of AR is likely indirect as the varied structures of these compounds would not suggest that they are competitive antagonists. Indeed, we found that FOXM1 expression correlates with cell-type selectivity, FOXM1 co-localizes with AR in the nucleus, and that shRNA-mediated knock down of FOXM1 reduces AR activity and thiostrepton sensitivity in a neuronal cell line. Thiostrepton treatment reduces FOXM1 levels and the nuclear localization of beta-catenin, a known co-activator of both FOXM1 and AR, and reduces the association between beta-catenin and AR. Treatment of rats with thiostrepton demonstrated AR signaling inhibition in neurons, but not muscles.

CONCLUSION

Our results suggest that thiazole antibiotics, or other inhibitors of the AR-FOXM1 axis, can inhibit AR signaling selectively in motor neurons and may be useful in the treatment or prevention of SBMA symptoms.

Key words: Androgen receptor; Selective androgen receptor modulator; Spinal and bulbar muscular atrophy; Kennedy's disease

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Core tip: Kennedy's disease is caused by genetic expansion of the polyglutamine tract in the androgen receptor (AR). There is debate over whether the toxicity is due to expression of this mutant AR in motor neurons, muscle cells, or both. We have identified neuron-specific AR inhibitors, which could be used to help answer this question, and might be useful in the treatment or prevention of Kennedy's disease. These inhibitors function *via* FOXM1 and beta-catenin, which are shown to have important roles in the regulation of AR in neurons.

Otto-Duessel M, Tew BY, Vonderfecht S, Moore R, Jones JO. Identification of neuron selective androgen receptor inhibitors. *World J Biol Chem* 2017; 8(2): 138-150 Available from: URL: <http://www.wjgnet.com/1949-8454/full/v8/i2/138.htm> DOI: <http://dx.doi.org/10.4331/wjbc.v8.i2.138>

INTRODUCTION

Spinal and bulbar muscular atrophy (SBMA), or Kennedy's disease, is a neuromuscular disorder of males with a prevalence of approximately 1/50000^[1]. The symptoms typically begin in the 4th or 5th decade and include progressive weakness due to degeneration of motor

neurons in the brain stem and spinal cord^[1]. Currently there is no means by which to prevent or treat the symptoms of SBMA. SBMA manifestations are dependent on androgen activation of a mutant androgen receptor (AR) with an expanded N-terminal polyglutamine tract^[2]. Although the exact mechanism of toxicity is still under investigation, activation of AR in motor neurons causes them to die, leading to muscle atrophy. While studies in animal models demonstrate that inhibition of AR through androgen deprivation strategies (castration or suppression of testicular testosterone production) can ameliorate the disease manifestations associated with SBMA^[3,4], similar studies in humans have not produced analogous results^[5-7].

One reason for the lack of efficacy in humans may be that systemic AR inhibition can also lead to muscle mass decrease by inhibiting anabolic AR activity in muscle cells^[8]. In essence, systemic AR inhibition might improve motor neuron disease but prevent rescue of muscle symptoms. Therefore, the ability to inhibit AR selectively in the motor neurons could ameliorate the symptoms of SBMA. However, there is an ongoing debate about the contribution of mutant AR in motor neurons vs muscle cells in the pathogenesis of SBMA^[9-12]. Systemic treatment of BAC f_xAR121 and AR113Q mice with antisense oligonucleotides (ASO) that silence mutant AR led to improvement of SBMA symptoms, suggesting a direct effect on mutant AR in muscle cells as ASO cannot cross the blood-brain barrier. Indeed, when administered directly into the CNS amelioration of the pathological phenotype was not achieved^[9]. However, using a different transgenic mouse model of SBMA, AR97Q mice showed significant improvement after administration of ASO into the brain^[13]. Thus, a neuron-selective AR inhibitor might not only have therapeutic application, but will also help to differentiate the tissue-specific role of AR in the etiology of the disease. Therefore, our goal was to screen for a drug that has cell specific antagonistic effects on AR. We applied our FRET-based AR conformation reporter assay^[14] in several cell lines to screen for a selective AR modulator. We identified siomycin A and thiostrepton, thiazole antibiotics, as compounds with the ability to inhibit AR activity in a neuronal but not a muscle cell line.

Thiostrepton has been shown to down-regulate the transcription factor FOXM1, which is a member of the forkhead box (FOX) protein family. Since FOX proteins have been reported to interact with hormone receptors^[15,16], we speculated that thiostrepton-induced AR inhibition may occur *via* FOXM1 regulation. In this study we demonstrate that FOXM1 expression correlates with the ability of thiostrepton to inhibit AR activity in cells lines and that the mechanism of AR inhibition involves disruption of a FOXM1/β-catenin/AR transcriptional complex. Additionally, we demonstrate that thiostrepton can cause decreased expression of FOXM1 and may inhibit AR activity selectively in motor neurons *in vivo* using our previously described panel of tissue-selective androgen-regulated genes^[17].

MATERIALS AND METHODS

Cell lines

Most cells were obtained through the ATCC. The GT1-7 neuronal cell line was kindly provided by Dr. Mellon (UCSD). GT1-7 cells were maintained in DMEM (Hydrex) media enriched with 10% fetal bovine serum (FBS), 1% sodium pyruvate (CellGro, Mediatech, VA) and 1% non-essential amino acids (Irvine Scientific, CA). 293, 3T3L, L6, and C2C12 cells (all obtained from the ATCC) were cultured in DMEM supplemented with 10% FBS. PC12 (ARQ112) cells, which were a gift of Dr. Diane Merry, were cultured in DMEM supplemented with 10% FBS and 250 ng/mL doxycyclin to induce AR expression. LNCaP C4 cells were grown in RPMI-1640 (CellGro) containing 10% FBS. TM4 cells were grown in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium with 1.2 g/L sodium bicarbonate and 15 mmol/L HEPES, 92.5%; horse serum, 5%; fetal bovine serum, 2.5%. ReNcell CX neural progenitor cells (Millipore) were grown on laminin coated plates in NSC maintenance media (Millipore) supplemented with EGF and FGF. All cultures were supplemented with 1% penicillin/streptomycin (Gibco, Life Technologies) and grown at 37 °C in humidified atmosphere containing 5% CO₂.

Creation of stable C-AR-Y reporter cell lines

Cell lines listed above were either transfected with a plasmid or a virus containing the CFP-AR-YFP construct preceding an IRES and hygromycin resistance gene and clonal stable cell lines selected. Qualification criteria for the transfected cell lines were described previously^[14].

Screen for AR-modulators

Screening was performed essentially as described previously^[14]. Briefly, C-AR-Y reporter GT1-7 were transferred to 96 well plates and treated with library compounds for 24 h. Each compound was tested on four different plates at a concentration of 1 µmol/L. To half of the plates DHT (5 nmol/L) was added in order to identify AR antagonists. The compounds to be tested were from the NCI/DTP Open Chemical Repository's Approved Oncology Drugs Set II, Diversity Set II and Mechanistic Set. Control wells on each plate included non-transfected cells, no DHT (baseline signal), DHT (5 nmol/L) without library compound (maximal signal) plus DHT combined with hydroxyflutamide (1 µmol/L) (AR antagonist). After 24 h, medium was aspirated and FRET was measured as previously described using a monochromator-based fluorescence plate reader (M1000, Tecan, Inc.)^[14]. We used GT1-7 cells for primary screening and used other cell lines mentioned above for secondary evaluation of candidate hits.

Luciferase reporter assay

Cells were transfected using Lipofectamine Plus (Invitrogen) with a normalization plasmid (pRL-SV40, Promega) and

an androgen responsive plasmid (MMTV-luciferase or PSA-luciferase) as previously described^[14], and where indicated, with an shRNA plasmid targeting FOXM1 (a gift of Dr. May^[18], adapted for mouse FOXM1 sequence targeting), a scrambled control shRNA plasmid, or ARQ24 or ARQ65 plasmids (gifts of Dr. Fischbeck). The following day, cells and drugs were distributed to 96-well plates. Twenty-four hours later, luciferase activity was assessed by a dual luciferase assay kit (Promega) on the Tecan plate reader. Samples were run in quadruplicates and a Student's *t* test was performed to determine significance. *P* < 0.05 was considered significant.

Immunoprecipitation

The co-IP assay was performed as previously described^[19]. In brief, cells were lysed on ice for 20 min in co-IP buffer containing 10 mmol/L Hepes (pH8), 300 mmol/L NaCl, 0.1 mmol/L EDTA, 20% glycerol, 0.2% NP-40 and protease inhibitor (Roche Diagnostics). Lysate was centrifuged at 10000 g for 10 min at 4 °C. Lysate was incubated with primary antibody (β-catenin: rabbit, 9562, Cell Signaling Technology) overnight at 4 °C followed by incubation with 30 µL of Protein A/G beads for 2 h at 4 °C. Collected protein complexes were washed and analyzed by Western blotting.

Western blot

Cell lysates were separated on a 6% SDS page gel and transferred to a nitrocellulose membrane. The membrane was blocked for one hour in 5% skim milk dissolved in TBS-T and probed with the following antibodies (1:1000): FoxM1 (rabbit, GeneTex), AR: 441 (mouse, Santa Cruz Biotechnology), β-catenin (rabbit 9562, Cell Signaling Technology), p84(mouse, GeneTex) and actin (mouse, Santa Cruz Biotechnology). Horse radish peroxidase linked secondary antibodies were used along with ECL reagents for visualization. Western blots were quantified using Quantity One analysis software (BioRad).

Animals and drugs

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments were performed under the IUCAC approval of City of Hope. Animals were purchased from Jackson Laboratory (Bar Harbor, Maine). For the pilot pharmacokinetic experiment, nine male Sprague Dawley rats (*n* = 3) were administered 1, 10, or 100 mg/kg intraperitoneal injections thrice per week for four weeks. One hour following the final dose, blood and spinal fluid were collected and analyzed by mass spectrometry. For the efficacy experiment, rats were divided into 3 groups (*n* = 7) as follows: Intact, castrated and thio-strepton. Thiostrepton was dissolved in 20% DMSO and 80% PEG400 (Sigma Aldrich) and administered at a concentration of 50 mg/kg per day *via* an IP osmotic pump implant (Alzet). Following a treatment period of 7 d, rats were euthanized and organs harvested, weighed

and processed for quantitative RT-PCR. One-way analysis of variance was applied to determine statistical differences among groups using *post-hoc* Dunnett's test. $P < 0.05$ was considered significant.

qRT-PCR

The quantitative RT-PCR method has been described previously^[14]. In brief, dissected tissue was placed in RNA Later (Qiagen) before being homogenized using a bead homogenizer (TissueLyser, Qiagen). Following homogenization, RNA was isolated with an RNeasy kit (Qiagen). RNA then was reverse transcribed (Promega, WI) and amplified (Qiagen Taq and reagents) on a StepOne Plus Real Time PCR System (Applied Biosystems), using SYBR green (Invitrogen) as the detecting dye and Rox (Invitrogen, CA) as the reference dye. Differences between experimental (x) and control (y) samples were normalized to RPL19 transcript levels (androgen unresponsive) and determined with the following calculation: $[2^{-(Ct_{xgene1} - Ct_{ygene1})}] / [2^{-(Ct_{xRPL19} - Ct_{yRPL19})}]$. One-way analysis of variance was applied to determine statistical differences among groups using *post-hoc* Dunnett's test. $P < 0.05$ was considered significant.

Immunofluorescence

Cells were fixed in 2% paraformaldehyde prior to incubating with primary antibody (FOXM1:mouse, Ab Cam,) overnight at 4 °C. Following washing, secondary antibody (Alexa 954 goat anti-mouse, Invitrogen) was applied for 45 min at 37 °C. After washing, slides were mounted and analyzed using a confocal microscope (Zeiss). The coloc-2 Image J plug-in (Fiji) was used to quantify co-localization of AR and FOXM1 from three independent samples using both Li's Intensity Correlation Quotient (ICQ) and Manders split coefficients co-localization values. One-way analysis of variance was applied to determine statistical differences among groups. $P < 0.05$ was considered significant.

Immunohistochemistry

Spinal cords were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned. Following de-waxing, slides were blocked with 10% normal goat serum for 30 min. The primary antibody (1:500) (AR: PG21, Millipore; FoxM1: GeneTex) was applied and incubated overnight at 4 °C. Following three washes with TBS-T plus 0.1% Tween, slides were incubated with goat anti-rabbit antibody (1:200) for 30 min. Following three washes slides were incubated with Vectastain ABC reagent for 10 min at room temperature, then developed with DAB and counterstained with hematoxylin, dehydrated and mounted. A veterinary pathologist, blinded to the identity of the treatment groups, scored the spinal cord sections based on the intensity of FOXM1 expression, which was typically uniform across the entirety of a section, creating a scale ranging from 0-2 in both white matter and grey matter. Because few spinal cords were available for analysis, results from intact and castrated

were grouped for analysis as "untreated". The staining between castrate and intact animals was very similar and distinct from thioestrepton treated animals. As a secondary quantification method, Image J was used to determine pixel density of FOXM1 staining. Images of ten sections from four thioestrepton treated and four untreated rat slides were captured. Integrated density values were averaged ($n = 40$) and a Student's *t* test was performed to determine significance. $P < 0.05$ was considered significant.

LC-MS/MS methods

Dried samples were resuspended in tetrahydrofuran spiked with 5 pg/ μ L reserpine, which was used as an instrument response control. Resuspended samples were separated with an Agilent 1290 UHPLC system (Agilent Technologies, Santa Clara, CA) and a Waters Acquity CSH C₁₈ column (Waters, Millford, MA) using a linear gradient from 95% buffer A (0.1% formic acid in 60% water/40% acetonitrile) to 100% buffer B (0.1% formic acid in 90% isopropanol/10% acetonitrile) over 2.5 min at 380 μ L/min. The UHPLC column eluent was sprayed into an Agilent 6490 triple quadrupole mass spectrometer equipped with a JetStream source (Agilent Technologies). Thioestrepton was detected by monitoring fragments from the doubly charged ion (m/z 832.9) at m/z 1247.4 and 1230.3. Data was analyzed using Agilent MassHunter quantitative analysis software.

RESULTS

Identification of GT1-7 specific AR inhibitors by high-throughput screening

In order to identify potential cell-type selective AR modulators, we built upon our FRET-based assay of AR conformation change. AR signaling is a complex process involving many steps in addition to ligand binding. One crucial step is the ligand-induced conformational change of AR, which brings the carboxy (C)- and amino (N)-termini of the receptor together. Full length AR with cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) fused to either end (CARY) produces a FRET signal upon AR conformation change (Figure 1A). This produces an accurate and quantifiable read-out of AR activity^[14]. Because we were interested in the potential of neuron selective AR inhibitors for treatment of SBMA, we carried out our initial screen in CARY-transfected GT1-7 cells, which are a mouse model of hypothalamic neurons^[20]. Cells were plated in 96 wells and treated with library compounds 24 h prior to accessing conformational changes by FRET. Non-CARY cells, CARY cells without DHT (baseline signal), with DHT (5 nmol/L) only (maximal signal) and DHT combined with 1 μ mol/L hydroxyflutamide (OHF) (AR antagonist) served as controls (Figure 1A). Candidate hits were then further evaluated by dose response. Figure 1B shows a dose dependent inhibition of DHT-induced AR conformation change by the compound NSC285116 (siomycin A) in GT1-7 cells. Once a dose response was observed,

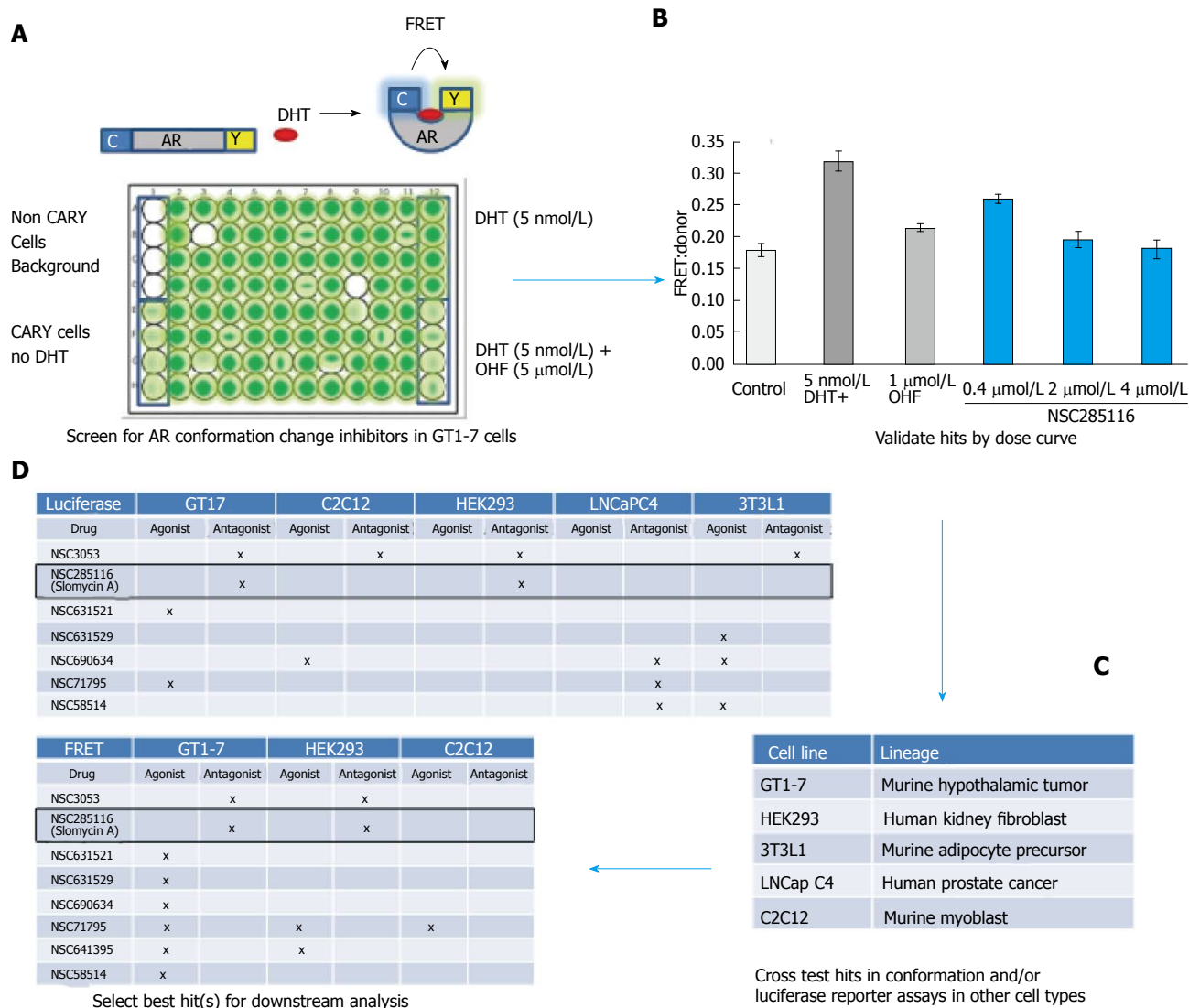


Figure 1 Screening for neuron-selective androgen receptor inhibitors. A: AR conformation change can be monitored by the CFP-AR-YFP FRET reporter (top). Screening for compounds that affect AR conformation change in GT1-7 cells was accomplished in 96 well plates; B: Hits were assessed for a dose response in the same assay. Shown is the dose response to NSC285116 (siomycin A); C, D: Hits were then assessed for agonist or antagonist activity in the indicated cell lines in a FRET or an androgen-responsive luciferase reporter assay. Siomycin A demonstrated antagonist activity in GT1-7 neuron cells but not C2C12 muscle cells and was therefore selected for further analysis. AR: Androgen receptor.

candidate compounds were then cross examined in CARY-expressing cell lines representing other tissues of interest, including muscle (C2C12), testes (TM4), kidney (HEK293), prostate (LNCaP C4), and adipose (3T3L1) (Figure 1C). In addition to FRET, AR transcriptional activity was also assessed in several cell lines by transfecting cells with a luciferase reporter driven by the human PSA promoter^[21]. After extensive testing in multiple cells lines (Figure 1D), we identified siomycin A as a potential neuron-selective AR inhibitor. It showed greatest potency in neuronal GT1-7 cells, with some activity in HEK293 kidney cells, but little to no activity in LNCaP C4 prostate cancer, C2C12 muscle or 3T3L1 adipose-derived cells.

AR inhibition by thiazole antibiotics

Siomycin A is a thiazole class antibiotic. It has been shown to inhibit transcriptional activity and expression

of the FOXM1 transcription factor^[22-24]. Thiostrepton belongs to the same class of antibiotics and has been reported to function similarly to siomycin^[25-27]. To test whether thiostrepton possesses similar AR antagonistic properties as siomycin, we assessed its ability to inhibit AR transcriptional activity *via* luciferase assay in GT1-7 and C2C12 cells (Figure 2A). Both thiostrepton and siomycin were found to selectively inhibit AR activity in GT1-7 cells, with thiostrepton having a greater potency. As thiazole antibiotics have also been found to have effects on proteasome function, we examined the activity of a recently developed, selective FOXM1 inhibitor, FDI-6^[28]. We found this compound also inhibited AR activity in GT1-7 cells but not C2C12 cells, suggesting that anti-FOXM1 activity is the key to the ability to inhibit AR activity in these cells. Further experimentation was conducted primarily with thiostrepton as it was the most

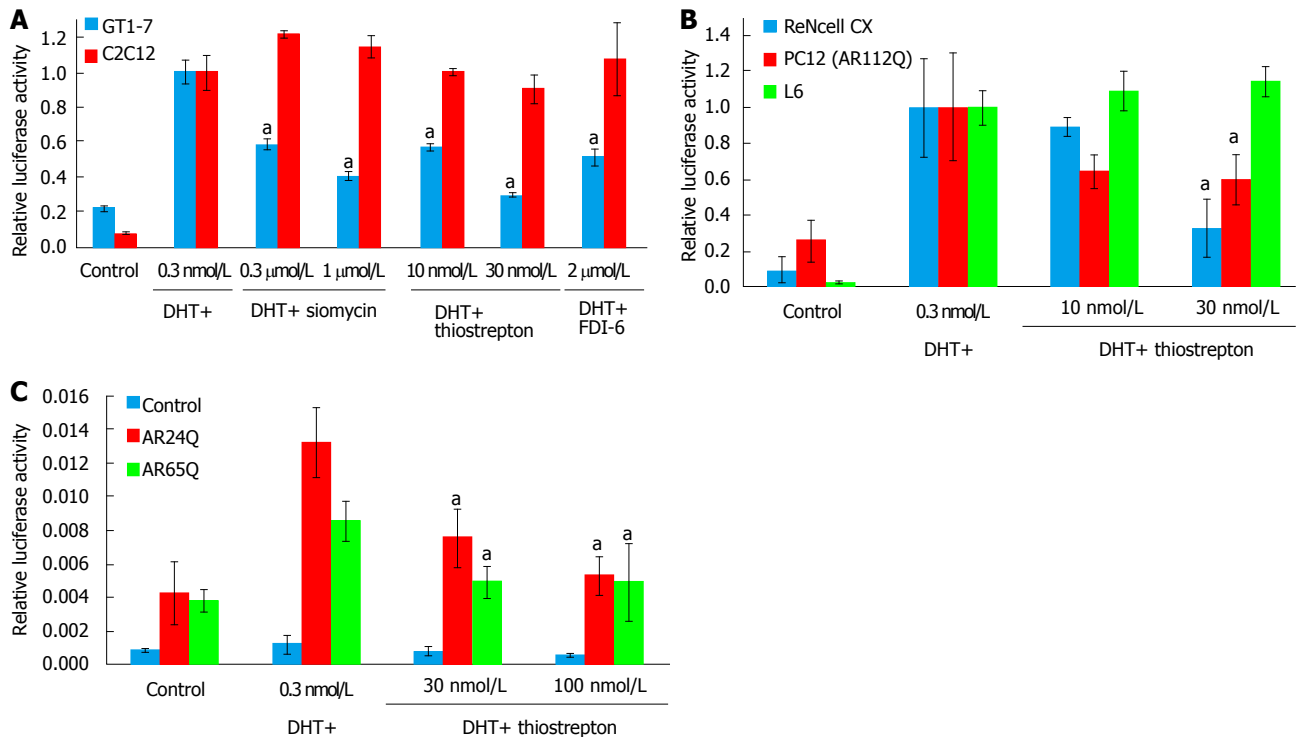


Figure 2 Thiazole antibiotics selectively inhibit androgen receptor in neuronal cells and have activity against polyQ-expanded androgen receptor. A: GT1-7 and C2C12 cells were transfected with androgen-responsive and control luciferase reporters and treated with the indicated drugs overnight. Both thiazole antibiotics siomycin and thiostrepton, as well as the selective FOXM1 inhibitor FDI-6, inhibited AR activity in GT1-7 but not C2C12 cells, with thiostrepton being the most potent of the drugs; B: Rat L6 skeletal muscle cells and ReNcell CX and PC12 (ARQ112) neuronal cells were transfected with androgen-responsive and control luciferase reporters and treated with thiostrepton overnight. Thiostrepton inhibited AR luciferase activity in neuronal, but not muscle cells; C: 293 cells were transfected with androgen-responsive and control luciferase reporters as well as plasmids containing AR with 24 (normal state) or 65 (disease state) glutamines and treated with drugs mentioned above overnight. Both normal and disease state AR activity was reduced by thiostrepton treatment. Error bars represent the mean $\pm$ SEM, ^a $P < 0.05$. AR: Androgen receptor.

potent of the FOXM1 inhibitors. The ability of thiostrepton to inhibit AR activity in additional neuronal cells, including ReNcell CX cells^[29] and PC12 cells^[30], and in rat L6 skeletal muscle cells^[31] was examined (Figure 2B). We again found that thiostrepton inhibited AR activity in neuronal but not muscle cells, strongly suggesting that AR inhibition by FOXM1 inhibitors is neuron-selective.

SBMA is caused by the activity of polyglutamine-expanded AR. To investigate the effects of thiostrepton against polyglutamine-expanded AR we transfected AR-negative HEK293 cells with plasmids encoding AR with a 24 glutamine tract (normal) or with a 65 glutamine tract (disease associated). The polyglutamine-expanded AR was less transcriptionally active than the normal AR, as has been previously documented for such constructs^[32]. Thiostrepton was able to inhibit the polyglutamine-expanded AR as well as the non-expanded AR (Figure 2C), suggesting that thiostrepton can inhibit the activity of a clinically relevant AR. In further support of this, the PC12 cells in Figure 2B express an AR with a 112 glutamine tract, and thiostrepton efficiently inhibited AR activity in those cells as well.

FOXM1 mediates AR inhibition by thiostrepton

FOX proteins can act as transcription factors and co-activators and have been shown to regulate AR tran-

scriptional activity^[33,34]. For instance, FOXA1 has been shown to interact and regulate transcription of both estrogen receptors and AR^[35]. Because thiazole antibiotics are known to inhibit FOXM1, we speculated that the thiostrepton-induced AR antagonistic effects might be mediated *via* FOXM1. We first examined FOXM1 expression levels in different cell lines (Figure 3A) and found that FOXM1 expression levels were highest in GT1-7, followed by 293HEK and LNCaP C4 cells. Little or no expression was observed in the remaining cell lines, suggesting a correlation between FOXM1 expression and the inhibitory effect of thiostrepton on AR activity. Expression levels of the CARY construct did not correlate with sensitivity (Figure 3A).

To further test the role of FOXM1 in association with AR activity, we decreased FOXM1 expression in GT1-7 cells using a lentiviral shRNA plasmid transfection. FOXM1 knock-down decreased the DHT-induced activity of AR, suggesting FOXM1 is an important AR coactivator in GT1-7 cells (Figure 3B). More importantly, decreasing FOXM1 expression diminished the potency of thiostrepton (compare DHT + thio/DHT alone ratio in control vs FOXM1-silenced cells). This strongly suggests that FOXM1 mediates the ability of thiostrepton to inhibit AR activity.

It has been previously reported that thiostrepton causes decreased levels of FOXM1^[25,36]. Treatment of GT1-7 CARY cells with thiostrepton resulted in slightly

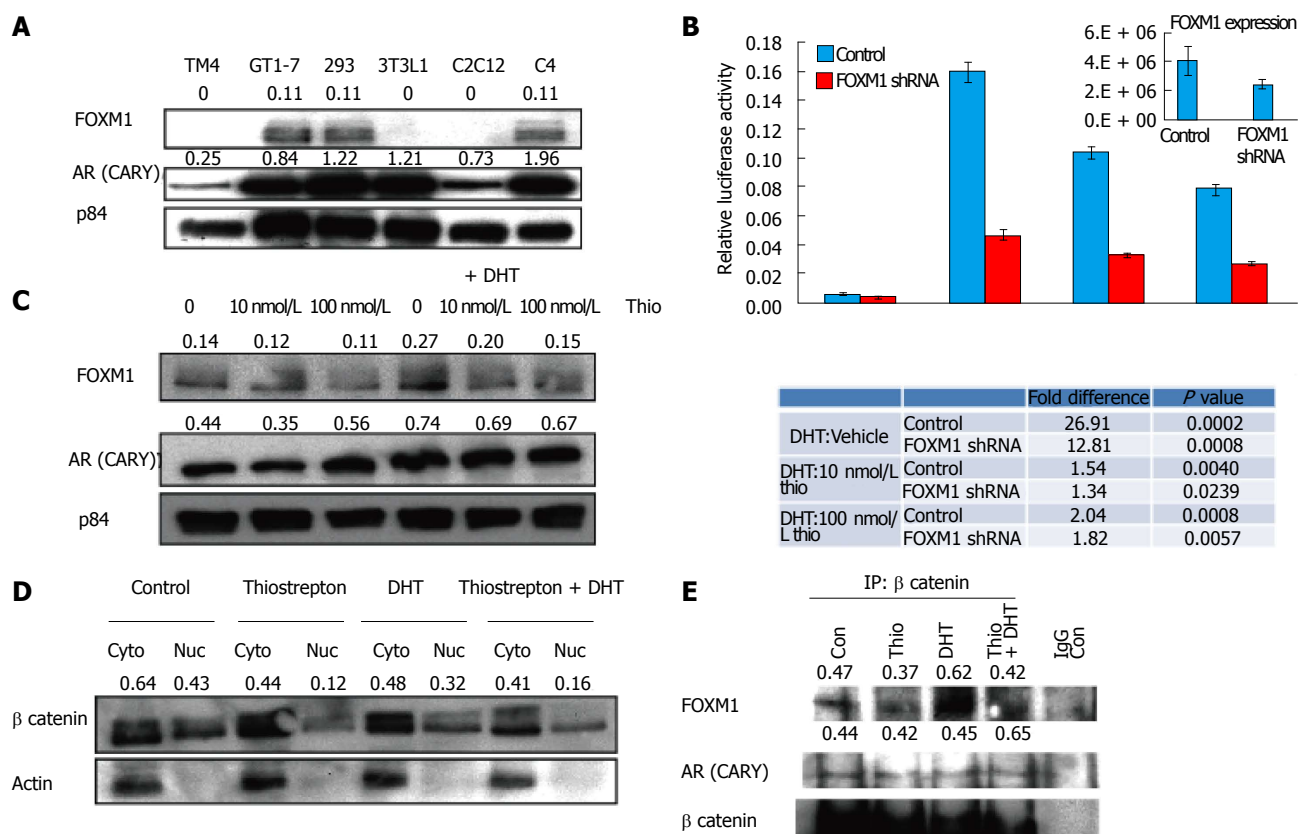


Figure 3 FOXM1 expression correlates with androgen receptor activity. A: Expression of FOXM1 and AR was assessed by Western blot in indicated cell lines; B: GT1-7 cells were transfected with a lentiviral plasmid shRNA targeting FOXM1 as well as luciferase plasmids, treated with drugs listed above, then assessed by luciferase assay. Decreased expression of FOXM1 (RT-qPCR in inset) reduced AR activity and diminished sensitivity to thio. Fold changes and *P* values are shown; C: Western blot analysis demonstrates that thio reduces FOXM1 levels in GT1-7 cells while DHT increases FOXM1 levels; D: GT1-7 cells were treated with the indicated drugs overnight, and nuclear and cytoplasmic fractions were probed by Western blot for the indicated proteins. β -catenin levels in nuclear fraction of thio treated cells are lower compared to untreated ones. Quantification of β -catenin levels relative to cytoplasmic actin expression is shown; E: β -catenin was immunoprecipitated from GT1-7 cell lysates and Western blot was performed to detect AR and FOXM1. Quantification of AR and FOXM1 levels relative to β -catenin levels in IP is shown. AR: Androgen receptor.

decreased FOXM1 levels compared to p84 loading control protein levels, both in the presence and absence of DHT (Figure 3C), as shown from quantification of representative blots. In contrast to thio, DHT treatment increased FOXM1 levels, implying interplay between AR signaling pathway with FOXM1. AR levels themselves were not consistently decreased in response to thio treatment. FOXM1 has been shown to bind directly to β -catenin and facilitate its nuclear localization. The nuclear association between FOXM1 and β -catenin increases the transcriptional activity of both factors^[19]. β -catenin is a key AR coactivator in a variety of cells^[37,38]. We therefore reasoned that thio might inhibit AR activity by limiting access to nuclear β -catenin via FOXM1 depletion. Nuclear and cytoplasmic fractions were isolated from GT1-7 cells treated with or without thio and DHT. Thio treatment caused lower levels of nuclear β -catenin, both phosphorylated and unphosphorylated (active) fractions (see doublet in Figure 3D), suggesting that thio treatment leads to β -catenin inactivation via its effects on FOXM1. Control actin protein is observed primarily in cytoplasmic fraction, as expected.

Co-immunoprecipitation confirmed a direct interaction between β -catenin and FOXM1 as well as between β -catenin and AR (Figure 3E). The amount of FOXM1 pulled down was reduced in thio-treated cells. While this may be due in part to the effects of thio on total FOXM1 levels, which is difficult to control for in these experiments, the decreased interaction between FOXM1 and β -catenin could also have an effect on AR activity.

Using a co-IP assay in GT1-7 CARY cell lysates, we were unable to detect an association between AR and FOXM1 (data not shown). However, immunofluorescence staining demonstrated significant AR-FOXM1 co-localization in GT1-7 CARY cells (Figure 4). Untreated cells predominantly expressed AR in the cytoplasm, while DHT treatment caused a shift to the nucleus. FOXM1 expression was mainly confined to the nucleus, which was true for untreated and treated cells. Image analysis revealed little to no co-localization between FOXM1 and AR in untreated cells (Figure 4B). Addition of DHT increased association between FOXM1 and AR, which was not significantly affected by addition of thio. Although co-localization is not evidence of an interaction,

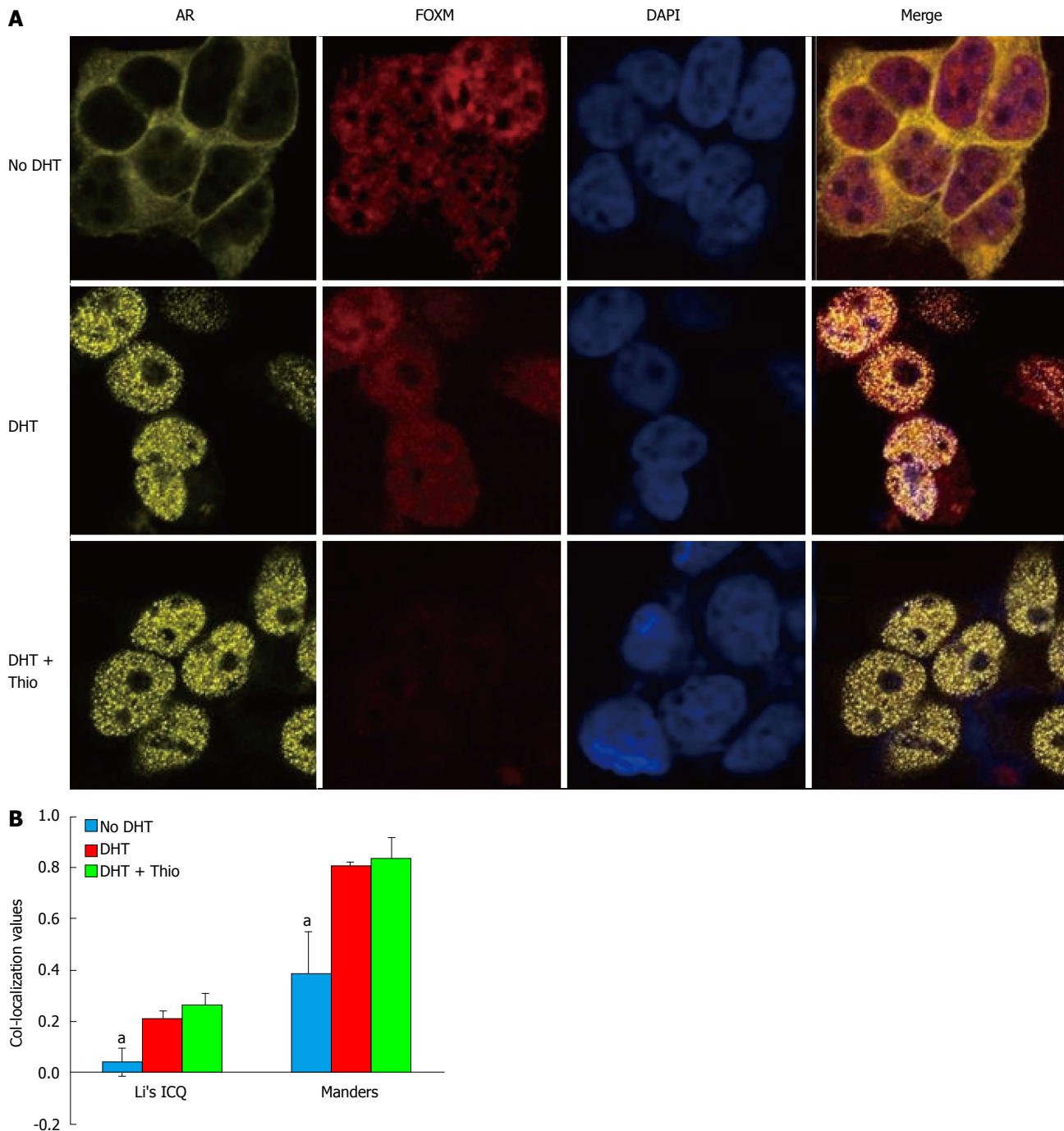


Figure 4 DHT promotes nuclear co-localization of androgen receptor and FOXM1. A: GT1-7 cells expressing a YFP-tagged AR were treated as indicated for 24 h prior to staining with anti-FOXM1 (red) and DAPI (blue); B: Quantification of co-localization using Image J demonstrates significant co-localization of FOXM1 and AR in the presence of DHT and DHT + thioestron by both Manders split coefficient and Li's ICQ value. Error bars represent the mean $\pm$ SEM, $^aP < 0.05$. AR: Androgen receptor; ICQ: Intensity Correlation Quotient.

our results suggest that FOXM1 and AR could plausibly function together in the presence of DHT. In conclusion, our data suggest a model in which thioestron decreases the levels of FOXM1, which leads to lower levels of nuclear β -catenin, an important AR coactivator, causing decreased AR transcriptional activity.

Thioestron treatment inhibits *in vivo* AR activity preferentially in rat spinal cord compared to skeletal muscle

In order to achieve desired dosing and to determine

if thioestron crosses the blood brain barrier, we first conducted a pilot pharmacokinetic study in rats. Rats ($n = 3/\text{group}$) received low (1 mg/kg), medium (10 mg/kg), or high (100 mg/kg) doses of thioestron by intraperitoneal (IP) injection, three times a week for four weeks. One hour after the final dose, animals were euthanized and blood as well as spinal fluid was collected and analyzed by LC-MS/MS (Figure 5A). We found a dose-dependent increase in plasma levels. Only the high dose of thioestron resulted in measurable levels of drug in the spinal fluid, suggesting that while

the drug could cross the blood brain barrier, it exists in that compartment at much lower levels than the plasma. However, it did reach concentrations above its IC₅₀ as determined by luciferase assay in cultured cells, suggesting that it could still be active in motor neurons of the spinal cord.

Based on our initial results, rats ($n = 7$) were treated with 100 mg/kg per day thioestrepton or vehicle for four weeks using IP implanted osmotic pumps. Intact and castrate cohorts were included as controls. To test the efficacy of the thioestrepton in the motor neurons, we examined the expression of FOXM1 in tissue sections of the spinal cord. FOXM1 was located in both gray and white matter of the spinal cord (Figure 5B), primarily in the nucleus, as had been shown previously^[39]. In the grey matter, FOXM1 was mainly found in the neurons. In the white matter, FOXM1 was found in glial cells, at least some of which appeared to be astrocytes; however, further immunohistochemistry would be necessary to confirm the identity of the cells. As scored by a veterinary pathologist blinded to treatment groups, treatment with thioestrepton significantly reduced the expression of FOXM1 in the spinal cord (Figure 5C). As a secondary quantification method, Image J was used to determine pixel density of FOXM1 staining. Images of ten sections from four thioestrepton treated and four untreated slides were captured. Integrated density values were averaged and displayed relative to untreated control values (Figure 5C). Thioestrepton treatment caused lower intensity FOXM1 staining ($P = 0.0036$) by this method as well. This data suggest that we achieved functional levels of thioestrepton in the spinal cord and that it decreased FOXM1 levels *in vivo*, as it did in cultured cells. AR was clearly observed in the nucleus and cytoplasm of neurons in both white and gray matter, and levels were not affected by thioestrepton, which parallels our cell culture data. Immunohistochemistry also revealed low to no expression of FOXM1 in intact muscle tissue (Figure 5D), corroborating our cell culture data and in accordance with published literature where it has been reported that FOXM1 is highly expressed in embryonic muscle tissue, but is drastically reduced in adult skeletal muscle tissue^[40]. AR expression is evident in the striated muscle tissue nuclei as expected and is not affected by thioestrepton treatment.

Androgens are known to control the growth and maintenance of the skeletal muscle and seminal vesicles^[41]. In order to investigate the impact of thioestrepton on these tissues, we assessed their weights at the end of the study. Compared to castrated rats, which displayed significantly reduced levator ani (LA) muscle ($P < 0.01$) and seminal vesicles (SV) ($P < 0.01$) weights, thioestrepton did not change LA and SV weights when compared to controls (Figure 5B). This data suggest that thioestrepton does not significantly affect AR activity in muscle or SV, in line with our cell culture findings. Because an equivalent androgen-dependent change in weight cannot be tested in motor neurons, we assessed the levels of tissue-selective androgen regulate genes as a marker of AR activity.

These genes were identified in our previous studies^[17]. RNA was isolated from spinal cord and LA muscle tissue and transcript levels were measured by RT-qPCR (Figure 5F and H). Both castration and thioestrepton treatment caused decreases in three androgen-dependent genes, two of which were statistically significant with thioestrepton treatment, in the spinal cord compared to control treatment. In LA muscle, castration had a much greater effect than thioestrepton treatment at the four androgen-dependent genes analyzed, significantly altering the expression of 3 of 4 genes examined, while thioestrepton did not significantly affect the levels of any genes. Importantly, the transcript levels of FOXM1 target genes *Cnd1* and *Sox2*^[42,43] were decreased in the spinal cords of thioestrepton-treated animals compared to intact controls, demonstrating on-target efficacy of the drug (Figure 5G). Our combined data suggest that thioestrepton inhibits AR activity in motor neurons but not LA muscle.

DISCUSSION

We originally created the FRET-based AR conformation change reporter assay to identify novel AR inhibitors for use in prostate cancer^[14]. Our initial studies identified cell type selective AR inhibitors. Here, we demonstrate the utility of the assay on a broader scale to identify cell type selective AR modulators with potential for clinical use in realms other than prostate cancer. Our focus was to find a selective neuronal AR inhibitor which might have potential in treating SBMA. Our data demonstrate that thioestrepton selectively inhibits AR in different cell lines *in vitro*. Based on extensive literature demonstrating FOXM1 as a target of thioestrepton and other thiazole antibiotics^[25,36,44], we speculated that the decreased AR activity is mediated by decreased levels of FOXM1, a transcription factor involved in many different molecular pathways. We showed that FOXM1 is differentially expressed in a panel of cell lines and that this expression mostly correlates with the ability of thiazole antibiotics to inhibit AR activity. LNCaP C4 cells, which expressed FOXM1, were not as sensitive to siomycin in our initial screen. This could be due perhaps to the expression of a mutant AR in these cells^[45]; further investigation is warranted. We also showed that a chemically distinct FOXM1 inhibitor, FDI-6, inhibited AR activity in neuronal but not muscle cells. Furthermore, manipulation of FOXM1 levels affected AR activity and sensitivity to thioestrepton, strongly suggesting that thioestrepton acts through FOXM1 to inhibit AR.

We showed thioestrepton reduced FOXM1 levels, in accordance with published literature. While the effect was not as strong as some published data, FOXM1 levels were quantifiably lower. Likewise, we showed that thioestrepton quantifiably reduced the nuclear levels of β -catenin in GT1-7 cells. Although only one blot is shown in each figure, each of the IP and Western blot experiments was repeated several times, with high reproducibility. While we were unable to detect an interaction between AR and FOXM1 by co-IP, confocal imaging demonstrates the

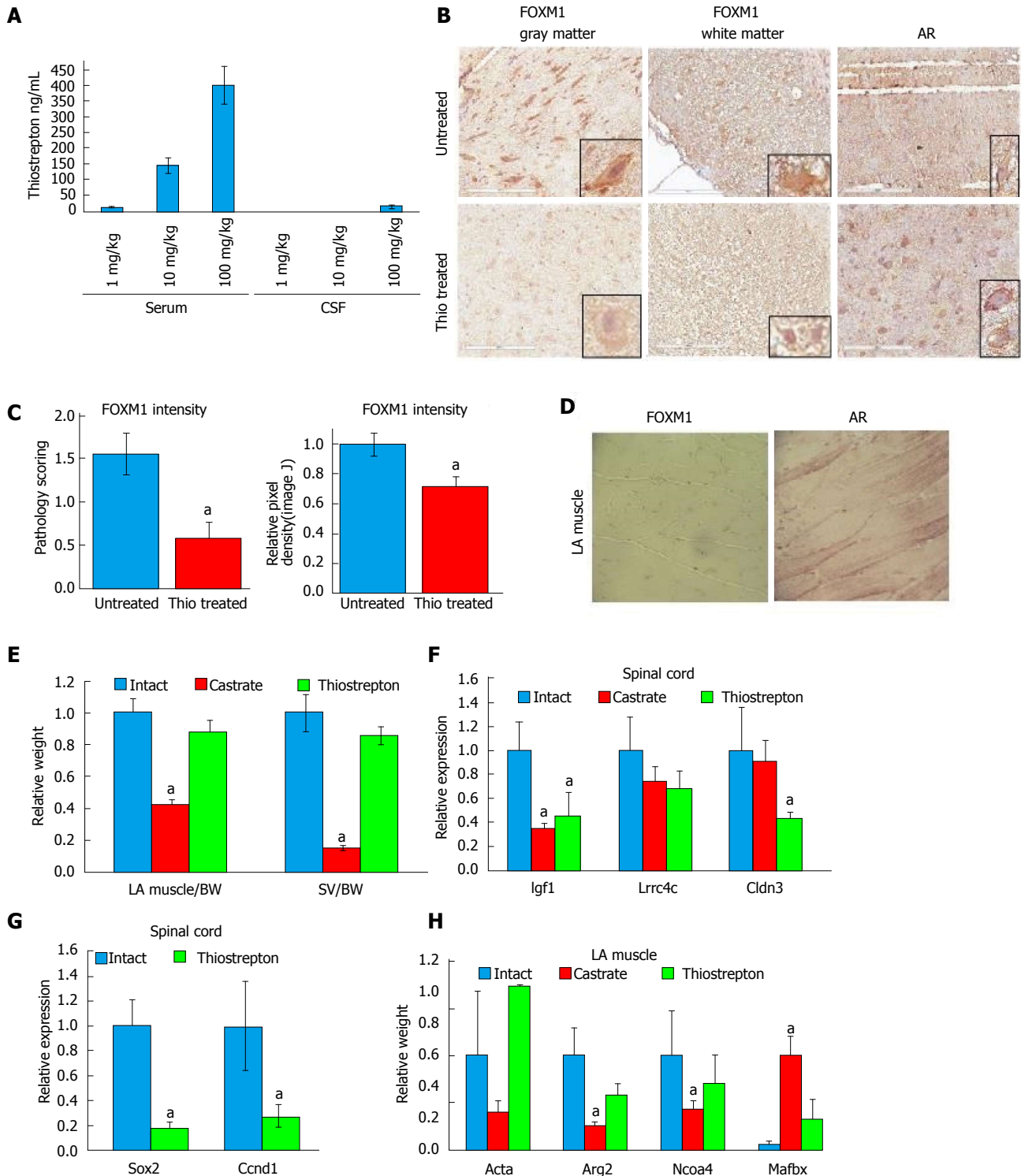


Figure 5 Thiostrepton treatment inhibits androgen receptor activity in rat spinal cord but not muscle. A: Rats ($n = 3$) were administered 1, 10, or 100 mg/kg intraperitoneal injections thrice per week for four weeks. One hour following the final dose, blood and spinal fluid were collected and analyzed by mass spectrometry. Thiostrepton displayed the expected dose response in the serum, but much less was found in the cerebral spinal fluid; B-F: Rats ($n = 7$) were treated with 100 mg/kg per day thiostrepton or vehicle for four weeks using intraperitoneally implanted osmotic pumps. Intact and castrate cohorts were included as controls; B, C: Immunohistochemistry revealed a reduction in the expression of FOXM1 in spinal cords compared to untreated (intact or castrate) animals. AR staining was also strong in the neurons; C: FOXM1 staining intensity was quantified by a veterinary pathologist (left) or by ImageJ (right) and found to be significantly reduced by thiostrepton treatment; D: IHC revealed weak to no FOXM1 staining in LA muscle tissue while AR staining was prevalent; E: Tissues were harvested and weighed. Castration significantly reduced the weight of the LA muscle ($P < 0.01$) and seminal vesicles (SV) ($P < 0.01$) compared to intact animals while thiostrepton treatment did not. (F-H) RT-qPCR was used to quantify the levels of androgen-regulated transcripts in spinal cord (F) or LA muscle tissue (H) or FOXM1-regulated transcripts in spinal cord (G). Thiostrepton was able to decrease the levels of androgen-regulated transcripts in spinal cords to a similar or greater extent than castration, suggesting inhibition of AR activity, while in LA muscle, castration had a much stronger effect on AR activity than thiostrepton treatment. Two FOXM1 target gene transcripts were decreased with thiostrepton treatment, demonstrating an on target effect of the drug. Error bars represent the mean $\pm$ SEM, $^aP < 0.05$ ($P < 0.08$ in G). AR: Androgen receptor.

plausibility of these proteins functioning together in the nucleus in the presence of DHT. Our co-IP experiments also demonstrate interactions between β -catenin and both AR and FOXM1, both of which have been previously reported^[19,37]. Our data suggest a model in which AR, FOXM1, and β -catenin form an active transcriptional complex in the nuclei of GT1-7 cells that is essential for full androgen-responsive transcription. Thioestrepton treatment reduces FOXM1 levels, which leads to lower levels of nuclear β -catenin, and thus reduced AR transcriptional activity.

Thioestrepton's selective inhibition of AR appears to apply *in vivo* as well. Thioestrepton treatment did not inhibit AR activity in LA muscle or SV, as measured by changes in organ weights. It did cause decreased FOXM1 levels and inhibit androgen-regulated gene expression in spinal cord neurons. Although many of the RT-qPCR changes were not significant, this is likely due to the small number of animals in each group. While further testing is necessary to determine exactly how selective thioestrepton and other thiazole antibiotics are *in vivo*, our initial data suggest that we have identified a compound that inhibits AR in spinal cord neurons but not muscle, which was the goal of our study. Such a drug could be useful in the treatment of SBMA. In mouse models of SBMA, castration by physical or chemical means can prevent and/or ameliorate disease symptoms^[39,40]. However, trials of androgen reducing agents in symptomatic humans have not had the same effect^[42-44]. This could be in part because muscle atrophy has already occurred and androgens are needed to rebuild muscle tissue. Thus, a neuron selective AR inhibitor might inhibit degenerative AR activity while allowing the body's natural androgens to rebuild atrophied muscle tissue. As mentioned above, there is evidence that polyglutamine expanded AR has direct toxic effects in muscle cells^[10], in which case, a neuron-selective AR inhibitor would not benefit SBMA patients. However, other studies show that knocking down neuronal AR while keeping muscle levels intact rescues the disease phenotype^[13]; genetic and RNA silencing approaches have not been able to settle the debate. Testing thioestrepton or similar compounds in mouse models of SBMA^[46] could help answer this question.

There are several limitations to our study. The largest is the lack of testing of FOXM1 inhibitors in animal models of SBMA. We are working to establish collaborations to test these compounds in such models. The use of a phenotypic screen also presents challenges, especially in the identification of a specific mechanism of action. However, it is clear from our studies that FOXM1 is involved in the neuron-selective inhibition of AR that was originally observed in our screen. Finally, thioestrepton is a natural product, and its large, complex structure makes synthesis difficult. It is also highly hydrophobic, typically being used as a topical cream, and therefore not ideal for *in vivo* administration. Some work has been done to improve the synthesis and pharmacokinetics of thioestrepton, but the development of specific FOXM1/AR inhibitors with better drug like

properties, including the ability to cross the blood brain barrier, would be beneficial. It is possible that FDI-6 and related compounds could be improved to become viable treatments for SBMA.

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COMMENTS

Background

Kennedy's disease is a neuromuscular disorder of males with a prevalence of approximately 1/50000 that is caused by genetic expansion of the polyglutamine tract in the androgen receptor (AR). The authors have identified neuron-specific AR inhibitors, which could be used to help answer this question, and might be useful in the treatment or prevention of Kennedy's disease. These inhibitors function via FOXM1 and beta-catenin, which are shown to have important roles in the regulation of AR in neurons.

Research frontiers

There is debate over whether the toxicity of polyglutamine expanded AR in Kennedy's disease is due to expression of this mutant AR in motor neurons, muscle cells, or both. There are also no effective treatments for this disease.

Innovations and breakthroughs

This is the first report of an *in vitro* screen for selective AR modulators and the first report of a neuron-selective AR inhibitor. It also finds FOXM1 as an important co-factor for AR activity in neurons, and suggests that FOXM1 and beta-catenin may play a role in Kennedy's disease.

Applications

The neuron-specific AR inhibitors the authors identified could be used to help determine the contribution of AR activity in neurons vs muscle to Kennedy's disease and they might be useful in the treatment or prevention of this disease.

Terminology

Polyglutamine expansion refers to the genetic process in which the CAG repeat in genes, which corresponds to the amino acid glutamine, is inappropriately copied, resulting in an increasing number of glutamine residues in the corresponding protein. At a certain length, which varies by gene, this expansion at the protein level causes disease.

Peer-review

This is an interesting study, the paper is well written and results could be potentially relevant.

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Basic Study

B-1 cells modulate the murine macrophage response to *Leishmania major* infection

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Abstract**AIM**

To investigate the modulatory effect of B-1 cells on murine peritoneal macrophages infected with *Leishmania major* (*L. major*) *in vitro*.

METHODS

Peritoneal macrophages obtained from BALB/c and

BALB/c XID mice were infected with *L. major* and cultured in the presence or absence of B-1 cells obtained from wild-type BALB/c mice. Intracellular amastigotes were counted, and interleukin-10 (IL-10) production was quantified in the cellular supernatants using an enzyme-linked immunosorbent assay. The levels of the lipid mediator prostaglandin E2 (PGE₂) were determined using a PGE₂ enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI), and the number of lipid bodies was quantified in the cytoplasm of infected macrophages in the presence and absence of B-1 cells. Culturing the cells with selective PGE₂-neutralizing drugs inhibited PGE₂ production and confirmed the role of this lipid mediator in IL-10 production. In contrast, we demonstrated that B-1 cells derived from IL-10 KO mice did not favor the intracellular growth of *L. major*.

RESULTS

We report that B-1 cells promote the growth of *L. major* amastigotes inside peritoneal murine macrophages. We demonstrated that the modulatory effect was independent of physical contact between the cells, suggesting that soluble factor(s) were released into the cultures. We demonstrated in our co-culture system that B-1 cells trigger IL-10 production by *L. major*-infected macrophages. Furthermore, the increased secretion of IL-10 was attributed to the presence of the lipid mediator PGE₂ in supernatants of *L. major*-infected macrophages. The presence of B-1 cells also favors the production of lipid bodies by infected macrophages. In contrast, we failed to obtain the same effect on parasite replication inside *L. major*-infected macrophages when the B-1 cells were isolated from IL-10 knockout mice.

CONCLUSION

Our results show that elevated levels of PGE₂ and IL-10 produced by B-1 cells increase *L. major* growth, as indicated by the number of parasites in cell cultures.

Key words: *Leishmania major*; Macrophages; B-1 cells; Interleukin-10; Prostaglandin E2; Infection

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Core tip: This original manuscript describes the modulatory effect of B-1 cells on *Leishmania major*-infected macrophages. We demonstrated the participation of soluble mediators in a co-culture system and characterized prostaglandin E2 and interleukin-10 (IL-10) as key factors involved in increased intracellular parasite replication. We also demonstrated that cell-cell contact is not important. The same effect was not observed when we used B-1 cells from IL-10 knockout mice, as no significant difference in parasite multiplication was observed. Thus, the current manuscript may be of interest for scientists working in the fields of immunoparasitology or immunomodulation.

Arcanjo AF, Nunes MP, Silva-Junior EB, Leandro M, Rocha JDB, Morrot A, Decote-Ricardo D, Freire-de-Lima CG. B-1 cells

modulate the murine macrophage response to *Leishmania major* infection. *World J Biol Chem* 2017; 8(2): 151-162 Available from: URL: <http://www.wjgnet.com/1949-8454/full/v8/i2/151.htm> DOI: <http://dx.doi.org/10.4331/wjbc.v8.i2.151>

INTRODUCTION

B-1 lymphocytes are murine cellular subpopulations that have their development and other characteristics that are distinct from the conventional B-2 cell populations^[1]. The B-1 cell population is a minor B-cell compartment that has been identified in mice and is involved primarily in innate immune responses^[2]. The interest in this cellular population has increased considerably due to its relationship with leukemia, autoimmunity and auto-reactivity^[3-6]. Recently, several manuscripts demonstrated that B-1 cells are able to transform into a type of phagocyte called a B-1 cell-derived phagocyte (B-1CDP) and play a role as antigen presenting cells (APCs)^[7-10]. Borrello and Phipps^[11] demonstrated that B-1 lymphocytes from the peritoneum of mice differentiate into phagocytic cells that are similar to macrophages.

Leishmania major (*L. major*) is the causative agent of cutaneous leishmaniasis (CL) in the Old World and is transmitted by the bite of the female phlebotomine sandfly, which injects the infective metacyclic promastigote forms into the dermis of the host^[12]. Cutaneous leishmaniasis is characterized by an ulcerative lesion that appears on the skin at the site of the sandfly bite and generally heals spontaneously. In experimental models of susceptibility, *L. major* infection induces a Th2-type immune response. In models of susceptibility to *L. major* infection, there is a production of anti-inflammatory mediators, which negatively modulate the response of the vertebrate host, favoring the establishment of infection^[13-15]. Graf and collaborators demonstrated that B-1 cells express COX-1 and up-regulate COX-2 and prostaglandin production in response to inflammatory signals. Our group recently demonstrated that B-1 CDP cells are easily infected by *L. major* and exhibit high susceptibility to infection and that this mechanism is dependent on prostaglandin E2 (PGE₂)/interleukin-10 (IL-10) production^[16].

Based on these data, we investigated the interaction between B-1 cells and *L. major*-infected macrophages from BALB/c mice and BALB/c XID mice (a lineage that is genetically depleted of B-1 cells) to elucidate the possible influence of this minor B-cell population on the progression of infection *in vitro*. We performed experiments to investigate whether B-1 cells could mediate any effect on macrophage susceptibility to infection based on cell direct interaction or cytokine-driven modulation.

MATERIALS AND METHODS

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of

Laboratory Animals of the National Institutes of Health (United States). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Health Science Center of the Federal University of Rio de Janeiro (CEUA-CCS, Permit Number: IBCCF 062/14), and all efforts were made to minimize suffering.

Mice and parasites

BALB/c and BALB XID (a lineage that is genetically depleted of B-1 cells) mice of both sexes, aged 6–8 wk, were obtained from the Oswaldo Cruz Institute Animal Care Facility (Fiocruz, Rio de Janeiro, Brazil). C57BL/6 IL-10 knockout (KO) and C57BL/6 wild-type mice were kindly donated by Professor João Santana Silva from the Department of Pharmacology, School of Medicine, USP, Ribeirão Preto. *L. major* strain LV39 (MRHO/Sv/59/P) was isolated each month from the footpads of infected BALB/c mice and maintained *in vitro* as proliferating promastigotes. The parasites were maintained in Schneider's medium (Gibco, Life Technologies) supplemented with 10% FCS, 1% glutamine and 2% human urine maintained in the animal facility at the Federal University of Rio de Janeiro (UFRJ).

B-1 cells

B-1 cells were isolated from BALB/c mice using the protocol described by Abrahão *et al.*^[1]. Briefly, macrophages were harvested *via* peritoneal lavage of BALB/c mice using cold DMEM medium (Gibco, Life Technologies). The total population of cells from the peritoneum was plated into 25 cm² tissue culture flasks (Corning) and incubated at 37 °C in a 5% CO₂ atmosphere for 120 min. The non-adherent cells were discarded, and DMEM medium containing 2 mmol/L glutamine, 50 µmol/L 2-ME, 10 µg/mL of gentamicin, 1 mmol/L sodium pyruvate, and 100 µmol/L MEM nonessential amino acids plus 10% fetal calf serum (FCS) was added to the adherent monolayer. The cultures were maintained for 5 d without changing the medium under the conditions described above. The non-adherent cell population was composed primarily of B-1 cells, as indicated by flow cytometry (results not shown), whereas the adherent cells represented an enriched macrophage population.

Macrophages and infection

Primary BALB/c or BALB/c XID peritoneal macrophages (2×10^5 cells/well) were cultured in 24-well plates (Corning) containing sterile round glass coverslips (13 mm) and allowed to attach for 2 h at 37 °C in 5% CO₂. The adherent macrophages were infected for 24 h with stationary phase *L. major* promastigotes at a multiplicity of infection of 10:1 (parasite/macrophage) and were then incubated at 37 °C in 5% CO₂. After 24 h, the monolayers were washed extensively with warm HBSS (Gibco, Life Technologies) to remove extracellular parasites. All cultures were maintained in medium containing 1% Nutridoma-SP (Roche, Basel, Switzerland) instead of FCS. B-1 cells were added at a 10:1 ratio (B-1 cell/macrophage) in the presence or absence of antibodies, solvents and reagents. After 3 d, infected

macrophages monolayers were extensively washed to remove non-phagocytosed promastigotes, and medium was replaced by Schneider medium (Life Technologies), supplemented with 20% FCS and 2% human urine. Infected monolayers were cultured at 26 °C for additional 3 d. The number of motile promastigotes released into the cellular supernatant was evaluated using a Neubauer chamber.

Assessment of the intracellular load of *L. major*

The relative intracellular load of *L. major* was assessed by counting the number of motile extracellular promastigotes released in each well. Infected peritoneal macrophages cultured on glass coverslips in the presence or absence of B-1 cells for 3 d at 37 °C. After this time the cultures were washed and stained with May-Grunwald Giemsa (Sigma-Aldrich), and intracellular amastigotes were counted in 100 infected macrophages. The results are shown as the number of intracellular amastigotes per macrophages and as the percentage of infected macrophages. All results are presented as the mean and SE of triplicate cultures.

Antibodies, cytokines and inhibitors

Peritoneal macrophage monolayers were treated with 10 µg/mL of aspirin (Sigma-Aldrich). Aspirin acts as an inhibitor of PGE₂ production^[17,18]. Neutralizing anti-transforming growth factor (TGF)-β and normal chicken IgY (R and D System), anti-IL-10 and rat IgG1 isotype control (BioSource Europe, Nivelles) antibodies were used at a concentration of 10 µg/mL.

Cytokine and PGE₂ measurement

The concentrations of cytokines in the supernatants obtained from infected cell cultures were quantified after 24 h of incubation using the sandwich immunoassay (ELISA) method according to the methodology recommended by the manufacturer (R and D). The optical density was obtained by reading the absorbance in a plate spectrophotometer (Versamax Microplates Reader Molecular Devices, United States), with a filter of 405 nm. The concentrations of cytokines were calculated from a standard curve of recombinant cytokines. PGE₂ was quantified using a PGE₂-specific EIA kit, according to the methodology recommended by the manufacturer (Cayman Chemical, Ann Arbor, MI).

Lipid body evaluation

To observe lipid bodies (LBs), macrophages were fixed with 3.7% formaldehyde for 10 min and stained by osmium tetroxide. The morphology of fixed cells was observed, and osmium-stained lipid bodies were counted by light microscopy with a 100 × objective lens in 50 consecutively scanned leukocytes.

Statistical analysis

Statistical analysis was performed using the program GraphPad InStat version 3.01 (San Diego, CA, United States). The data were analyzed using a *t*-test. Differences

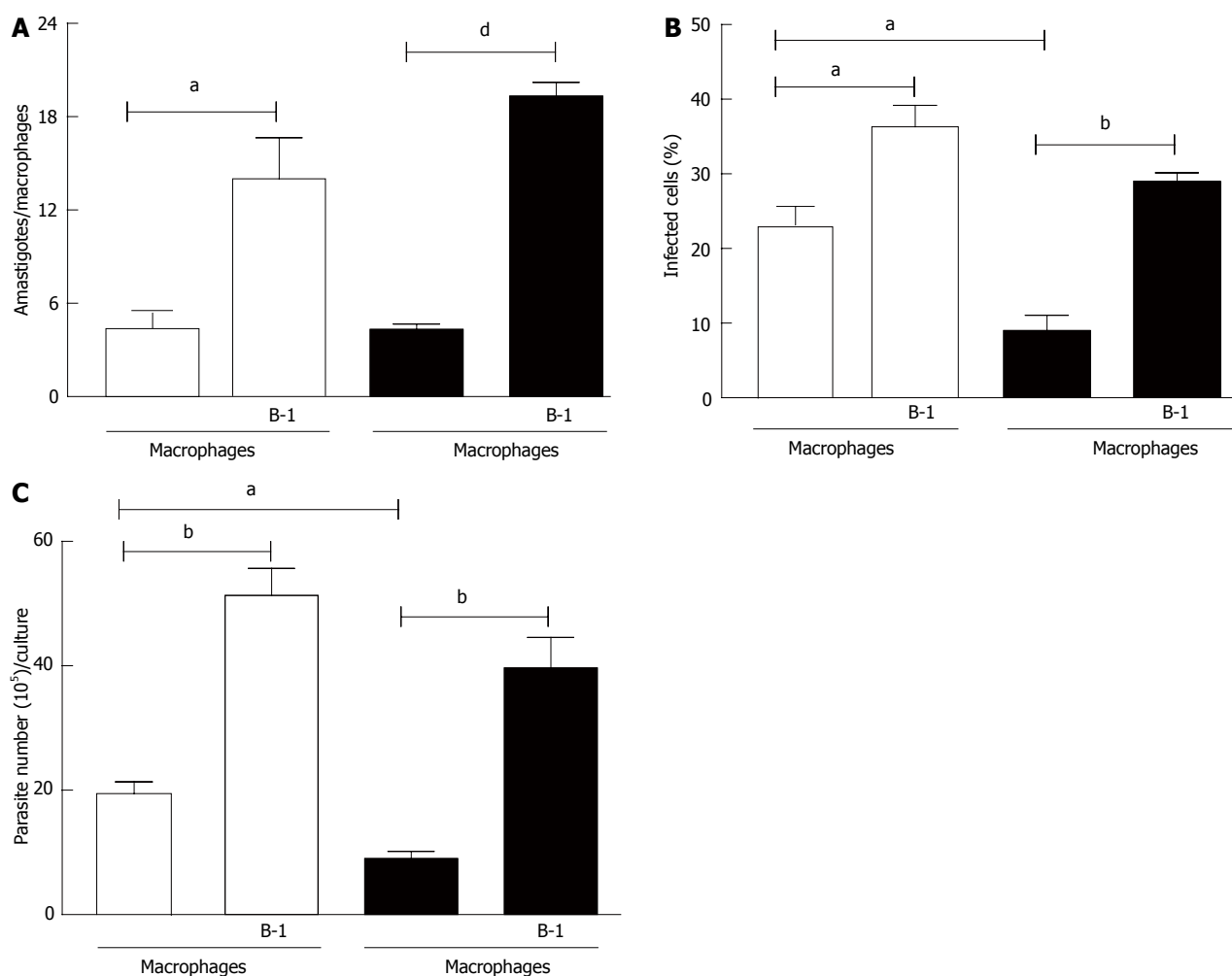


Figure 1 Effect of B-1 cells co-cultured with macrophages infected with *Leishmania major*. B-1 cells and peritoneal macrophages from BALB/c mice (white bars) or XID mice (black bars) were cultured (105/well) and infected with metacyclic promastigotes of *L. major*. After 24 h, the cell culture was washed and phagocytes were cultured for another 3 d with DMEM supplemented with 10% FBS at 37 °C. After this period, cells were stained and amastigotes inside the phagocytes were counted under the light microscope (A) and set the percentage of infected cells (B). To quantify promastigotes forms in the supernatants, the cells were cultures and infected with *L. major*. After 24 h of infection, so were washed and cultured at a temperature of 27 °C in Schneider medium 20% FBS during 5 d. After this period, the promastigotes were quantified in the supernatant of the cultures of infected phagocytes (C). All cultures were performed in triplicate and bars show the mean $\pm$ SD. Statistical analysis were performed by t-test from representative results of three different experiments. ^a $P < 0.05$, ^b $P < 0.005$ and ^d $P < 0.0001$.

with a P value of 0.05 or lower were considered to be significant.

RESULTS

Macrophage interactions with B-1 cells induce the growth of *L. major*

In this study, we analyzed the role of B-1 lymphocytes in modulating the replication of *L. major* in infected peritoneal macrophages *in vitro*. B-1 cells obtained from wild-type BALB/c mice and co-cultured with peritoneal macrophages from BALB/c and BALB XID mice infected with *L. major* promastigotes induced not only accentuated intracellular amastigote growth (Figure 1A) but also an increase in the percentage of infected cells (Figure 1B). The results obtained by counting the number of amastigotes in infected macrophages were confirmed *via* the quantification of motile promastigote forms in the supernatants after 5 d of interaction between B-1

cells and *L. major*-infected macrophages (Figure 1C). It is important to mention that XID macrophages were more resistant to infection with *L. major* than BALB/c macrophages (Figure 1).

The immunomodulatory factor of B-1 cells is not dependent on physical contact

We then assessed whether B-1/macrophage physical contact was required for the increased intracellular amastigote replication that was observed in our co-cultures. Increasing *T. cruzi* replication in macrophages requires recognition with apoptotic cells, thus directing the process of adhesion and phagocytosis^[19-21]. However, when using a Trans-well system in our model of intracellular *L. major* growth, we found that parasite multiplication was independent of cell contact between B-1 cells and infected macrophages from both types of mice (Figure 2). These results indicate that in our system, the observed parasite growth is mediated by a soluble factor(s) that is released

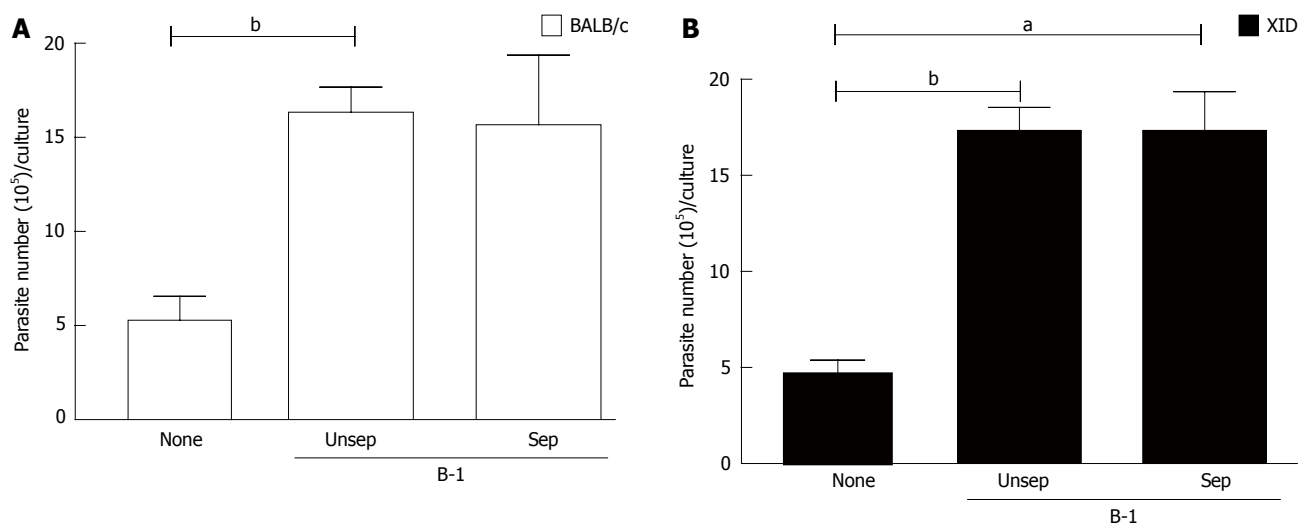


Figure 2 The immunomodulatory effect of co-cultured infected macrophages and B-1 cells is independent of contact. Peritoneal macrophages from BALB/c (A) and XID (B) mice were plated in 24 wells vessels at 105 cells/well in complete culture medium and infected with *L. major* (10⁶/well). After 24 h of infection, cells were washed and B-1 cells were added at same compartment (Unsep), or separated (Sep) by a cell-impermeable culture insert. The cells were cultured in Schneider medium for 5 d at a temperature of 27 °C. After this period, the promastigotes were quantified in the supernatant of the cultures of infected phagocytes. All cultures were performed in triplicate and bars show the mean $\pm$ SD. Statistical analysis were performed by *t*-test from representative results of three different experiments. ^a*P* < 0.05 and ^b*P* < 0.005.

into the cultures.

IL-10 released into the culture medium by B-1 cells controls *L. major* growth

B-1 cells are known to produce high levels of IL-10^[13,22,23]. However, the importance of B-1-derived IL-10 in infected macrophages must be clarified. Recently, our group demonstrated that the IL-10 produced by B-1 CDP cells is important to facilitate intracellular infection and increase the number of motile *L. major* promastigotes in the supernatants^[13]. Based on this information, we investigated whether B-1 cell-derived IL-10 should be related in the susceptibility of macrophages to intracellular infection.

Our data show that the amount of IL-10 in the supernatants of macrophages infected with *L. major* is increased in the presence of B-1 cells (Figure 3A). The cytokine TGF- β , which is another important mediator involved in the modulation of macrophages infected with intracellular parasites^[19-20], was not detected in the present study model (data not shown).

To determine the real importance of B-1-derived IL-10 in *L. major*-infected macrophages, we added a neutralizing anti-IL-10 antibody to the cell cultures. Our results demonstrated that the neutralization of IL-10 induced an important reduction in the number of promastigotes released into the supernatant (Figure 3B and C). The data previously described demonstrate that the production of this cytokine by B-1 lymphocytes is relevant for increasing parasite growth in peritoneal macrophages.

The presence of B-1 cells induced lipid body formation and PGE₂ production in infected macrophages

Lipid bodies (LBs) are organelles that are related in arachidonic acid metabolism and produce lipid mediators

in response to inflammatory stimulus^[24,25]. Infection with intracellular pathogens alters and increases the number of the lipid bodies^[26,27] in infected cells. In recent years, we have described a progress of the number of lipid bodies in phagocytic cells infected with *Trypanosoma cruzi* and *L. major* and evidenced the role of these lipid bodies in disease development^[13,26].

The results shown in Figure 4 demonstrate that infected macrophages co-cultured with B-1 cells contain a large number of lipid bodies in comparison to infected macrophages cultivated without B-1 cells (Figure 4). Our data demonstrate that macrophages from BALB/c mice contain more lipid bodies than XID macrophages, even in absence of a stimulus (Figure 4).

Production of PGE₂

The mechanism of LB formation and PGE₂ synthesis during *L. major* infection in co-cultures of macrophages and B-1 cells was investigated. Phagocytes derived from B-1 cells (B-1CDP cells) constitutively express COX enzymes and produce large amounts of PGE₂ in response to inflammatory signals or infection with *Leishmania* spp.^[13]. However, the production of the lipid mediator PGE₂ by B-1 cells has not yet been described. Based on this information, we investigated the production of PGE₂ by *L. major*-infected peritoneal macrophages obtained from BALB/c and XID mice. As described in Figure 5, the presence of B-1 cells induced considerable amounts of PGE₂ in both types of infected macrophages. Our data also demonstrate that infected macrophages from XID mice produce less PGE₂ than macrophages from BALB/c mice, even in the absence of B-1 cells (Figures 5 and 6).

To characterize the importance of PGE₂ for parasite load, we added two different non-steroidal anti-inflammatory drugs (NSAIDs) to the B-1 cell/macrophage cultures:

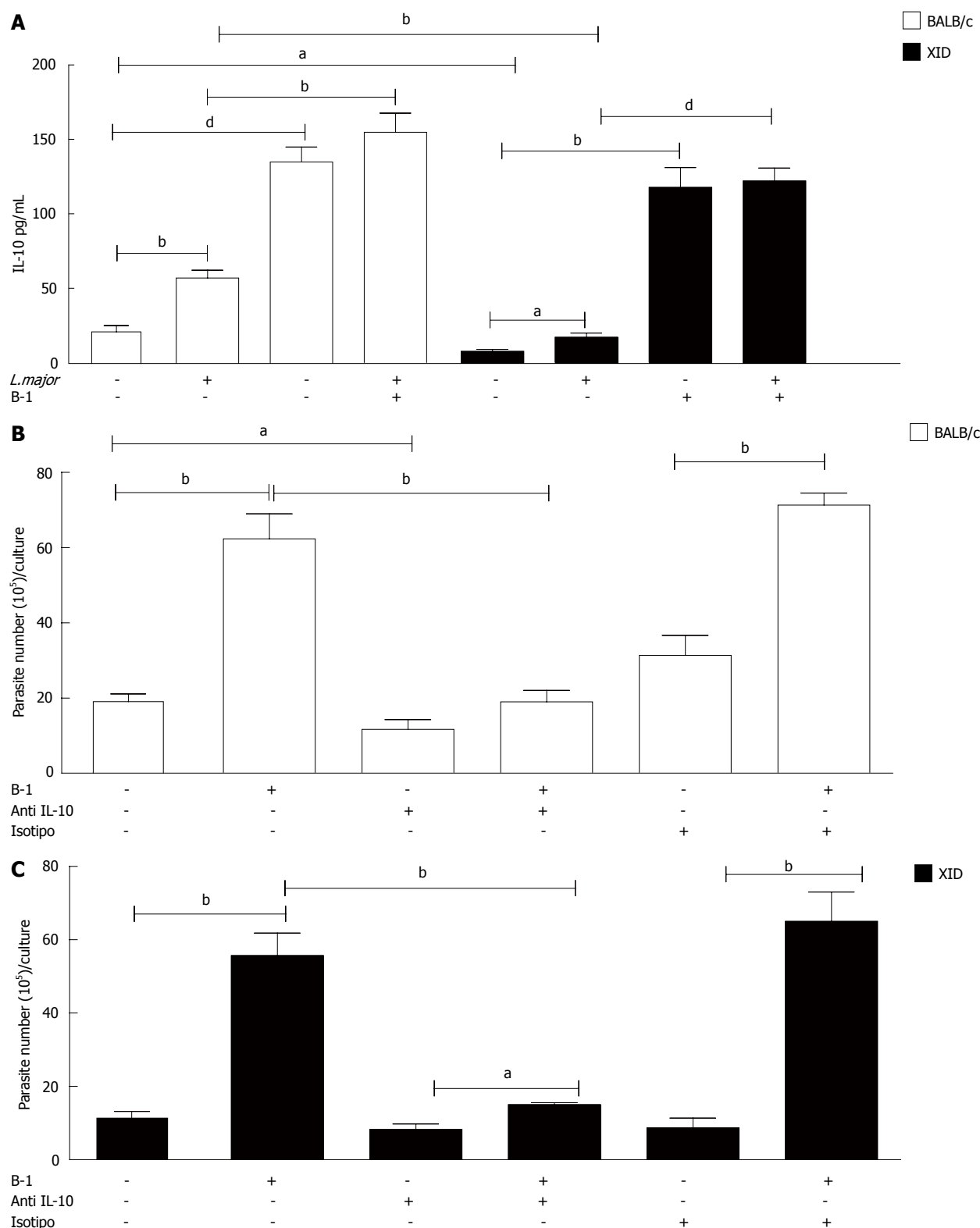


Figure 3 Interleukin-10 is a determinant factor for the increased parasite load in macrophages. Peritoneal macrophages from BALB/c or XID mice co-cultured with B-1 cells were cultured in the presence or absence of *L. major* (MOI 10:1). After 24 h, the supernatant was collected and IL-10 was measured by ELISA (A). All cultures were performed in triplicate and bars show the mean $\pm$ SD. Representative result of three different experiments. ^a $P < 0.05$, ^b $P < 0.005$ and ^c $P < 0.0001$. Co-cultures of B-1 cells and infected macrophages from BALB/c or XID mice were treated or not with doses of monoclonal neutralizing anti-IL-10 or control isotype. Once were infected with *L. major*, after 24 h, the cell cultures were washed with DMEM and incubated 3 d and then passed to Schneider medium. After 5 d in medium Schneider, promastigotes were counted in the supernatant (B). Statistical analysis were performed by *t*-test from representative results of three different experiments and bars show the mean $\pm$ SD. ^a $P < 0.05$ and ^b $P < 0.005$.

aspirin and indomethacin. These two drugs are recognized as potent inhibitors of PGE₂ production^[19,20]. Our data

demonstrate that the blockade of PGE₂ production affected the parasite burden inside the macrophages (Figure 6).

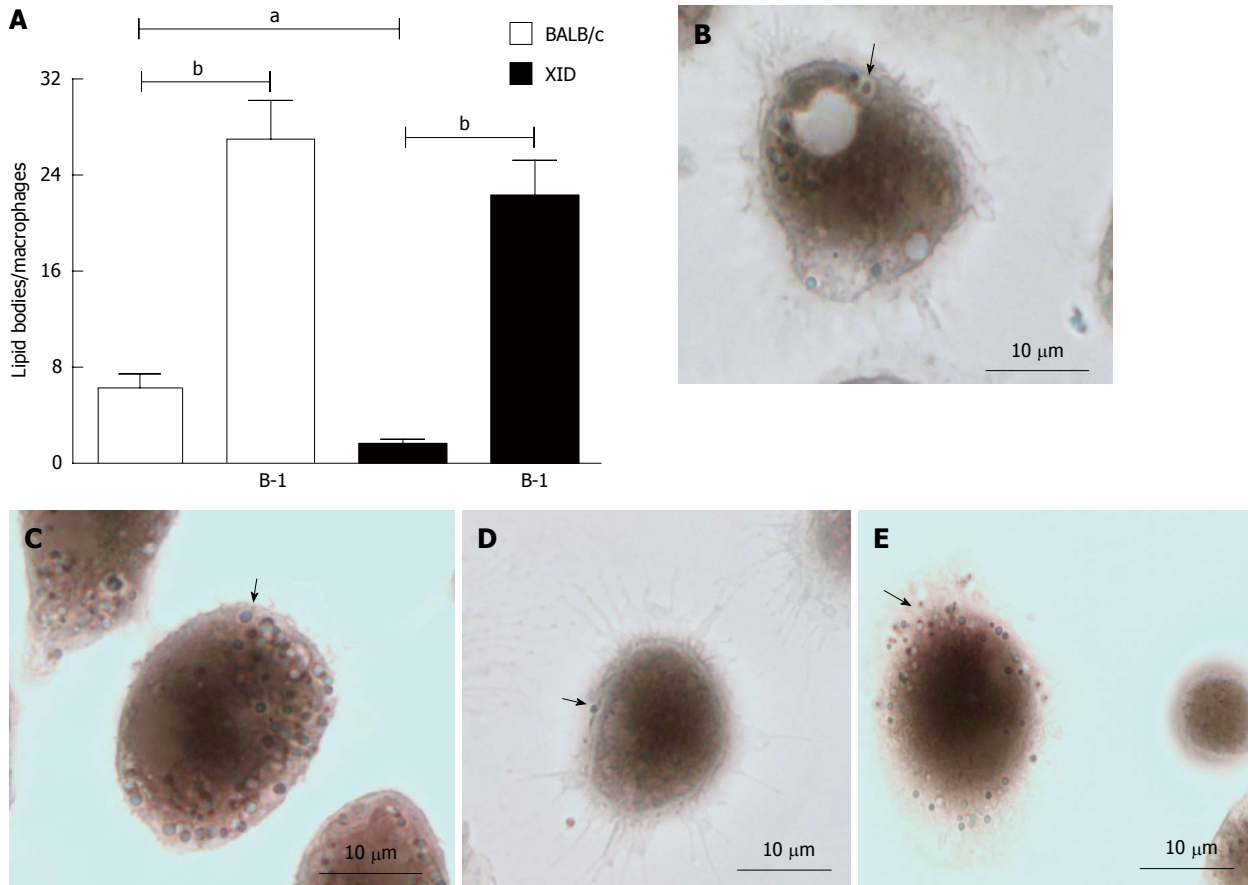


Figure 4 Presence of B-1 cells increased numbers of lipid bodies in infected macrophages. Peritoneal macrophages from BALB/c or XID mice were incubated with glass coverslips; some cultures were infected with *L. major*. Stained with Osmium tetroxide, the slides were washed and stained with DAPI (Sigma). The morphology of fixed cells was observed, and Nile red LBs were counted by light microscopy with a 100 × objective lens in 50 consecutively scanned leukocytes (A). Representative images of lipid body formation in infected macrophages from BALB/c mice (B), infected macrophages from BALB/c mice cultured in the presence of B-1 cells (C), infected macrophages from XID mice (D) and infected macrophages from XID mice co-cultured with B-1 cells (E). Black arrows pointing to the LB. Statistical analysis were performed by *t*-test from representative results of three different experiments. ^aP < 0.05 and ^bP < 0.005.

Blockade of PGE₂ by NSAIDs reduced IL-10 production by infected macrophages

Following the rationale that the inhibition of PGE₂ decreased the release of promastigotes into the supernatant of *L. major*-infected macrophages co-cultured with B-1 cells, we investigated whether that lipid mediator has an effect on IL-10 production. The results shown in Figure 7 reveal that an important reduction of IL-10 production occurred when the cultures were treated with NSAIDs (Figure 7).

B-1 cells isolated from IL-10 KO mice do not stimulate *L. major* growth

To demonstrate that the presence of the soluble cytokine IL-10 was essential for favoring intracellular infection, we used murine B-1 lymphocytes from IL-10 KO animals. When *L. major*-infected macrophages were co-cultured with B-1 cells from wild-type mice, the number of intracellular amastigotes (Figure 8A), the percentage of infected macrophages (Figure 8B) and the number of promastigotes released (Figure 8C) all increased. On the other hand, when infected macrophages were co-cultivated with B-1 cells from IL-10 KO mice, the modulatory effect that favors the parasite was not

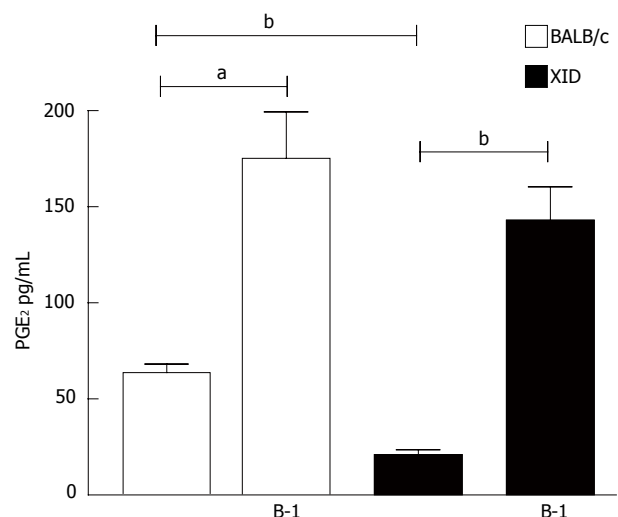


Figure 5 Infected macrophages co-cultured with B-1 cells secrete high levels of prostaglandin E₂. Infected macrophages from BALB/c or XID mice were incubated in the presence or absence of *L. major* (MOI 10:1). After 24 h of infection the supernatant was collected, and B-1 cells were added to the culture. After 24 h the level of PGE₂ secreted was measured by EIA. All cultures were performed in triplicate and bars show the mean ± SD. Statistical analysis were performed by *t*-test from representative results of three different experiments and bars show the mean ± SD. ^aP < 0.05 and ^bP < 0.005.

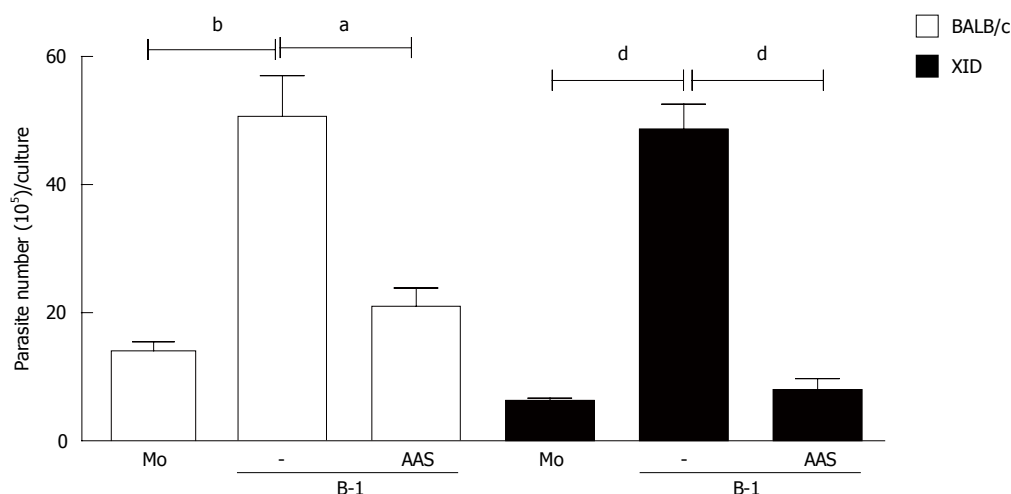


Figure 6 Blockage of the cyclooxygenase pathway induces protective phenotype in the *Leishmania major* infection. Infected macrophages from BALB/c or XID mice macrophages were incubated in the presence or absence of *L. major*, 24 h after the cellular cultures were washed and B-1 cells were added. The co-culture was treated or not with aspirin (AAS) (10 mg/mL). After 24 h of incubation, the cells were washed and incubated again for 3 d. After this time, the promastigotes were counted in the culture supernatant. All cultures were performed in triplicate and bars show the mean $\pm$ SD. Statistical analysis were performed by *t*-test from representative results of three different experiments and bars show the mean $\pm$ SD. ^a*P* < 0.05, ^b*P* < 0.005 and ^d*P* < 0.0001.

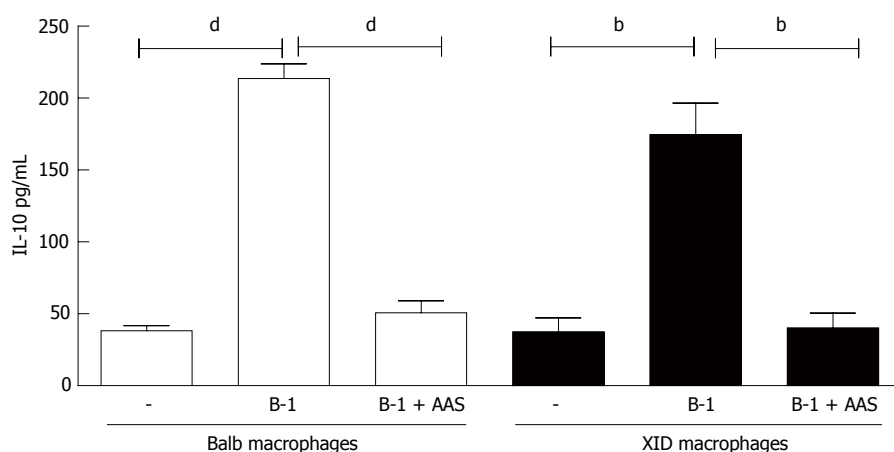


Figure 7 Effect of prostaglandin E2 inhibition on interleukin-10 production. Infected peritoneal macrophages from BALB/c or XID mice were co-cultured with B-1 cells and incubated with or without aspirin (AAS) (10 μ g/mL). After 24 h of incubation, the supernatant was collected and interleukin-10 measured by enzyme-linked immunosorbent assay. All cultures were performed in triplicate and bars show the mean $\pm$ SD. Statistical analysis were performed by *t*-test from representative results of three different experiments and bars show the mean $\pm$ SD. ^b*P* < 0.005 and ^d*P* < 0.0001.

observed. These results confirm the importance of IL-10 released by B-1 cells in the modulation of macrophage infection by *L. major*.

DISCUSSION

The findings reported in this manuscript are strengthened by experimental data. We co-cultivated B-1 cells with *L. major*-infected peritoneal macrophages from BALB/c mice and BALB XID mice, which are devoid of B-1 cells.

B-1 cells are of interest due to the immunomodulatory effects that these cells have exerted in different models of infection and inflammation^[2,13,23,28-31]. B-1 cells from the peritoneal cavity migrate to inflammatory and/or infectious sites and release a series of immunomodulatory factors into the environment and also have the ability to differentiate into phagocytes^[2,32]. Our group recently

demonstrated a role for B-1 cell-derived phagocytes (B-1CDP) in *L. major* infection *in vitro*^[13].

In the present study, we demonstrate that B-1 lymphocytes are efficient at inducing parasite replication when co-cultured with *L. major*-infected macrophages. The increased number of amastigotes observed in infected macrophages co-cultured with B-1 cells in our system is related to the number of motile promastigotes in the supernatants. Peritoneal macrophages isolated from XID mice exhibit resistance to infection with *L. major* when compared to BALB/c mice. However, when B-1 lymphocytes from BALB/c were added to the cultures of infected XID peritoneal macrophages *in vitro*, the cells became susceptible to infection, in comparison to co-cultures without B-1 cells. B-1 cells are known to produce large amounts of cytokines that could modulate macrophages and promote the intracellular infection of

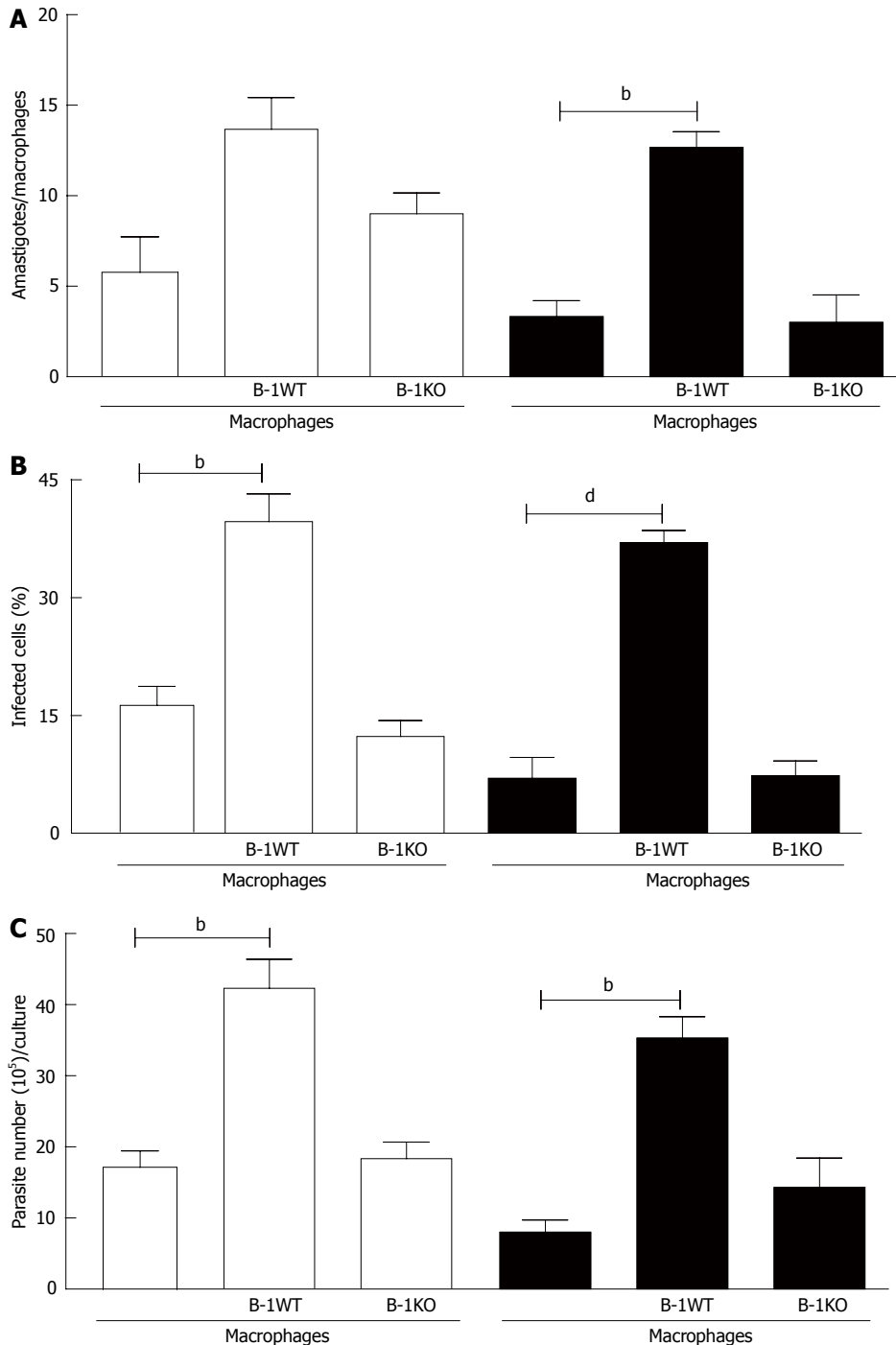


Figure 8 B-1 cells from interleukin-10 deficient mice are more competent to control *Leishmania major* infection in macrophages. To confirm the production of IL-10 is involved in the susceptibility to infection by *L. major*, we used B-1 cells from BALB/c (white bars) and from IL-10 KO mice (black bars). Our data demonstrate decreased in the number of intracellular amastigotes (A) and percentage of infected cells (B). We also observed the significant decrease in the liberated promastigotes forms by infected macrophages co-cultured with B-1 cells from IL-10 KO mice (C). Statistical analysis was performed by *t*-test from representative results of three different experiments and bars show the mean $\pm$ SD. ^b*P* < 0.005 and ^c*P* < 0.0001.

macrophages^[13,23]. To determine if cell-cell physical contact was responsible for macrophage infection and parasite replication, we used a cell-impermeable membrane and observed that macrophage infection occurred in the absence of cell contact. This result demonstrates that soluble factors were being secreted into the medium by B-1 cells.

As reported in several studies, B-1 cells can produce large amounts of IL-10, which plays an important immunomodulatory action in different cell types^[13,23,33]. IL-10 is an important mediator, which when released acts directly on different cell populations and is known to be an inhibitor of different types of cytokines, which are

important factors for the activation of phagocytes^[34-37] and consequently suppress the secretion of nitric oxide (NO). NO is an important factor that has leishmanicidal activity and favors the arginase pathway, which is involved in the activity of ornithine decarboxylase^[19,38-40]. Arginase would facilitate the intracellular growth of pathogens^[19,20].

In addition to IL-10, TGF- β is another cytokine that is important in modulating the intracellular growth of parasites^[19,20,26]. TGF- β has been implicated in increased parasite replication in different *in vivo* and *in vitro* models of experimental *Leishmania* infection^[41-44]. The results reported in this manuscript strongly suggest that

macrophage susceptibility to infection with *L. major* was related to the production of IL-10 by B-1 cells. Our data also indicated that the TGF- β was not related in the increased intracellular infection (data not shown). The importance of IL-10 produced by B-1 cells was confirmed in our study when we added a neutralizing antibody anti-IL-10 and observed a marked decrease in the number of parasites released into the culture supernatants.

We also found a large number of lipid bodies in the cytoplasm of *L. major*-infected macrophages co-cultured with B-1 cells. Lipid bodies are lipid-rich cytoplasmic organelles that control the accumulation and hydrolysis of neutral lipids and are found primarily in adipocytes^[45,46]. Macrophages were previously reported to have few lipid bodies in their cytoplasm, but the number of lipid bodies in these phagocytic cells can increase after stimulation^[13,26]. *T. cruzi* and *Leishmania* are able to induce lipid bodies in the cytoplasm of infected macrophages^[13,26]. It is already known that lipid bodies can be used as lipid sources that favor parasite replication or contribute to the production of soluble mediators involved in inflammation^[47]. Lipid bodies store arachidonic acid, which is important for the production of lipid mediators^[47].

Our data demonstrate that the number of lipid bodies in the cytoplasm of infected macrophages increased when these cells were co-cultured with B-1 cells. In addition to the increased number of lipid bodies, we also observed strong production of PGE₂ in the co-cultures. Our data also show that the inhibition of PGE₂ modulated the infection, resulting in a decrease in the number of promastigote forms released in co-cultures of infected macrophages and B-1 cells. PGE₂ is a lipid mediator that plays an important role in the production of factors, such as TGF- β ^[19] and IL-10^[13].

Our group recently described the production of anti-inflammatory mediators in experimental *T. cruzi* and *Leishmania* models, resulting in the inhibition of leishmanicidal products^[13,19].

PGE₂ is an important modulator of the T lymphocytes activation and the production of NO by phagocytes and favors the infection of intracellular parasites^[19,21]. In addition, PGE₂ is essential for increasing parasite growth in macrophages that have ingested apoptotic cells^[19,21]. The presence of the lipid mediator PGE₂ acts as a potent inhibitor of not only the immune response mediated by T lymphocytes but also the production of microbicidal factors by macrophages, favoring the growth of intracellular parasites^[13,19,21]. In addition, PGE₂ is a key lipid mediator that has been implicated in *T. cruzi* amastigote proliferation inside macrophages that have phagocytosed apoptotic bodies^[19-21,26].

Our results demonstrate that the production of IL-10 by B-1 cells is involved in the immunomodulatory mechanism in infected macrophages and consequently favors the replication of intracellular parasites. This result clarifies the involvement of IL-10 produced by B-1 cells as the key factor for the modulation of macrophage

activation. A similar result was obtained by Arcanjo *et al.*^[13], who used B-1CDP phagocytes from IL-10 KO mice and observed that intracellular infection with *L. major* was impaired. Another elegant work reported similar data in the context of *in vitro* infection by *Coxiella burnetii*. The authors observed that the macrophages of XID mice, which present a defect in the production of B-1 lymphocytes, have a better resistance to intracellular infection by *C. burnetii* when compared to the macrophages of wild-type mice^[48].

In summary, our work demonstrates that one role of the B-1 cell population is to produce an anti-inflammatory cytokine and a lipid mediator that exert their effects on macrophages of the innate immune system, contributing to *L. major* growth and replication *in vitro*, probably by inhibition of NO and ROS production. Further studies should be addressed to investigate the importance of B-1 cells in the lesion sites highlighting a possible clinical significance of these cells in the infection.

COMMENTS

Background

During infections the pathogens subvert the immune responses to their purpose. This is well demonstrated in chronic persistent infection mediated by *Leishmania* species. This parasite is adapted to grow inside macrophage where they are maintained during the parasitism of the host. Herein, the authors show that infection by *Leishmania major* (*L. major*) triggers the suppressive role of B-1 cells responsible to secrete interleukin-10 (IL-10) cytokine upon contact with infected macrophages. This immunomodulatory cytokine is shown to deactivate macrophage innate responses thus favoring the parasite burst in the host.

Research frontiers

The findings shown in the present study are of relevance to the immuno-parasitology field.

Innovations and breakthroughs

The knowledge of the host-parasite interplay is crucial to design therapies aimed at controlling pathogen infections. Here the authors show that *L. major* parasites induce immunosuppression of macrophage cells which is important to their maintenance in the host.

Applications

The authors' main findings point to a role of B-1 cells mediating the parasite subversion of the host's immune defenses. Further studies should be focused to investigate the molecular mechanisms involved in this immunomodulatory response.

Terminology

Parasite subversion: Inhibition of protective immunity of the host to favor the parasitism; Chronic persistent infection: A sort of pathogens are able to subvert the host immune responses in order to establish latent infections; Immunomodulatory response: The immune system is counter balanced by homeostatic responses in order to avoid its overactivity; B-1 cells: B lymphocyte subset displaying functional properties different from the conventional B cell population (B-2 cells). B-1 cells are thought to mediate immunomodulatory responses by IL-10 and prostaglandin E₂ (PGE₂) secretion.

Peer-review

The authors investigate the immunomodulatory effect of B-1 cells in *L. major* infected macrophages. Results suggest that PGE₂ and IL-10 released from B-1 cells increase intracellular parasite replication. The manuscript is generally well-written.

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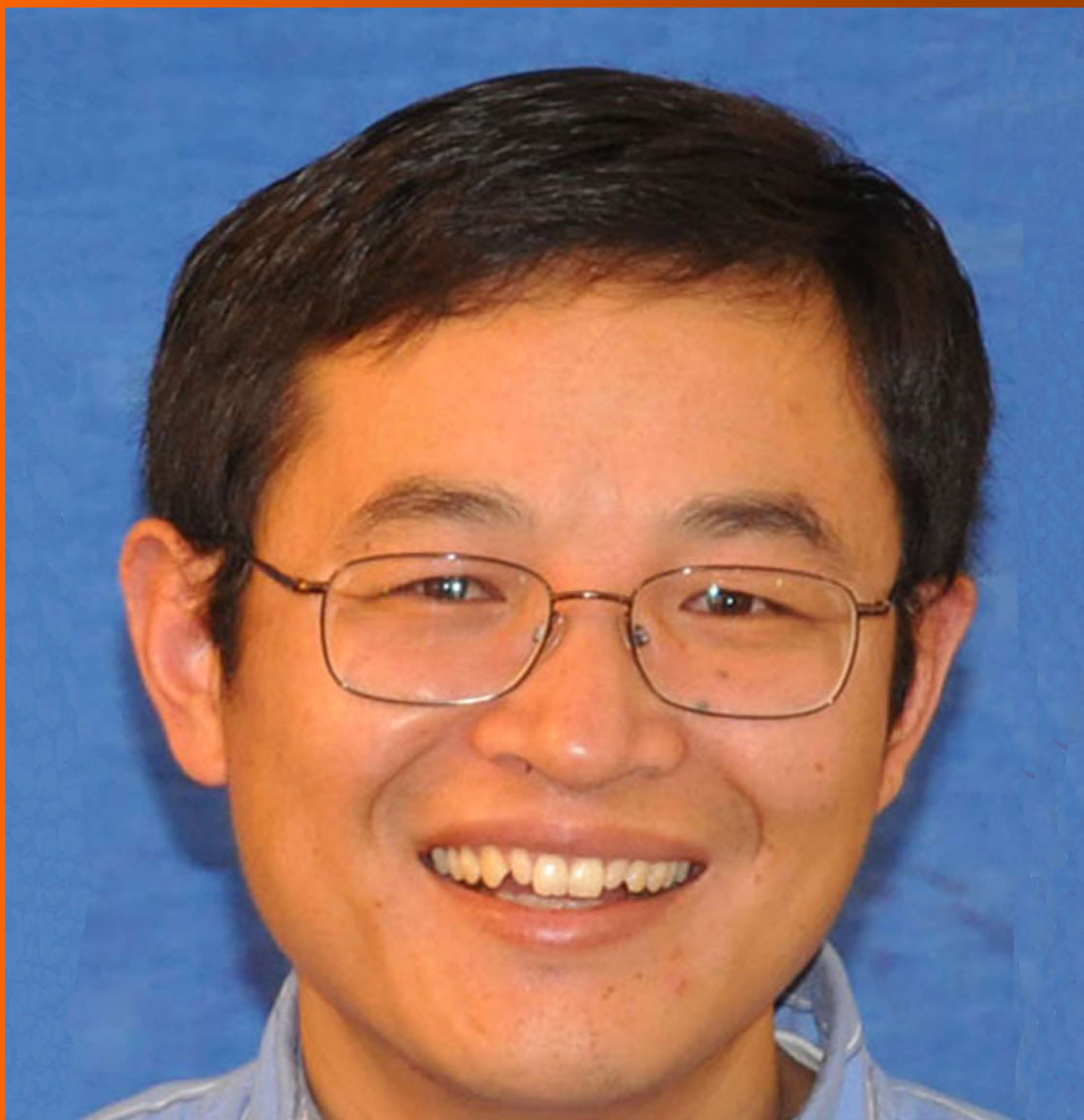


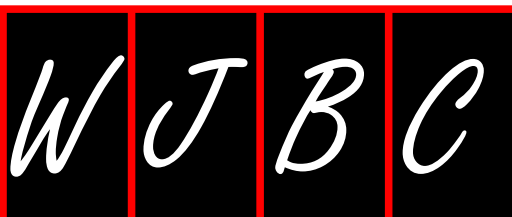
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ORIGINAL ARTICLE

Basic Study

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Samuels AL, Louw A, Zareie R, Ingley E

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Basic Study

Control of nuclear-cytoplasmic shuttling of Ankrd54 by PKC δ

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Abstract**AIM**

To identify and characterize the effect of phosphorylation on the subcellular localization of Ankrd54.

METHODS

HEK293T cells were treated with calyculin A, staurosporin or phorbol 12-myristate 13-acetate (PMA). Cells were transfected with eGFP-tagged Ankrd54 with or without Lyn tyrosine kinase (wild-type, Y397F mutant, or Y508F mutant). The subcellular localization was assessed by immunofluorescence imaging of cells, immunoblotting of subcellular fractionations. The phosphorylation of Ankrd54 was monitored using Phos-tagTM gel retardation. Phosphorylated peptides were analysed by multiple-reaction-monitoring (MRM) proteomic analysis.

RESULTS

Activation of PKC kinases using PMA promoted nuclear export of Ankrd54 and correlated with increased Ankrd54 phosphorylation, assayed using Phos-tagTM gel retardation. Co-expression of an active form of the PKC δ

isoform specifically promoted both phosphorylation and cytoplasmic localization of Ankrd54, while PKC δ , Akt and PKA did not. Alanine mutation of several serine residues in the amino-terminal region of Ankrd54 (Ser14, Ser17, Ser18, Ser19) reduced both PMA induced cytoplasmic localization and phosphorylation of Ankrd54. Using MRM proteomic analysis, phosphorylation of the Ser18 residue of Ankrd54 was readily detectable in response to PMA stimulation. PMA stimulation of cells co-expressing Ankrd54 and Lyn tyrosine kinase displayed increased co-immunoprecipitation and enhanced co-localization in the cytoplasm.

CONCLUSION

We identify phosphorylation by PKC δ as a major regulator of nuclear-cytoplasmic shuttling of Ankrd54, and its interaction with the tyrosine kinase Lyn.

Key words: Ankrd54; PKC δ ; Lyn; Btk; Phosphorylation

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Core tip: Ankrd54 is a nuclear-cytoplasmic shuttling adaptor that interacts with Lyn, Btk, Txk, and HCLS1. Activation of PKC kinases promoted nuclear export and phosphorylation of Ankrd54, and increased interaction and cytoplasmic co-localization with Lyn. Co-expression of an active form of the PKC δ isoform specifically promoted both phosphorylation and cytoplasmic localization of Ankrd54. Alanine mutation of several serine residues in the amino-terminal region of Ankrd54 reduced both phorbol 12-myristate 13-acetate induced cytoplasmic localization and phosphorylation of Ankrd54. These results identify PKC δ as a major regulator of nuclear-cytoplasmic shuttling and interaction of Ankrd54 with Lyn, through its phosphorylation of at least the Ser18 residue.

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INTRODUCTION

Protein nuclear-cytoplasmic compartmentalization and the shuttling of proteins between the nucleus and cytoplasm is regulated at multiple levels and is vitally important for many cellular processes (e.g., transcription). During interphase, when the nuclear envelope exists, the nuclear pore complex (NPC) controls the nuclear entry and exit of many proteins and protein complexes^[1]. While proteins/complexes less than approximately 40 kDa in size can typically diffuse through the NPC opening, larger proteins rely on an active process to traverse the NPC and enter/exit the nucleus. The primary control mechanism for most proteins passing through the NPC is the exposure of a nuclear localization sequence (NLS) and/or a nuclear

export sequence (NES)^[2]. The classical NLS is enriched in basic residues and mediates interaction with an importin α/β complex, which then guides the NLS containing cargo through the NPC, and it is released into the nucleus upon RanGTP binding^[3]. Proteins exiting the nucleus typically display a leucine-rich NES which interacts with Crm1 in the presence of RanGTP and mediates their export through the NPC. Many proteins that actively shuttle between the nucleus and cytoplasm contain both NLS and NES motifs^[4].

Posttranslational modifications of nuclear-cytoplasmic shuttling proteins have been shown to regulate the exposure and interaction of NLS and NES motifs with the importin α/β and Crm1 complexes, respectively^[5]. Phosphorylation appears to be a major regulator and mediator of both NLS and NES activity, with examples of phosphorylation promoting nuclear import (e.g., STATs and Erk1/2) as well as nuclear export (e.g., NFAT)^[6-8].

Previously we identified Ankrd54 (also called Liar) as a novel nuclear-cytoplasmic (containing functional NLS and NES motifs) shuttling adaptor of the Src family tyrosine kinase Lyn and other signalling molecules (e.g., HCLS1), that regulates down-stream signalling in erythropoietin-responsive erythroid cells^[9]. Ankrd54 was also identified as an adaptor for the Tec family tyrosine kinases Btk and Txk^[10]. Both these studies illustrated that Ankrd54 interacts with the SH3 domains of these kinases/signalling molecules *via* its ankyrin repeats and this interaction can regulate the nuclear-cytoplasmic compartmentalization of Lyn/Btk^[9,10]. While we identified that erythropoietin stimulation of erythroid cells promoted nuclear speckle accumulation of both endogenous Ankrd54 and Lyn^[9], the molecular mechanism controlling Ankrd54 nuclear-cytoplasmic shuttling remained unclear. Consequently, we investigated the role of phosphorylation in regulating Ankrd54 localization to nuclear/cytoplasmic compartments and identified an amino-terminal serine cluster that is required for phorbol ester (PMA)-stimulated cytoplasmic accumulation of Ankrd54, mediated by the serine/threonine kinase PKC δ isoform. Intriguingly, tyrosine phosphorylation of PKC δ by Src family kinases activates the serine/threonine kinase, which could then promote cytosolic accumulation of Ankrd54 and cytoplasmic interaction with Lyn.

MATERIALS AND METHODS

Cell culture, antibodies and reagents

HEK293T cells were maintained in Delbocco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). Polyclonal rabbit antibodies were raised against murine Ankrd54 using the purified full-length protein fused to GST, as the antigen, and high titer serum was purified on protein-A beads as described previously^[9]. Additional antibodies used for immunoblotting were anti-Lyn (Santa-Cruz Biotechnology, sc-15 and sc-7274); anti- β -actin (AC-15, ab6276, Abcam, Cambridge, United Kingdom); anti-Histone H3 (H0164, Sigma-Aldrich); and anti-14-3-3 ζ (ab51129, Abcam). TRITC-labeled phalloidin

(P1591, Sigma-Aldrich) was used to visualize filamentous actin (F-actin). Phorbol 12-myristate 13-acetate (PMA, P8139), staurosporine (S4400) and leptomycin B (L2913) were purchased from Sigma-Aldrich, calyculin A (#9902) was purchased from Cell Signaling Technologies, and Phos-tag™-Acrylamide TM (ALL-107) was purchased from Wako Laboratory Chemicals (Japan).

Plasmid construction

All plasmid constructs were generated by site-directed mutagenesis using oligonucleotides (sequences available upon request), subcloning in frame into the appropriate vector and confirmation by sequencing. Murine Lyn, LynY397F and LynY508F expressing plasmids have previously been described^[11]. Full-length murine Ankrd54 cDNA was generated by incorporation of a 5'RACE product and a cDNA isolated from a cDNA library using site-directed mutagenesis as previously described^[9]. eGFP-tagged Ankrd54 was generated by subcloning murine Ankrd54 into the mammalian expression vector pEGFPC1 (Invitrogen, Carlsbad, CA, United States). This eGFP-Ankrd54 expression construct was then used as a substrate for site-directed mutagenesis to generate the NLS (Ankrd54KKRL111AALA, Ankrd54RR98AA, Ankrd54D98-114) and NES (Ankrd54LSL289ASA) mutants as previously described^[9]. The S14A/S17A/S18A/S19A mutant was generated by the same procedure using oligonucleotides that simultaneously introduced these serine-alanine mutations in one reaction. The mCherry-SH3 domain fusion was generated by subcloning the SH3 domain from murine Lyn cDNA into the pmCherry-C1 (Invitrogen) vector as an in-frame C-terminal fusion of mCherry. The Akt mammalian expression vectors were a kind gift from Professor Brian A. Hemmings (FMI, Switzerland) and have been previously described^[12]. The PKC mammalian expression vectors for PKC α and PKC δ isoforms were a generous gift from Professor Jae-Won Soh (Inha University, South Korea) and have been described previously^[13,14]. Expression vectors for the catalytic domain of cAMP-dependent protein kinase (PKA) were a kind gift from Professor G. Stanley McKnight (University of Washington, Seattle, WA, United States) and have previously been described^[15].

Cell lysis, fractionation, immunoblotting, and immunoprecipitation

Cells (HEK293T) were transiently transfected using Lipofectamine 2000 (Invitrogen) and harvested 24-48 h post transfection. Protein lysates were prepared in 1% nonidet P40, 0.1% SDS, 150 mmol/L NaCl, 50 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA, 1 mmol/L EGTA, 25 mmol/L sodium fluoride, 25 mmol/L β -glycerophosphate, 1 mmol/L Na_2VO_4 , 1 $\times$ protease inhibitor cocktail (Complete, ROCHE) or 1 mmol/L benzamidine/1 mmol/L phenylmethylsulphonyl fluoride (PMSF)/1 $\mu\text{g}/\text{mL}$ aprotinin, for 30 min on ice, followed by centrifugation at $10000 \times g$ for 20 min. Protein concentration was estimated using the BioRad Dc protein assay according to manufacturer's instructions (BioRad), using bovine serum albumin as a

standard.

For immunoprecipitation, 5 mg of total protein from clarified cell lysates were incubated with specific antibodies (10 μg) for 2 h at 4 °C, collected with protein G-Sepharose beads (Sigma-Aldrich) for 16 h (4 °C) before washing in lysis buffer, and subsequent analysis by SDS-PAGE.

For separation of nuclear and cytoplasmic compartments subcellular fractionation was performed. Cells were washed (twice) with cold PBS, and lysed in cell lysis buffer (20 mmol/L Tris-HCl pH 8, 0.5% NP40, 10 mmol/L NaCl, 3 mmol/L MgCl_2 supplemented with protease inhibitors) for 30 min on ice with vigorous pipetting every 5 min. Nuclei were sedimented by centrifugation at $5000 \times g$ for 5 min at 4 °C and the supernatant (cytoplasmic fraction) was collected. The nuclear pellet was washed 3 times in cell lysis buffer to remove cytoplasmic traces and the nuclei were lysed in nuclear lysis buffer (50 mmol/L Tris-HCl, 1% SDS) on ice for 20 min followed by brief sonication to ensure nuclei rupture. Nuclear debris was collected by centrifugation at $12500 \times g$ for 10 min at 4 °C and the supernatant (nuclear fraction) was collected. Nuclear and cytoplasmic fractions were stored at -80 °C prior to analysis by SDS-PAGE and Western blotting.

After probing with specific primary antibodies, proteins were revealed using either secondary antibody coupled to horseradish peroxidase (GE Healthcare or Cell Signaling Technology) and detection by enhanced chemiluminescence (GE Healthcare), or with fluorescently labeled secondary antibodies and an Odyssey scanner (LI-COR Biosciences, Lincoln, NE, United States). Quantitation of immunoblot analysis was performed using on Odyssey scanned blots using Image Studio Light (V3.1.4, LI-COR Biosciences).

Immunofluorescent microscopy

Glass coverslips (No. 1.5H, Marienfeld) were coated with either poly-L-lysine (Sigma-Aldrich) or serum before seeding cells (HEK293T). At the end of specific cell treatments of specific time points, coverslips were washed with PBS (37 °C) then fixed in 4% paraformaldehyde/PBS for 15 min at 37 °C, followed by permeabilization with 0.5% triton X-100 in PBS (5 min). Coverslips were then either processed immediately or stored at -80 °C with minimal PBS media. Coverslips were blocked for 2 h at 25 °C in 3% BSA/PBS/Tween-20 (0.1%), then incubated with primary antibody diluted in blocking buffer at 4 °C for 16 h, then washed 3 times in PBS/Tween-20 (15 min) before incubation with fluorophore-conjugated secondary antibodies (Alexa-Fluor-labeled-488/594, Life-Technologies), with Hoechst 33342 at 0.01 $\mu\text{g}/\text{mL}$, for 1 h at 25 °C, then washed in PBS/Tween-20 before mounting in Vectashield (H-1000, Vector Labs). For some experiments TRITC-labeled phalloidin (P1591, Sigma-Aldrich) at 50 $\mu\text{g}/\text{mL}$ was added to the secondary antibody/Hoechst solution to visualize filamentous actin (F-actin). For experiments where only transfected fluorescent protein was being analysed then permeabilized cells were incubated directly with Hoechst with or without

TRITC-phalloidin. For cells co-transfected with eGFP-Ankrd54 and Lyn expressing plasmids, Lyn was detected using anti-Lyn antibodies (Sant-Cruz Biotechnology, sc-15) and Alexa-Fluor-546 donkey anti-rabbit IgG (ThermoFisher Scientific). Fluorescent microscopy was performed using three different instruments: An MRC 1024 UV laser scanning confocal microscope (Bio-Rad) using a Nikon Diaphot 300 microscope fitted with a 40 × /1.15 oil objective and analysed using Confocal Assistant (v4.02, Bio-Rad); an × 60 plan-Apo 1.40 oil objective on an inverted Elcips-Ti microscope (Nikon, Japan), fitted with a CoolSNAP HQ² CCD camera (Photometrics), images analysed using NIS Elements 4.x (Nikon); or on a DeltaVision Elite system and analysed with softWoRx suite 2.0 (GE Healthcare). Generally, images were processed as z-stacks (deconvolution software used for the non-confocal systems) and maximum image projections generated. Quantitation of subcellular localization was performed on 50-100 individual cells for each experiment by analyzing 10 nuclear and 10 cytoplasmic sub-compartments per individual cell. Ratios of nuclear:cytoplasmic amounts of fluorescence (eGFP channel) were then collated. For statistical analysis experiments were repeated as three biological replicates and analysed by students *t*-test or ANOVA with two-tailed analysis, with orthogonal comparisons (using Prism v7.0b.154, Graphpad). Data are presented as mean ± SD, *P* < 0.05, *P* < 0.01, ns *P* > 0.05. The statistical methods of this study were reviewed by Michael Phillips from the Harry Perkins Institute of Medical Research.

Multiple reaction monitoring mass spectrometry

Protein bands obtained following SDS-PAGE were reduced and alkylated using DTT and iodoacetamide before digestion with chymotrypsin at pH approximately 8.0. Peptides were extracted as previously described^[16] and analysed by electrospray ionisation mass spectrometry using an Ultimate 3000 nano HPLC system (Dionex) coupled to a 4000 Q TRAP mass spectrometer (Applied Biosystems). Peptides were resolved using a C18 PepMap100 column (LC Packings) running a linear gradient of water/acetonitrile/with 0.1% formic acid. Multiple reaction monitoring (MRM) transitions for the putative phosphopeptides (flanking Ser18 of ANKRD54) were generated using the Skyline software^[17]. Both singly-phosphorylated and nonphosphorylated forms of the peptides, with one optional missed cleavage, were targeted. A non-phosphorylated peptide (sequence: QQDVEPRDEL, corresponding to amino acid 72-81) was also included in the MRM experiment as an internal normalization control. Spectra were imported back into the Skyline software for interpretation. Peak heights from each sample were divided by the relative peak height of the internal normalization control in the same sample to obtain normalized quantities. Normalized values associated with each hypermass were summed to obtain an overall quantity indicator for that hypermass. The overall hypermass quantities were then divided over the

maximum observed for that hypermass to obtain the overall percentage for the hypermass.

RESULTS

Ankrd54 contains a functional NLS and NES when expressed as an eGFP fusion, co-localizes with active Lyn and interacts with the SH3 domain of Lyn

We have previously shown that myc/HA-tagged and untagged Ankrd54 contain functional NLS and NES sequences^[9]. For this study, we generated an N-terminal eGFP fusion of Ankrd54 and confirmed that the NLS and NES were still functional in this fusion construct (Figure 1A). Further, the Crm1 inhibitor Leptomycin B promoted strong nuclear accumulation of eGFP-Ankrd54 (Figure 1B) as has been observed with myc/HA-tagged Ankrd54 previously^[9]. Interaction of eGFP-Ankrd54 and Lyn was also analogous to that previously delineated with the strongest co-localization seen with constitutively active (Y508F) Lyn and minimal with inactive (Y397F) Lyn (Figure 1C-E).

In erythroid cells Lyn and Ankrd54 both co-localize to nuclear speckles after erythropoietin stimulation^[9], while when overexpressed in HEK293 cells active Lyn and Ankrd54 co-localize in the cytoplasm/plasma-membrane (Figure 1E). To ascertain if Ankrd54 could co-localize with Lyn in the nucleus *via* their Ankyrin repeat-SH3 interaction, we co-expressed an mCherry fusion of the SH3 domain of Lyn with eGFP-tagged Ankrd54 (Figure 1F). Here eGFP-Ankrd54 was predominantly nuclear and localized to nuclear speckles. The SH3 domain of Lyn localized to both the cytoplasm and nucleus with stronger accumulation in the nucleus and specifically within Ankrd54 co-localized nuclear speckles (Figure 1F).

Modulation of phosphorylation via CalyculinA and staurosporine regulates nuclear-cytoplasmic shuttling of Ankrd54

Having established that the eGFP fusion of Ankrd54 retained functional NLS and NES, localization dynamics and Lyn/SH3 domain interactions, we then sought to delineate what signals could influence its nuclear-cytoplasmic shuttling. Modulation of phosphorylation using the phosphatase inhibitor CalyculinA and the kinase inhibitor Staurosporine produced profound effects on the subcellular localization of eGFP-Ankrd54 (Figure 1G and H). Immunofluorescence microscopy revealed that activation of kinases through inhibition of phosphatases with CalyculinA promoted cytoplasmic accumulation of eGFP-Ankrd54, while inhibition of kinases with Staurosporine significantly increased nuclear accumulation (Figure 1G). Biochemical subcellular fractionation and immunoblot analysis also showed that CalyculinA significantly promoted cytoplasmic accumulation of eGFP-Ankrd54 (Figure 1H). Further, addition of CalyculinA was unable to influence the compartmentalization of either NLS or NES deletion mutants of eGFP-Ankrd54, which accumulate in the

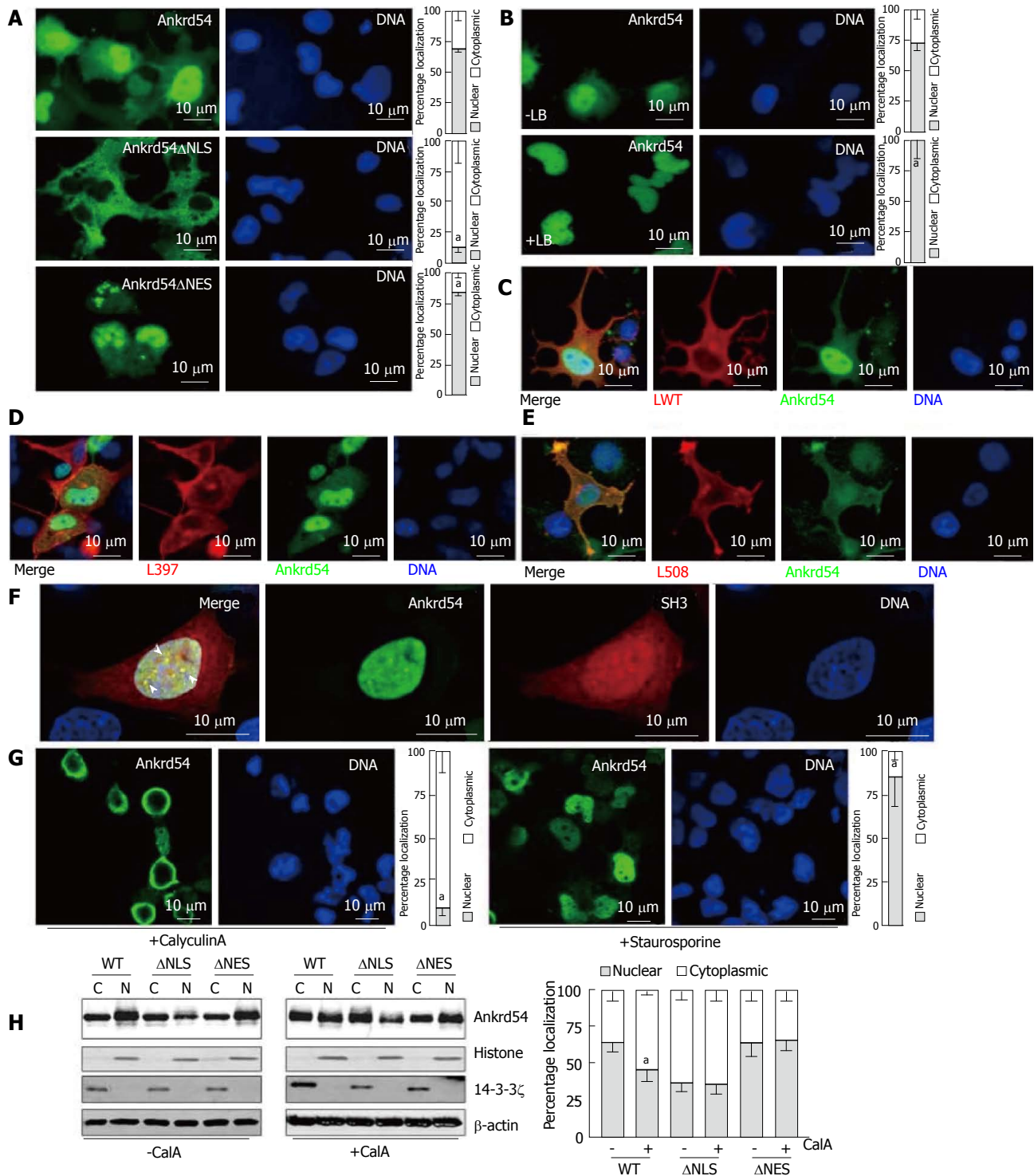


Figure 1 Ankrd54 shuttles between nuclear and cytoplasmic compartments and phosphorylation regulates this subcellular compartmentalization. **A:** Localization analysis of eGFP-tagged Ankrd54 (top panel), eGFP-Ankrd54 with the NLS deleted (Ankrd54 Δ NLS, middle panel), and eGFP-Ankrd54 with the NES deleted (Ankrd54 Δ NES, bottom panel) in HEK293 cells. Delineation of the nucleus by Hoechst staining of the DNA is on the right, eGFP fluorescence is on the left. Nuclear and cytoplasmic localization of Ankrd54 was enumerated (graph at right), $^aP < 0.05$; **B:** Wild-type eGFP-Ankrd54 expressing HEK293 cells were analysed after treatment with Leptomycin B (+LB) for 2 h (0.4 ng/mL), eGFP fluorescence (left) DNA counter staining (right), and quantitation of nuclear/cytoplasmic localization (graph at right), $^aP < 0.05$; **C:** Localization analysis of co-expressed eGFP-Ankrd54 (green) and Lyn wild-type (LWT, red), with DNA counterstained (blue). Merged and individual channels are shown; **D:** Localization analysis of co-expressed eGFP-Ankrd54 (green) and kinase inactive Lyn (L397, red), with DNA counterstained (blue). Merged and individual channels are shown; **E:** Localization analysis of co-expressed eGFP-Ankrd54 (green) and dominant active Lyn (L598, red), with DNA counterstained (blue). Merged and individual channels are shown; **F:** Localization analysis of eGFP-Ankrd54 and an mCherry-fusion of the SH3 domain of Lyn, with DNA counterstained. Merged image (left panel) illustrates co-localizing nuclear puncta (arrow heads); **G:** Effect of Calyculin A (50 nmol/L, 60 min) or Staurosporine (100 nmol/L, 60 min) on eGFP-Ankrd54 subcellular localization. DNA counterstained (blue), eGFP fluorescence (green). Quantitation of nuclear/cytoplasmic localization (graph at right), $^aP < 0.05$; **H:** Immunoblot of subcellular fractionation analysis of eGFP-Ankrd54 (WT), eGFP-Ankrd54 Δ NLS (Δ NLS) and eGFP-Ankrd54 Δ NES (Δ NES), without (left) and with (right) the addition of Calyculin A (50 nmol/L, 60min). Nuclear (N) and cytoplasmic (C) fractions of transfected HEK293 cells were immunoblotted using anti-Ankrd54, anti-Histone (H3), anti-14-3-3 ζ , and anti- β -actin antibodies. Quantitation of nuclear/cytoplasmic localization of Ankrd54 bands depicted in graph at right, $^aP < 0.05$.

cytoplasm or nucleus, respectively (Figure 1H).

Identification of PKC δ as mediating phosphorylation and cytoplasmic accumulation of Ankrd54

Having established that global phosphorylation is important for NES mediated cytoplasmic accumulation of Ankrd54 (Figure 1G and H), we then investigated the direct effect of phosphorylation on Ankrd54 subcellular localization. Analysis of phosphorylation databases (PhosphoSitePlus[®], Cell Signaling Technologies; PhosphoNET, Kinexus Bioinformatics Corporation, Canada; and PHOSIDA, Max Planck Institute of Biochemistry, Germany), indicated that Ankrd54 is a phosphoprotein with several residues identified in mass spectrometric data. Utilizing Phos-Tag[™] SDS-PAGE to detect phosphorylation-mediated mobility shifted Ankrd54, we discovered that CalyculinA promoted phosphorylation of Ankrd54 in the cytoplasmic fraction (Figure 2A). Using this Phos-Tag mobility shift assay we then analysed the ability of co-expressing candidate kinases (predicted from database searches, PhosphoNET, PHOSIDA) and kinase activators for their ability to mimic the CalyculinA effect. While co-expression of constitutively active forms of Akt or the catalytic subunit of PKA failed to promote a band-shift of Ankrd54, the addition of the PKC activator PMA did produce a clear phospho-Ankrd54 shifted band (Figure 2B and C). Consequently, we then tested the ability of two widely expressed PMA activated PKC isoforms (a conventional PKC, PKC α ; and a novel PKC, PKC δ) to promote cytoplasmic phosphorylation of Ankrd54 (Figure 2D and E). This clearly demonstrated that PKC δ could promote phosphorylation of cytoplasmic Ankrd54, especially when expressed as a constitutively active form (Δ NPS, N-terminal pseudo-substrate domain deletion), while PKC δ failed to promote Ankrd54 phosphorylation (Figure 2D and E). Further, quantitation of the PKC δ NPS promoted phospho-Ankrd54 revealed that PKC δ also promoted cytoplasmic accumulation of Ankrd54 (Cytoplasmic: nuclear ratio; Ankrd54 1.0:1.5; Ankrd54 + PKC δ Δ NPS 1.0:0.3). In addition, the CalyculinA mediated band-shift of cytoplasmic Ankrd54 was enhanced with NLS deleted, but strongly reduced in NES deleted mutants of Ankrd54 (Figure 2F). Consequently, we then ascertained the effect of co-expressing active PKC δ (PKC δ Δ NPS) with Ankrd54 on the subcellular localization of Ankrd54 by immunofluorescent microscopy (Figure 2G). This revealed, in agreement with the biochemical analysis (Figure 2E), that PKC δ significantly promotes cytoplasmic accumulation of Ankrd54.

Utilizing predictive algorithms (NetPho3.1)^[18,19] and interpretation of potential site of PKC phosphorylation (Figure 2H), identified four regions of potential phosphorylation; an amino-terminal cluster of serines (Ser14, Ser17, Ser18, Ser19), Thr103, Ser116 and Ser271. The C-terminal site Ser271, close to the NES motif, showed low probability of PKC phosphorylation due to a lack of N-terminal basic residues within the site. The Thr103 and Ser116 sites are located within the NLS motif (Thr103) and immediately C-terminal

(Ser116) to the NLS motif, and the N-terminal cluster (Ser14, Ser17, Ser18, Ser19) all have potential for PKC-mediated phosphorylation.

Mass spectrometric analysis of Ser18 of Ankrd54 correlates its phosphorylation status with PMA stimulation

While we identified several potential PKC phosphorylation sites, only the N-terminal Ser14/Ser17/Ser18/Ser19 cluster has been robustly detected in mass-spectrometry datasets (PhosphositePlus[®], PhosphoNET, PHOSIDA), in addition to several phosphorylated residues located close to the P-loop (Thr31, Ser38, Ser44, Ser54, Ser58, Ser63), with the Thr103, Ser116 and Ser271 motifs not robustly detected in these global phosphorylation analysis datasets. Consequently, we focused our initial attention on the amino-terminal Ser14/Ser17/Ser18/Ser19 cluster and its phosphorylation in response to PMA treatment. Using MRM of singly phosphorylated peptides encompassing the N-terminal serine cluster (Table 1) we identified this region as showing strong correlation of its phosphorylation with exposure of cells to PMA. Significantly, this included an inverse correlation of PMA stimulation with the non-phosphorylated peptide.

Mutation of amino-terminal serine cluster (Ser14, Ser17, Ser18, Ser19) mitigates PMA stimulated cytoplasmic accumulation of Ankrd54

Stimulation of eGFP-Ankrd54 transfected HEK293 cells with PMA promotes significant cytoplasmic accumulation of Ankrd54 by 1 h post phorbol ester addition, assayed by immunofluorescent microscopy (Figure 3A). Consequently, we used this time point of PMA stimulation to analyse the effect of mutagenesis of the N-terminal serine cluster (S14A, S17A, S18A, S19A) on cytoplasmic accumulation of Ankrd54 (Figure 3B-E). While wild-type Ankrd54 (Figure 3B) showed significant cytoplasmic accumulation after PMA stimulation in addition to phosphorylation (assayed by Phos-Tag[®] SDS-PAGE), the S14/17/18/19A mutant failed to show significant alteration to its predominantly nuclear subcellular localization, and failed to show a robust phosphorylation mobility shift (Figure 3C). Importantly, direct in-gel comparison of wild-type and the SA mutant of Ankrd54 showed a strong reduction in the band shift upon PMA stimulation of mutant Ankrd54 (Figure 3C, far right panel). Interestingly, mutagenesis of the NES motif (promoting nuclear localization) also disrupted the PMA-mediated cytoplasmic accumulation and phosphorylation of Ankrd54 (Figure 3D), while the NLS motif mutant (predominantly cytoplasmic) showed no significant further cytoplasmic accumulation upon PMA stimulation, but did show strong PMA-mediated phosphorylation (Figure 3E).

Modulation of phosphorylation by PMA stimulation regulates nuclear-cytoplasmic shuttling of Ankrd54 and its interaction with Lyn

Stimulation of eGFP-Ankrd54 and Lyn co-transfected HEK293 cells with PMA for 1 h promotes interaction

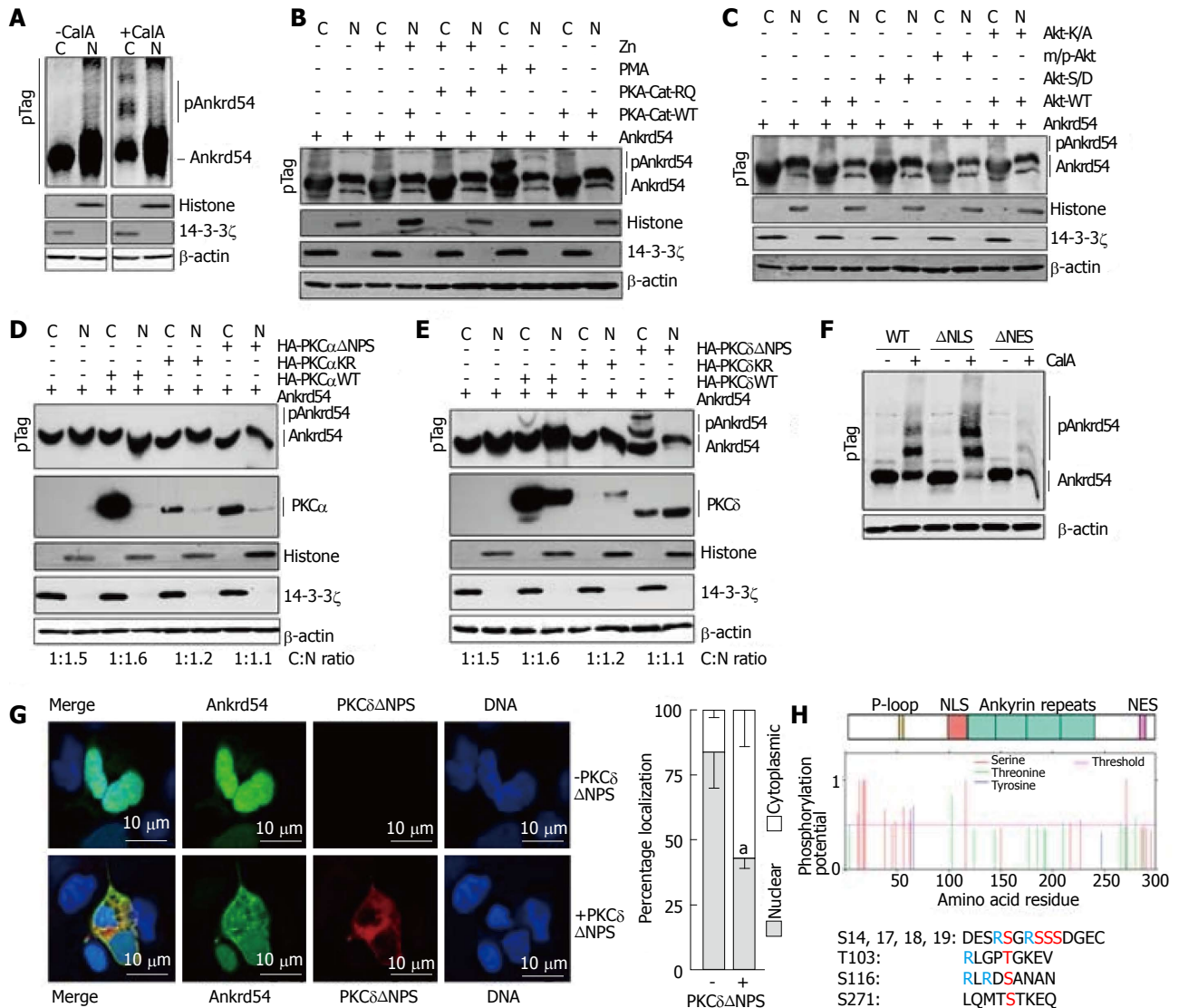


Figure 2 Phosphorylation by PKC δ regulates subcellular compartmentalization of Ankrd54. A: Immunoblot analysis of nuclear (N) and cytoplasmic (C) fractionation of eGFP-Ankrd54 using Phos-Tag™ SDS-PAGE (pTag), with (+CalA) and without (-CalA) CalyculinA treatment (50 nmol/L, 60 min). Transfected HEK293 cells were immunoblotted using anti-Ankrd54, anti-Histone (H3), anti-14-3-3 ζ , and anti- β -actin antibodies. Mobility shifted Ankrd54, due to phosphorylation, is indicated (pAnkrd54); B: Immunoblot analysis of nuclear (N) and cytoplasmic (C) fractionation of eGFP-Ankrd54 using Phos-Tag™ SDS-PAGE (pTag), co-transfected with PKA expression plasmids, or with addition of PMA (200 nmol/L, 60 min). The wild-type (PKA-Cat-WT) catalytic subunit of PKA, or a dominant active mutant (PKA-Cat-RQ) were induced to express by the addition of Zn. Transfected HEK293 cells were immunoblotted using anti-Ankrd54, anti-Histone (H3), anti-14-3-3 ζ , and anti- β -actin antibodies. Mobility shifted Ankrd54, due to phosphorylation, is indicated (pAnkrd54); C: Immunoblot analysis of nuclear (N) and cytoplasmic (C) fractionation of eGFP-Ankrd54 using Phos-Tag™ SDS-PAGE (pTag), co-transfected with Akt expression plasmids. Plasmids expressed either wild-type Akt (Akt-WT), a kinase inactive mutant (Akt-K/A), an oncogenic myristoylated/palmitoylated Akt (m/p-Akt), or an activation phospho-mimic (Akt-S/D). Transfected HEK293 cells were immunoblotted using anti-Ankrd54, anti-Histone (H3), anti-14-3-3 ζ , and anti- β -actin antibodies. Mobility shifted Ankrd54, due to phosphorylation, is indicated (pAnkrd54); D: Immunoblot analysis of nuclear (N) and cytoplasmic (C) fractionation of eGFP-Ankrd54 using Phos-Tag™ SDS-PAGE (pTag), co-transfected with HA-tagged PKC α expression plasmids. Plasmids expressed either wild-type PKC α (HA-PKC α WT), a kinase inactive mutant (HA-PKC α KR), or an activation mutant (HA-PKC α ΔNPS). Transfected HEK293 cells were immunoblotted using anti-Ankrd54, anti-HA (PKC α), anti-Histone (H3), anti-14-3-3 ζ , and anti- β -actin antibodies. Mobility shifted Ankrd54, due to phosphorylation, is indicated (pAnkrd54). Quantitation of cytoplasmic:nuclear ratios are displayed; E: Immunoblot analysis of nuclear (N) and cytoplasmic (C) fractionation of eGFP-Ankrd54 using Phos-Tag™ SDS-PAGE (pTag), co-transfected with HA-tagged PKC δ expression plasmids. Plasmids expressed either wild-type PKC δ (HA-PKC δ WT), a kinase inactive mutant (HA-PKC δ KR), or an activation mutant (HA-PKC δ ΔNPS). Transfected HEK293 cells were immunoblotted using anti-Ankrd54, anti-HA (PKC δ), anti-Histone (H3), anti-14-3-3 ζ , and anti- β -actin antibodies. Mobility shifted Ankrd54, due to phosphorylation, is indicated (pAnkrd54). Quantitation of cytoplasmic:nuclear ratios are displayed; F: Immunoblot analysis of cytoplasmic fractionation of eGFP-Ankrd54 using Phos-Tag™ SDS-PAGE (pTag), with and without the addition of CalyculinA (50 nmol/L, 60 min). Transfected HEK293 cells were immunoblotted using anti-Ankrd54 and anti- β -actin antibodies. Mobility shifted Ankrd54, due to phosphorylation, is indicated (pAnkrd54); G: Localization analysis of eGFP-Ankrd54 with and without co-expression of dominant active PKC δ (PKC δ ΔNPS) in HEK293 cells. Delineation of the nucleus by Hoechst staining of the DNA is on the right, eGFP fluorescence (green), PKC δ (HA-tag, red), with merged image (left). Nuclear and cytoplasmic localization of eGFP-Ankrd54 was enumerated (graph at right), *P < 0.05; H: Prediction of phosphorylated residues in Ankrd54. Top, schematic of Ankrd54 domain structure showing location of P-loop (yellow), NLS (red), ankyrin repeats (green) and NES (purple). Middle, graph of potential phosphorylated residues, predicted by NetPhos3.1. Below, amino acid sequences surrounding top predicted motifs as indicated.

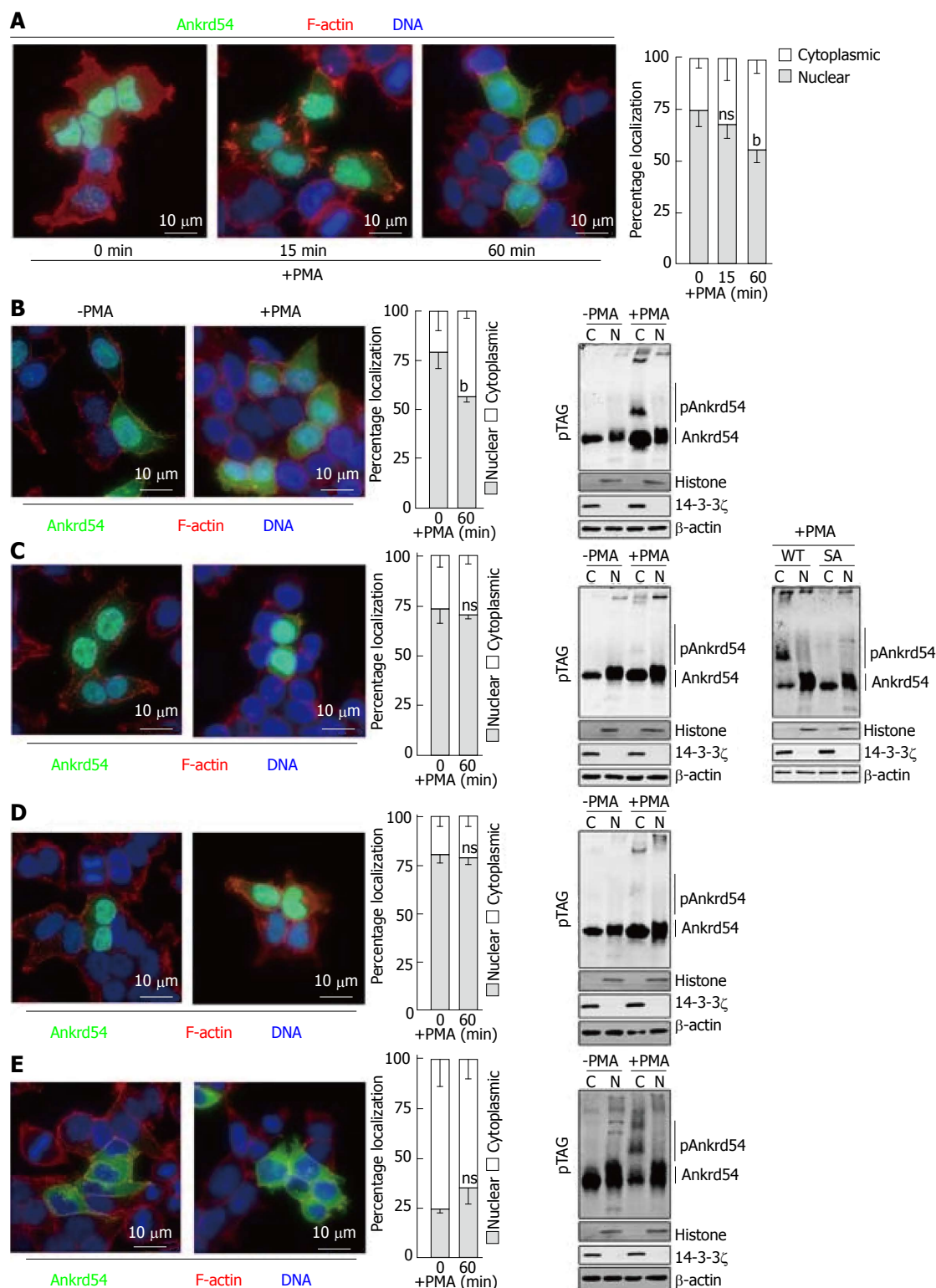


Figure 3 Amino terminal serine cluster (Ser14, Ser17, Ser18, Ser19) of Ankrd54 is required for phorbol 12-myristate 13-acetate stimulated nuclear export. A: Localization analysis of eGFP-tagged Ankrd54 (green) in HEK293 cells stimulated with PMA (200 nmol/L) for the indicated times. Delineation of the nucleus by Hoechst staining (blue), and F-actin with TRITC-phalloidin (red). Nuclear and cytoplasmic localization of Ankrd54 was enumerated (graph at right), ^b $P < 0.01$, ns: Not significant ($P > 0.05$); B-E: Localization analysis of eGFP-tagged Ankrd54 by immunofluorescence of cells (left panels) and by Phos-Tag™ SDS-PAGE (pTag) analysis (right panel), in HEK293 cells with and without PMA treatment (200 nmol/L, 60 min). Delineation of the nucleus by Hoechst staining (blue), and F-actin with TRITC-phalloidin (red). Nuclear and cytoplasmic localization of Ankrd54 was enumerated (graph in centre), ^b $P < 0.01$. Right panel, immunoblot analysis of nuclear (N) and cytoplasmic (C) fractionation of transfected HEK293 cells were immunoblotted using anti-Ankrd54, anti-Histone (H3), anti-14-3-3 ζ , and anti- β -actin antibodies. Mobility shifted Ankrd54, due to phosphorylation, is indicated (pAnkrd54). Cell immunofluorescence and immunoblot analysis was undertaken for cells expressing wild-type (B), serine-alanine (S14A, S17A, S18A, S19A) mutated (C), NLS deleted (D) and NES deleted (E) constructs of eGFP-tagged Ankrd54. For panel (C) a direct in-gel comparison of wild-type and SA mutant Ankrd54, before and after PMA stimulation is shown in the far-right panel. PMA: Phorbol 12-myristate 13-acetate.

Table 1 Multiple reaction monitoring of Ser18 of Ankrd54 with phorbol 12-myristate 13-acetate stimulation

Target peptide	m/z	Charge state	Transition	Normalized quantity		Overall percentage	
				+PMA	-PMA	+PMA	-PMA
Non-phosphorylated S18 peptide	936.078	3+	AATGGGADDESRSGRSSSDGECVAPEPL.y4+	4.2E+02	1.9E+03	26%	91%
			AATGGGADDESRSGRSSSDGECVAPEPL.y2+	8.5E+02	2.5E+03		
			AATGGGADDESRSGRSSSDGECVAPEPL.y15+++	2.6E+02	1.1E+03		
	702.310	4+	AATGGGADDESRSGRSSSDGECVAPEPL.y4+	ND	ND	ND	ND
			AATGGGADDESRSGRSSSDGECVAPEPL.y2+	ND	ND		
	826.871	4+	AATGGGADDESRSGRSSSDGECVAPEPLAEAGGL.y6+	2.0E+03	3.2E+03	61%	100%
			AATGGGADDESRSGRSSSDGECVAPEPLAEAGGL.y2+	2.0E+03	3.2E+03		
Normalization Control (Q72-L81)	614.794	2	QQDVEPRDEL.y8+	1	1	94%	100%
			QQDVEPRDEL.y6+	1	1		
			QQDVEPRDEL.y5+	1	1		
Singly-phosphorylated S18 peptide	962.733	3+	AATGGGADDESRSGRSSSDGECVAPEPL(p).y4+	1.3E+04	7.0E+03	70%	44%
			AATGGGADDESRSGRSSSDGECVAPEPL(p).y2+	1.1E+03	1.6E+03		
			AATGGGADDESRSGRSSSDGECVAPEPL(p).y15+++				
	722.302	4+	AATGGGADDESRSGRSSSDGECVAPEPL(p).y4+	5.5E+03	2.4E+03	71%	38%
			AATGGGADDESRSGRSSSDGECVAPEPL(p).y2+	5.8E+02	9.0E+02		
	846.862	4+	AATGGGADDESRSGRSSSDGECVAPEPLAEAGGL(p).y6+	ND	ND	100%	83%
			AATGGGADDESRSGRSSSDGECVAPEPLAEAGGL(p).y2+	1.1E+03	8.8E+02		

Singly-phosphorylated and nonphosphorylated forms of peptides encompassing the Ser18 residue (S18) were analysed and compared to the non-phosphorylated normalization control peptide of Ankrd54, before and after phorbol 12-myristate 13-acetate stimulation (1 μ mol/L, 1 h), of immunoprecipitated Ankrd54. ND: Not detected.

of Lyn and Ankrd54, assayed by immunoblotting of Ankrd54 immunoprecipitates (Figure 4A). Further, immunofluorescent microscopy (Figure 4B) of co-transfected cells illustrated strong co-localization in the cytoplasmic compartment of cells co-expressing Ankrd54 and Lyn, when the cells were stimulated with PMA for 1 h.

DISCUSSION

Here we describe the identification of an N-terminal serine cluster that can be phosphorylated by PKC δ to promote cytoplasmic accumulation of the nuclear-cytoplasmic shuttling adaptor Ankrd54 (binding partner of Lyn and Btk tyrosine kinases, and other signalling proteins)^[9,10,20,21] (summarized in Figure 5). These are important findings that help place the pathway context in which Ankrd54 complexes shuttle between the nuclear and cytoplasmic compartments, as being controlled (in part) by PKC δ (Figure 5).

Many of the binding partners of Ankrd54 have important functions both within the cytoplasm as well as the nucleus and enact these subcellular-specific functions in response to many different stimuli. Further, recent findings have identified the nuclear speckles that Ankrd54 can localize to^[9] as being PML (promyelocytic leukaemia) nuclear bodies and paraspeckles^[22], and the strong interaction of the SH3 domain of Lyn and Ankrd54 has been independently confirmed^[23,24]. PML nuclear bodies have a plethora of functions including cell cycle arrest, apoptosis, senescence and transcription and are regulated by signals that feed into these pathways. Paraspeckles function as regulators of gene expression, in response to cellular stress and differentiation, by controlling nuclear retention of RNA (containing double-stranded RNA regions) which have undergone adenosine-

to-inosine editing^[25-27]. The identification of Ankrd54 as a component of these two sub-nuclear structures, which contain many nuclear-cytoplasmic shuttling molecules, suggests the pathways that Ankrd54 (and its binding partners) is a part of are related to those that these two nuclear bodies/speckles are intimately involved with, *i.e.*, cell cycle, senescence, apoptosis, differentiation and transcription. Indeed, strong nuclear accumulation of Ankrd54 promotes apoptosis, and altering Ankrd54 levels and subcellular localization influences viability and differentiation pathways^[9,20].

PKC δ , a member of the novel (activated by diacylglycerol but not calcium) PKC subfamily has an intimate link with Src family kinases (SFK), including Lyn, as well as nuclear-cytoplasmic shuttling/signalling and the control of apoptosis in response to DNA damage^[28-31]. Activation of PKC δ involves diacylglycerol binding as well as tyrosine phosphorylation by SFK (as well as Abl and Syk), which can lead to activated PKC δ localizing to the plasma-membrane, mitochondria or the nucleus^[30]. Nuclear and mitochondrial localized PKC δ appear associated with induction of apoptosis^[29,32]. Differential tyrosine phosphorylation of PKC δ by SFKs/Abl mediates activation and either translocation to the nucleus or plasma-membrane^[29,31]. Interestingly, when active Lyn is co-expressed with Ankrd54 (Figure 1C-E)^[9], or Btk and Ankrd54 are co-expressed^[10], both accumulate in the cytoplasmic/plasma-membrane fraction. Consequently, Lyn/Btk tyrosine phosphorylated and activated PKC δ could be the driver of the cytoplasmic accumulation of Ankrd54 and interaction with Lyn. In addition, when an active form of PKC δ is co-expressed with Ankrd54 both localize outside of the nucleus (Figure 2G). This could also indicate that the activation of PKC δ within this context promotes a viability/anti-apoptotic response, due

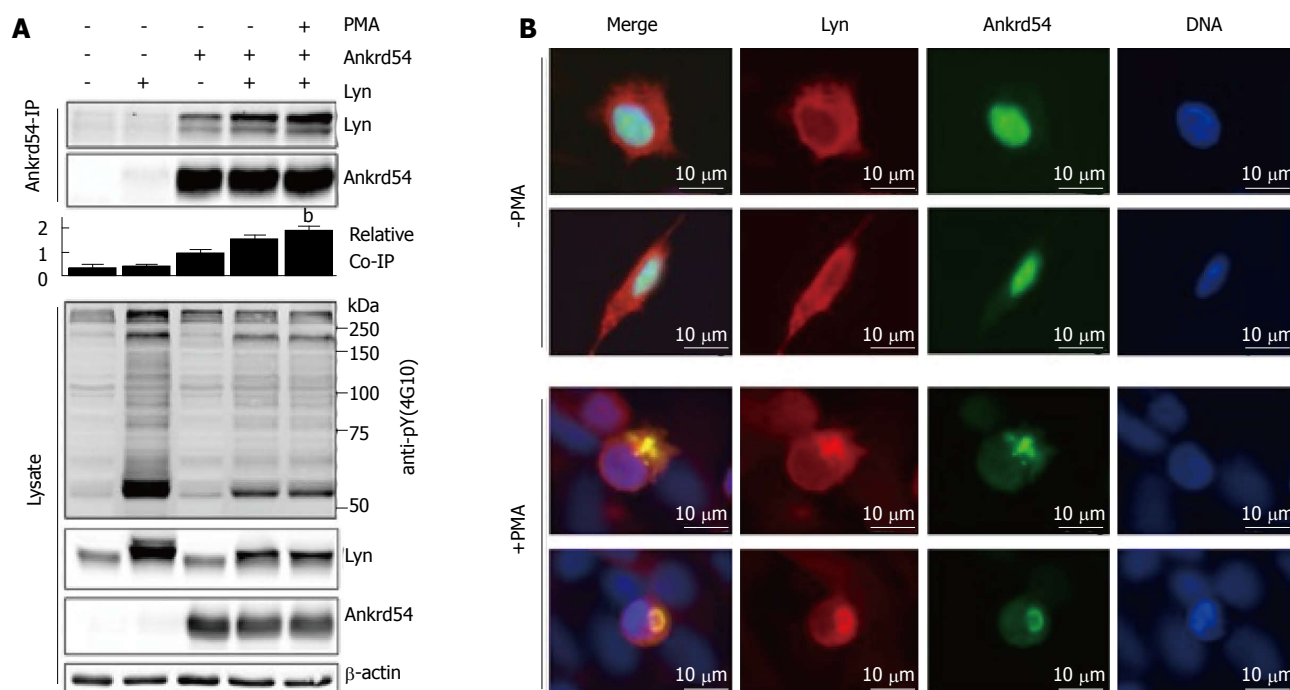


Figure 4 Phorbol 12-myristate 13-acetate stimulation of cells co-expressing Ankrd54 and Lyn promotes their co-immunoprecipitation and co-locations. A: Immunoblot analysis of Ankrd54 immunoprecipitates from cells transfected with eGFP-Ankrd54 and/or Lyn with or without PMA stimulation (200 nmol/L, 1 h). Immunoprecipitates and cell lysates were immunoblotted using anti-Ankrd54, anti-Lyn (sc-15, sc-7274), anti-pY-4G10, and anti- β -actin antibodies, $^bP < 0.01$; B: Localization analysis of eGFP-tagged Ankrd54 (green) and Lyn (sc-15, alexaFluor546) by immunofluorescence of HEK293 cells with and without PMA treatment (200 nmol/L, 1 h). Delineation of the nucleus by Hoechst staining (blue). Scale bar = 10 μ m; PMA: Phorbol 12-myristate 13-acetate.

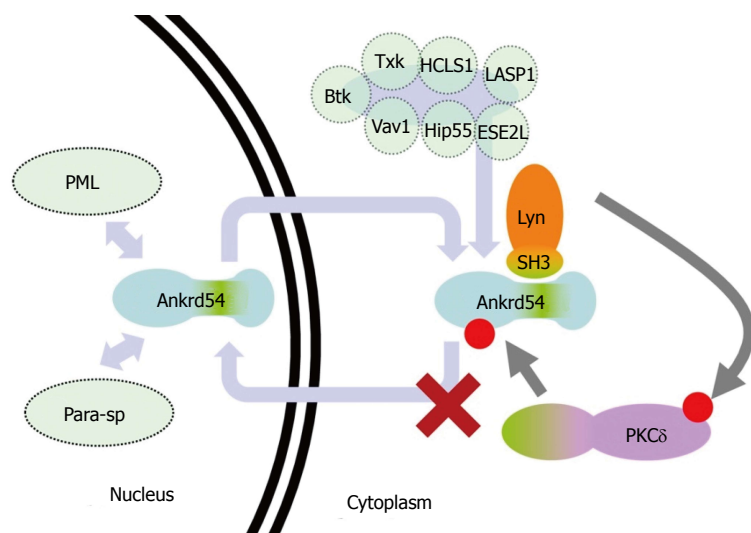


Figure 5 Schematic of proposed model of PKC δ regulation of Ankrd54 nuclear-cytoplasmic shuttling. Model of potential mechanism by which PKC δ regulated the cytoplasmic accumulation of Ankrd54. Ankrd54 shuttles between the nuclear and cytoplasmic compartments, promoted by its NLS and NES motifs. Ankrd54 can interact with Lyn, Btk, Txk, HCLS1, LASP1, Vav1, Hip55 and ESE2L. Nuclear Ankrd54 can localize to PML nuclear bodies and paraspeckles (Para-sp). Lyn, and potentially Btk/Txk, can phosphorylate and activate PKC δ , which can then phosphorylate Ankrd54, promoting cytoplasmic accumulation.

to the subcellular localization of PKC δ .

The mechanism by which phosphorylation of Ankrd54 by PKC δ enhances cytoplasmic accumulation of Ankrd54 and its interaction with Lyn remains to be determined. The sites of phosphorylation do not fit the consensus for 14-3-3 binding and so it is unlikely to be *via* 14-3-3 sequestration^[33]. Alternatively, it may interfere with the NLS function and/or enhance the NES function. However, it would be worth exploring the composition of cytoplasmic localized Ankrd54 to determine potential interactions (that may be phosphorylation dependent)

that mediate its cytoplasmic accumulation, as well as determining the influence of nuclear/cytoplasmic localized Ankrd54 on the subcellular distribution of its known binding partners. Further, studies on rates of nuclear/cytoplasmic shuttling for mutants of the N-terminal serine cluster could also identify whether there is a direct effect upon the ability of Ankrd54 to traverse the nuclear pore, *i.e.*, to influence the function of the NLS and/or NES.

In this manuscript, we have identified a mechanism that controls the subcellular localization of the nuclear-

cytoplasmic adaptor Ankrd54 and promotes its interaction with Lyn, through PKC δ mediated phosphorylation of an N-terminal serine cluster (Figure 5). Consequently, it will be important to define the consequences of this PKC δ mediated phosphorylation of Ankrd54 on its other binding partners, their subcellular compartmentalization as well as its influence on their biological function.

ACKNOWLEDGMENTS

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COMMENTS

Background

Protein nuclear-cytoplasmic compartmentalization and the shuttling of proteins between the nucleus and cytoplasm is regulated at multiple levels and is vitally important for many cellular processes. Phosphorylation is a major regulator of subcellular compartmentalization of proteins. Ankrd54 shuttles between the nucleus and cytoplasm and here the authors investigated the involvement of phosphorylation on subcellular compartmentalization of this protein.

Research frontiers

The regulation of nuclear-cytoplasmic transport and subcellular localization of signalling molecules such as Ankrd54 is critical for their functions in both normal and diseased cells. The identification of the kinases important for the regulation of subcellular localization of Ankrd54 was the objective of the authors.

Innovations and breakthroughs

This is the first discovery of the involvement of PKC kinases as being important in the nuclear-cytoplasmic shuttling of Ankrd54.

Applications

Knowing that PKCs can regulate the subcellular localization of Ankrd54 may find applications in identifying way to regulate its important interacting partners (*i.e.*, Lyn, Btk, Txk, HCLS1) in health and disease.

Terminology

Regulated subcellular localization: The localization of a protein inside a cell can have dramatic impact on its function, and in many disease incorrect location of proteins leads to specific pathologies. Further, many proteins have altered localization after specific stimulation the promotes of inhibits their function. A proteins localization within a cell is consequently critical for its function and is often regulated by post-translation modification, with phosphorylation being the most common.

Peer-review

In this manuscript, the authors characterized phosphorylation of Ankrd54, a nuclear-cytoplasmic shuttling adaptor protein. They provided good evidence that phosphorylation plays a role in regulating subcellular localization of Ankrd54. Specifically, phosphorylation of the N-terminal region of the protein promotes its accumulation in cytoplasm. They also demonstrate enhanced phosphorylation of Ankrd54 by activated version of PKC-delta, implying it as a potential kinase responsible for Ankrd54 phosphorylation. Using cutting-edge mass spec analysis, they nicely demonstrated that phosphorylation at Ser18 is responding to phorbol 12-myristate 13-acetate stimulation. Overall, the work is very carefully done and the data are with high quality.

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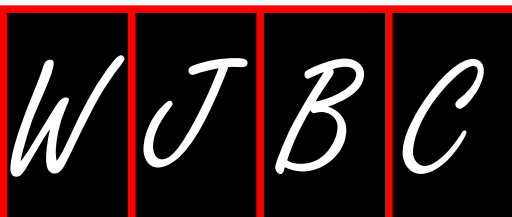
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PDRG1 at the interface between intermediary metabolism and oncogenesis

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Abstract

PDRG1 is a small oncogenic protein of 133 residues. In normal human tissues, the p53 and DNA damage-regulated gene 1 (*PDRG1*) gene exhibits maximal expression in the testis and minimal levels in the liver. Increased expression has been detected in several tumor cells and in response to genotoxic stress. High-throughput studies identified the PDRG1 protein in a variety of macromolecular complexes involved in processes that are altered in cancer cells. For example, this oncogene has been found as part of the RNA polymerase II complex, the splicing machinery and nutrient sensing machinery, although its role in these complexes remains unclear. More recently, the PDRG1 protein was found as an interaction target for the catalytic subunits of methionine adenosyltransferases. These enzymes synthesize S-adenosylmethionine, the methyl donor for, among others, epigenetic methylations that occur on the DNA and histones. In fact, downregulation of S-adenosylmethionine synthesis is the first functional effect directly ascribed to PDRG1. The existence of global DNA hypomethylation, together with increased PDRG1 expression, in many tumor cells highlights the importance of this interaction as one of the putative underlying causes for cell transformation. Here, we will review the accumulated knowledge on this oncogene, emphasizing the numerous aspects that remain to be explored.

Key words: Epigenetic modifications; Glutathione; Methylation; Oncogenes; Intermediary metabolism; p53 and DNA damage-regulated gene 1; Protein complexes; R2TP/prefoldin complex; S-adenosylmethionine synthesis; Redox stress

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Core tip: PDRG1 is an understudied protein of the R2TP/prefoldin-like complex that has been found as part of different multiprotein complexes. Increased

PDRG1 expression levels have been associated with several types of tumors that concomitantly show global DNA hypomethylation. More recently, this protein has been uncovered as an interaction target for methionine adenosyltransferase catalytic subunits MAT α 1 and MAT α 2. Through this interaction, PDRG1 downregulates nuclear S-adenosylmethionine synthesis, hence impacting epigenetic methylations.

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INTRODUCTION

Late in the 20th century, global DNA hypomethylation was described as a main characteristic of cancer cells and as the underlying cause of their altered expression pattern^[1,2]. Moreover, tumor cell growth was found to be totally dependent on glutamine and methionine, and the latter amino acid could not be replaced by homocysteine^[3]. Methionine is one of the substrates required for the synthesis of the main cellular methyl donor, S-adenosylmethionine (AdoMet), which is used for epigenetic methylations occurring at both the DNA and its associated proteins for the regulation of gene expression. These modifications are among the dozens of methylation reactions that occur in a cell using S-adenosylmethionine (AdoMet) as a methyl donor^[4,5]. However, the methyltransferases catalyzing these modifications are not the main consumers of AdoMet, despite their importance in regulating cell function. This fact is especially clear in the liver, where three methyltransferases involved in the synthesis of small compounds use the majority of the methyl donor synthesized^[6,7]. Those are glycine N-methyltransferase (GNMT), guanidinoacetate N-methyltransferase (GAMT) and phosphatidylethanolamine N-methyltransferase (PEMT), which produce sarcosine, creatine and phosphatidylcholine, respectively^[8-11]. Nevertheless, these estimations were made on the basis that AdoMet synthesis occurs exclusively in the cytoplasm and that the methyl donor would be transported to other subcellular localizations as required. This assumption has been challenged lately by reports describing the existence of nuclear pools of several enzymes of the methionine cycle, the pathway where AdoMet is produced, in turn suggesting the existence of two independent AdoMet stocks in the cytoplasm and the nucleus^[12-16]. The existence of a nuclear branch of this pathway is supported by the importance of epigenetic methylations and the need to guarantee their AdoMet supply.

To date, information regarding the cytoplasmic and nuclear branches of the methionine cycle has been mainly obtained in liver, and the accumulated knowledge can be summarized as follows. The cytoplasmic

methionine cycle involves the following (Figure 1): (1) methionine adenosyltransferases (MATs) that synthesize AdoMet; (2) a large number of methyltransferases, which methylate many types of substrates, also rendering S-adenosylhomocysteine (AdoHcy); (3) AdoHcy hydrolase (AHCY), which eliminates AdoHcy leading to adenosine and homocysteine (Hcy); and (4) betaine homocysteine S-methyltransferases (BHMT and BHMT2) and methionine synthase (MTR), which methylate Hcy to obtain methionine using betaine or S-methylmethionine and 5-methyltetrahydrofolate, respectively^[17,18]. In contrast, the nuclear branch of the pathway involves a more reduced set of enzymes as follows (Figure 1): (1) MATs; (2) methyltransferases such as those involved in DNA and histone methylations, GNMT and GAMT; (3) AHCY^[12,13,15,16]; and (4) BHMT^[19]. Of note, levels of MATs, GNMT and AHCY are markedly higher in the cytoplasm than in the nucleus, but increases in the latter compartment can be observed as a consequence of pathological changes in glutathione concentrations and, precisely, decreases in the GSH/GSSG ratio^[14]. More recently, PDRG1 [Consensus nomenclature for the human (*PDRG1*) and mouse genes (*Pdrg1*) is used throughout the text. The protein is in all the cases referred to as PDRG1] was added to this nuclear branch of the cycle as an interaction target and regulator of MATs, and hence, of AdoMet synthesis^[20,21].

PDRG1 stands for p53 and DNA damage-regulated gene 1 as it was discovered during studies devoted to the identification of components of the cellular response to DNA damage, or genotoxic stress, and it is regulated by p53^[22]. However, what do we really know about PDRG1? This is the question that will be addressed in the next sections of the present mini-review.

PDRG1 GENE STRUCTURE AND REGULATION

Studies devoted to the identification of components of the cellular response to DNA damage have been carried out for a number of years, and many of them used UV-irradiation as a stressor. Some of these investigations identified several novel UV-induced genes using a UV-treated low-ratio hybridization subtraction-enriched cDNA hamster library, and they were followed by screenings of partial-length expressed sequence tag (EST) clones regulated by this type of stress^[22-24]. This latter procedure rendered a UV-induced EST clone that hybridized with a 1.4 kb transcript, which was named *PDRG1*^[22]. Sequencing of the corresponding human and mouse EST clones from different tissues showed a single open reading frame coding for a protein of 133 amino acids.

The *PDRG1* gene is organized among five exons and four introns in both humans and mice (Figure 2)^[22]. The start codon is located at position 120 in exon 1 of the human gene, whereas the stop codon occurs at position 85 on exon 5. The mouse gene is located on chromosome 2, whereas the human gene appears on the

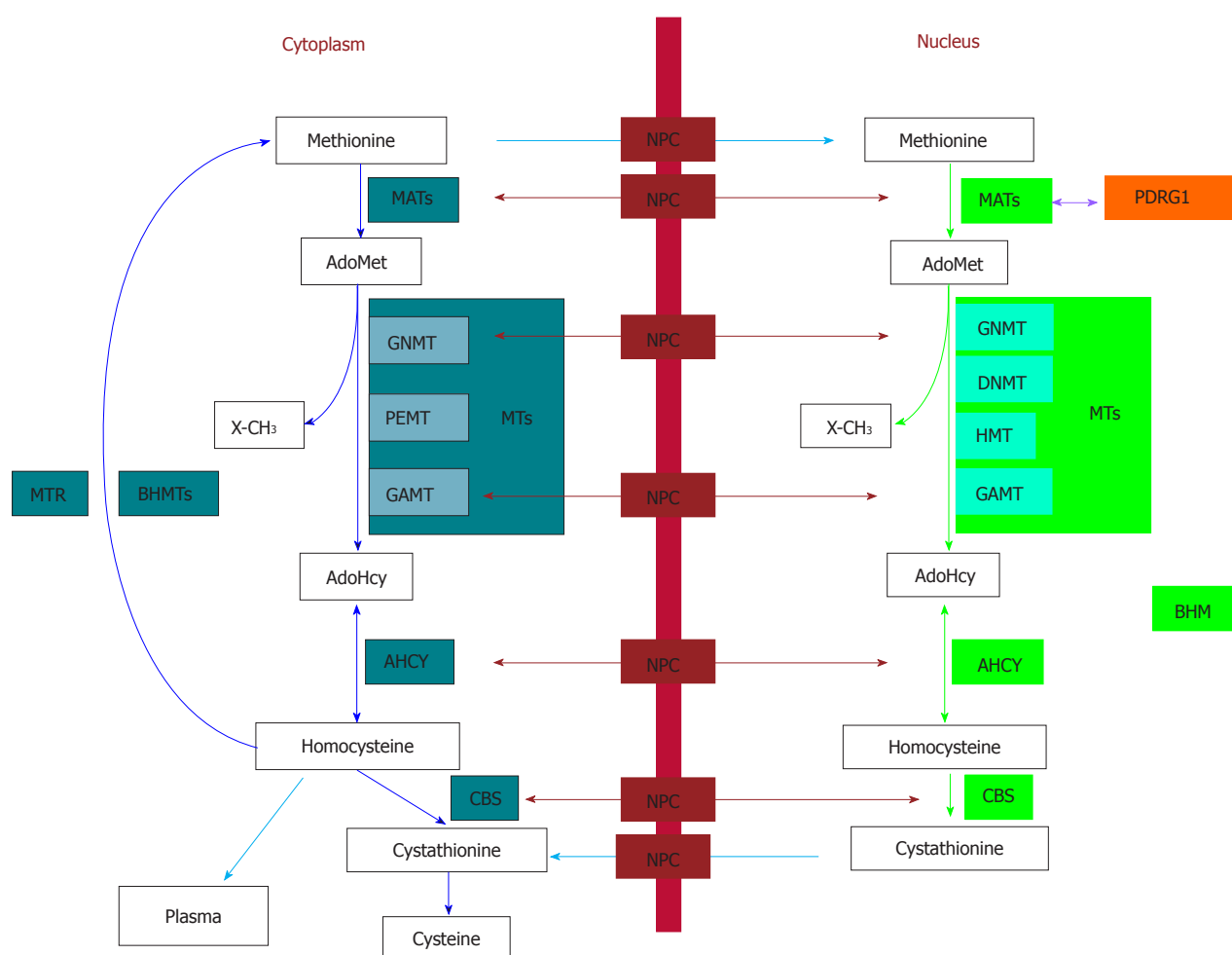


Figure 1 The cytoplasmic and nuclear branches of the hepatic methionine cycle and transsulfuration. Essential components (metabolites and enzymes) of the pathways are shown in the figure. Cytoplasmic enzymes appear in aquamarine boxes, their nuclear equivalents are shown in green boxes, and protein interaction targets are depicted in orange. Only key methyltransferases (MTs) mentioned in the text are illustrated in both subcellular compartments. The nucleocytoplasmic transport of metabolites (blue arrows) and enzymes (maroon arrows) is shown, whereas protein interactions are indicated by violet arrows. AdoHcy: S-adenosylhomocysteine; AdoMet: S-adenosylmethionine; AHCY: S-adenosylhomocysteine hydrolase; BHMT: Betaine homocysteine S-methyltransferase; CBS: Cystathionine β -synthase; DNMT: DNA methyltransferase; GAMT: Guanidoacetate N-methyltransferase; GNMT: Glycine N-methyltransferase; HMT: Histone methyltransferase; MATs: Methionine adenosyltransferases; MTR: Methionine synthase; NPC: Nuclear pore complex; *PDRG1*: p53 and DNA damage-regulated gene 1; PEMT: Phosphatidylethanolamine N-methyltransferase; X-CH₃: Methylated acceptors (metabolites, proteins, DNA, etc).

long arm of chromosome 20 at the 20q11.2 region^[22]. Interestingly, the human gene localization coincides with a position in which gains of DNA are detected in hepatocellular carcinoma (HCC) and dysplastic nodules, and where alterations in cirrhotic processes are also found^[25]. Moreover, these are pathologies in which impairment of the methionine cycle, especially of AdoMet production, have been described^[17].

Analysis of the human *PDRG1* promoter was initially carried out using a 1.7 kb fragment that included the 5'-UTR and part of the non-coding region of exon 1, and it showed high levels of promoter activity in breast cancer MCF-7 cells when fused to the luciferase gene^[22]. The gene contains a TATA-like element (TAATAA) localized at -268 from the start codon, but no canonical TATA box was identified^[22]. Promoter activity was eliminated by deletion of the -138/+1 region, in turn suggesting the presence of key regulatory elements in this segment^[22]. These same authors also detected two

putative downregulatory elements in the promoter. The first, a p53 binding element, was identified at position -240/-214 and includes two RRRCWWGYYY (R = purine, Y = pyrimidine, W = A or T, S = G or C, D = A, G or T) repeats arranged head to tail and separated by a 6 bp sequence^[26-28]. The second corresponds to a putative p53 transcriptional repressor element (TRE) located at -854/-834^[29]. Regulation of the *PDRG1* promoter by p53 was further proven when luciferase activity was abolished in the presence of this tumor suppressor^[22]. Basal *PDRG1* expression in p53 null HCT116 colon cancer cells was in fact higher than that in their wild-type counterparts, results that further supported repression of *PDRG1* by p53.

Additionally, the *PDRG1* promoter includes a canonical Oct-1 binding element located at -689/-683^[22,30]; this factor is known to upregulate expression of a number of genes when activated by UV irradiation^[31,32]. Cotransfection of a *PDRG1* promoter-luciferase construct and Oct-1 into

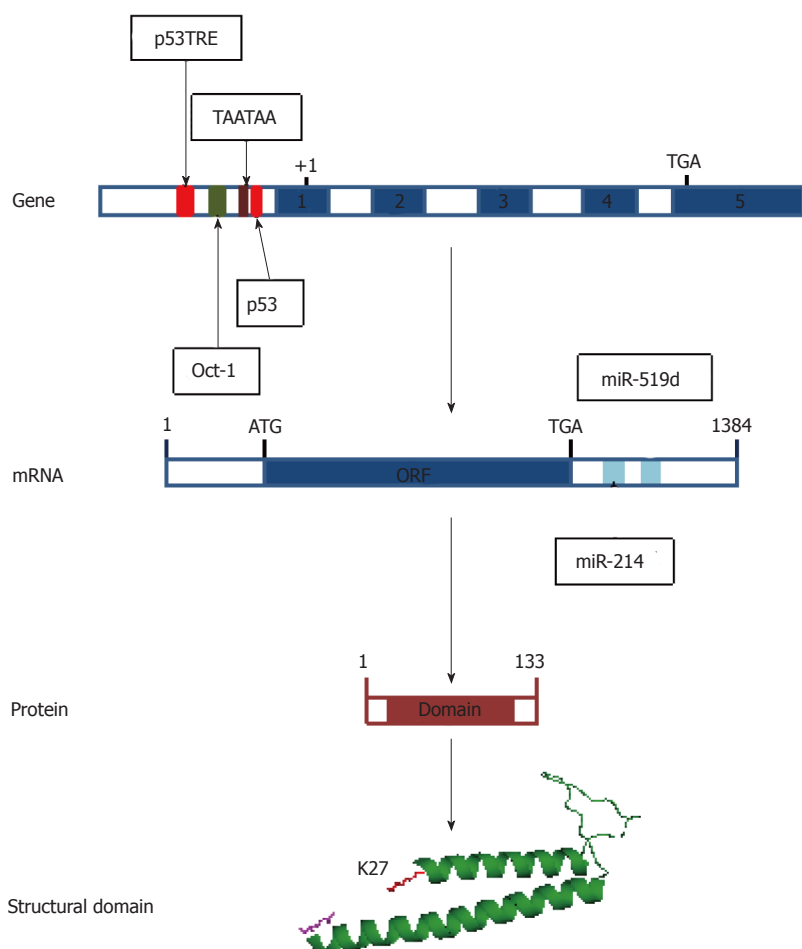


Figure 2 Regulatory elements on the human *PDRG1* gene and mRNA and structural features of the protein. From top to bottom, the figure shows first a scheme of the human *PDRG1* gene, indicating the regulatory elements on the promoter identified to date as follows: p53 and p53TRE elements (red), an Oct-1 binding element (green) and the TATA-box-like element (crimson). Exons appear as blue boxes. Second, a schematic representation of the mRNA is depicted, including the ORF (blue box) and the miRNA binding sites identified at the 3'-UTR (aquamarine boxes). Third, a scheme of the *PDRG1* protein is shown where the sequence encoding the prefoldin-like domain is indicated as a crimson box. Finally, a cartoon of the modeled prefoldin-like domain of rat *PDRG1* is included with the N-terminal K27 (red) and the C-terminal Q106 (magenta) depicted as sticks.

MCF-7 cells rendered approximately 8-fold induction of the luciferase activity, thus demonstrating the functionality of this Oct-1 binding element^[22]. This increased promoter activity was far greater than that caused exclusively by UV irradiation, a fact that Luo *et al*^[22] ascribed putatively to p53 activation in these cells.

More recently, the presence of complementary sequences for miR-214 (position 842-850) and miR-519d (position 1076-1082) in the 3'-UTR of the *PDRG1* mRNA was reported^[33,34]. Overexpression of miR-214, a miRNA with dual functionality as tumor suppressor or oncogene, in bladder cancer cell lines reduced *PDRG1* expression and protein levels^[33]. Similarly, overexpression of miR-519d leads to decreased *PDRG1* mRNA and protein levels in two nasopharyngeal carcinoma cell lines, which in turn correlates with a higher radiation-induced mortality (Figure 3)^[34].

Regarding the *PDRG1* expression profile, this aspect was initially studied using commercial human poly(A)⁺ Northern blots, where a 1.4 kb transcript was identified in brain, heart, liver, lung, spleen, stomach, testis and

skeletal muscle^[22]. More recently, real-time RTqPCR of rat tissues also demonstrated wide expression of *Pdrg1*, further including the cerebellum, intestine, pancreas, kidney and cava vein^[20]. In human tissues the highest levels were found in testis, whereas in the rat similar expression levels were detected in brain, cerebellum and testis. Nevertheless, both species showed very low *PDRG1* expression in the liver^[20,22].

PDRG1 PROTEIN

Mammalian *PDRG1* proteins share a high level of conservation, and most of them are over 90% identical (Figure 4). The sequences usually comprise 133 residues (15.5 kDa for the human protein), although larger proteins have been predicted in several marine mammals. In those cases, *PDRG1* presents with N-terminal extensions of variable length. The protein's isoelectric point is 5.81, and several post-translational modifications have been predicted and identified in high-throughput studies. Among them, phosphorylations at human serine residues

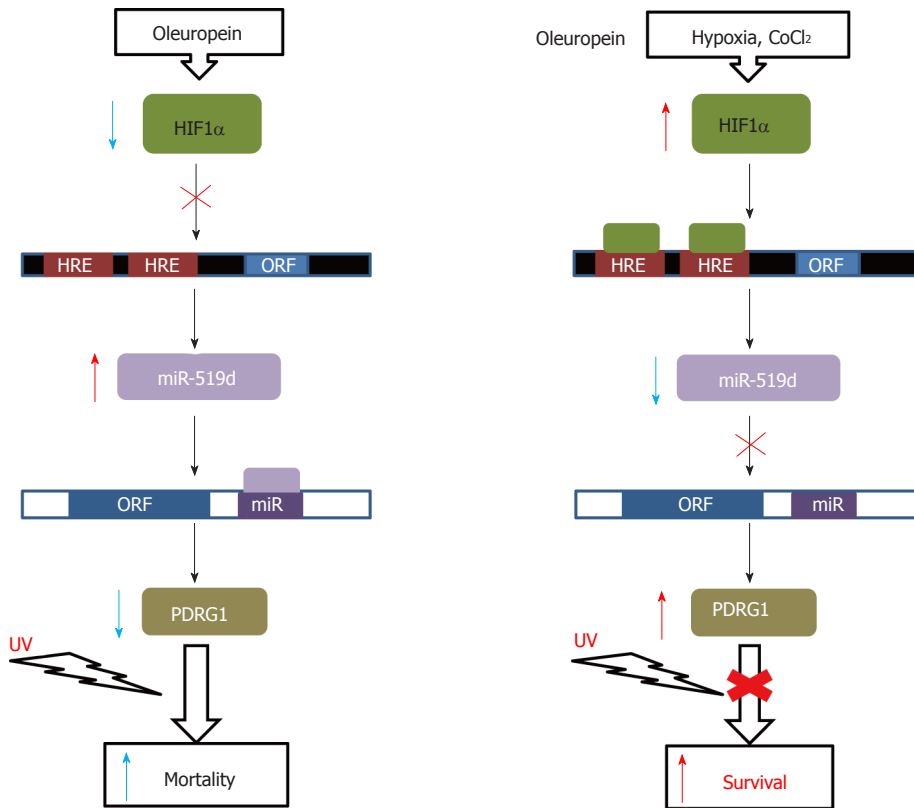


Figure 3 Regulation of cell survival. A schematic representation of the effects on cell survival exerted by PDRG1 regulation through miR-519d expression is shown. Binding sites for HIF1α (HRE) in the promoter of the *miR-519d* gene (black box) and for this miRNA on the *PDRG1* mRNA (white box) are indicated. Agents used for induction or blockade of UV-irradiation effects are indicated on the top.

Rattus norvegicus	¹ MVSP EADRVRLRYLVEVEELAEAVLSD ¹ KRQIVDLDTKRNQNGEGLRALQKDPVSSEDVMVC
Mus musculus	¹ MLSP EAEVRLRYLVEVEELAEAVLSD ¹ KRQIVDLDTKRNQNGEGLRALQKDL ¹ SVSEDMVC
Homo sapiens	¹ MLSP EAEVRLRYLVEVEELAE ¹ VLADKRQIVDLDTKRNQNGEGLRALQKDL ¹ SVSEDMVC
Bos taurus	¹ MLSP EAEVRLRYLVEVEELAE ¹ VLADKRQIVDLDTKRNQNGEGLRALQKDL ¹ SVSEDMVC
Cricetulus griseus*	¹ MLSP EAEVRLRYLVEVEELAEAVLSD ¹ KRQIVDLDTKRNQNGEGLRALQKDL ¹ SVSEDMVC
Microtus ochrogaster*	¹ MLSP EAEVRLRYLVEVEELAEAVLSD ¹ KRQIVDLDTKRNQNGEGLRALQKDL ¹ SVSEDMVC
Pteropus vampyrus*	¹ MLSP EAEVRLRYLVEVEELAEAVLSD ¹ KRQIVDLDTKRNQNGEGLRALQKDL ¹ SVSEDMVC
Canis lupus*	¹ MLSP EAEVRLRYLVEVEELAEAVLSD ¹ KRQIVDLDTKRNQNGEGLRALQKDL ¹ SVSEDMVC
Jaculus jaculus*	¹ MLSP EAEVRLRYLVEVEELAE ¹ VLADKRQIVDLDTKRNQNGEGLRALQKDL ¹ SVSEDMVC
Ictidomys tridecemlineatus*	¹ MLSP EAEVRLRYLVEVEELAE ¹ VLADKRQIVDLDTKRNQNGEGLRALQKDL ¹ SVSEDMVC
Erinaceus europaeus*	¹ MLSP EAEVRLRYLVEVEELAE ¹ VLADKRQIVDLDTKRNQNGEGLRALQKDL ¹ SVSEDMVC
Ursus maritimus*	¹ MLSP EAEVRLRYLVEVEELAE ¹ VLADKRQIVDLDTKRNQNGEGLRALQKDL ¹ SVSEDMVC
Physeter catodon*	¹ MDRGRTELEQVNTML ¹ SP EAEVRLRYLVEVEELAE ¹ VLADKRQIVDLDTKRNQNGEGLRALQKDL ¹ SVSEDMVC

Rattus norvegicus	⁶¹ FGNMF ⁶¹ IKMPHLKTKEMI ⁶¹ QKQD ⁶¹ EHLDKEIER ⁶¹ LRSQLKVKVNRL ⁶¹ LEAQ ⁶¹ GKPELKG ⁶¹ FNLN ⁶¹ PLNQDELKALQVILKG
Mus musculus	⁶¹ FGNMF ⁶¹ IKMPH ⁶¹ PKTKEMI ⁶¹ QKQD ⁶¹ EHLDKEIER ⁶¹ LRSQLKVKVNRL ⁶¹ FEAQ ⁶¹ GKPELKG ⁶¹ FNLN ⁶¹ PLNQDELKALQVILKG
Homo sapiens	⁶¹ FGNMF ⁶¹ IKMPH ⁶¹ PKTKEMI ⁶¹ QKQD ⁶¹ EHLDKEIER ⁶¹ LRSQLKVKVNRL ⁶¹ FEAQ ⁶¹ GKPELKG ⁶¹ FNLN ⁶¹ PLNQDELKALQVILKG
Bos taurus	⁶¹ FGNMF ⁶¹ IKMPH ⁶¹ PKTKEMI ⁶¹ QKQD ⁶¹ EHLDKEIER ⁶¹ LRSQLKVKVNRL ⁶¹ FEAQ ⁶¹ GKPELKG ⁶¹ FNLN ⁶¹ PLNQDELKALQVILKG
Cricetulus griseus*	⁶¹ FGNMF ⁶¹ IKMPH ⁶¹ PKTKEMI ⁶¹ QKQD ⁶¹ EHLDKEIER ⁶¹ LRSQLKVKVNRL ⁶¹ FEAQ ⁶¹ GKPELKG ⁶¹ FNLN ⁶¹ PLNQDELKALQVILKG
Microtus ochrogaster*	⁶¹ FGNMF ⁶¹ IKMPH ⁶¹ PKTKEMI ⁶¹ QKQD ⁶¹ EHLDKEIER ⁶¹ LRSQLKVKVNRL ⁶¹ FEAQ ⁶¹ GKPELKG ⁶¹ FNLN ⁶¹ PLNQDELKALQVILKG
Pteropus vampyrus*	⁶¹ FGNMF ⁶¹ IKMPH ⁶¹ PKTKEMI ⁶¹ QKQD ⁶¹ EHLDKEIER ⁶¹ LRSQLKVKVNRL ⁶¹ FEAQ ⁶¹ GKPELKG ⁶¹ FNLN ⁶¹ PLNQDELKALQVILKG
Canis lupus*	⁶¹ FGNMF ⁶¹ IKMPH ⁶¹ PKTKEMI ⁶¹ QKQD ⁶¹ EHLDKEIER ⁶¹ LRSQLKVKVNRL ⁶¹ FEAQ ⁶¹ GKPELKG ⁶¹ FNLN ⁶¹ PLNQDELKALQVILKG
Jaculus jaculus*	⁶¹ FGNMF ⁶¹ IKMPH ⁶¹ PKTKEMI ⁶¹ QKQD ⁶¹ EHLDKEIER ⁶¹ LRSQLKVKVNRL ⁶¹ FEAQ ⁶¹ GKPELKG ⁶¹ FNLN ⁶¹ PLNQDELKALQVILKG
Ictidomys tridecemlineatus*	⁶¹ FGNMF ⁶¹ IKMPH ⁶¹ PKTKEMI ⁶¹ QKQD ⁶¹ EHLDKEIER ⁶¹ LRSQLKVKVNRL ⁶¹ FEAQ ⁶¹ GKPELKG ⁶¹ FNLN ⁶¹ PLNQDELKALQVILKG
Erinaceus europaeus*	⁶¹ FGNMF ⁶¹ IKMPH ⁶¹ PKTKEMI ⁶¹ QKQD ⁶¹ EHLDKEIER ⁶¹ LRSQLKVKVNRL ⁶¹ FEAQ ⁶¹ GKPELKG ⁶¹ FNLN ⁶¹ PLNQDELKALQVILKG
Ursus maritimus*	⁶¹ FGNMF ⁶¹ IKMPH ⁶¹ PKTKEMI ⁶¹ QKQD ⁶¹ EHLDKEIER ⁶¹ LRSQLKVKVNRL ⁶¹ FEAQ ⁶¹ GKPELKG ⁶¹ FNLN ⁶¹ PLNQDELKALQVILKG
Physeter catodon*	⁷⁴ FGNMF ⁷⁴ IKMPH ⁷⁴ PKTKEMI ⁷⁴ QKQD ⁷⁴ EHLDKEIER ⁷⁴ LRSQLKVKVNRL ⁷⁴ FEAQ ⁷⁴ GKPELKG ⁷⁴ FNLN ⁷⁴ PLNQDELKALQVILKG

Figure 4 Alignment of mammalian PDRG1 proteins. The figure shows the BLASTP alignment of thirteen representative mammalian PDRG1 sequences using that of *Rattus norvegicus* as a reference. Most of the data correspond to predicted sequences (*). Conservative changes are highlighted in green, non-conservative substitutions appear in red, and N-terminal extensions are indicated in yellow. The C-terminal helix-turn-helix motif and the prefoldin-like domain are indicated in blue and crimson boxes, respectively.

3, 52, 54 and mouse serine 120 and ubiquitinations on human lysine residues 27, 108 and 125 have been postulated or found^[35-41]. Initial studies suggested

association of the protein into homo-oligomers, a fact that was later confirmed by detection of HA-PDRG1 hexamers in embryonic kidney HEK293T cells using analytical gel

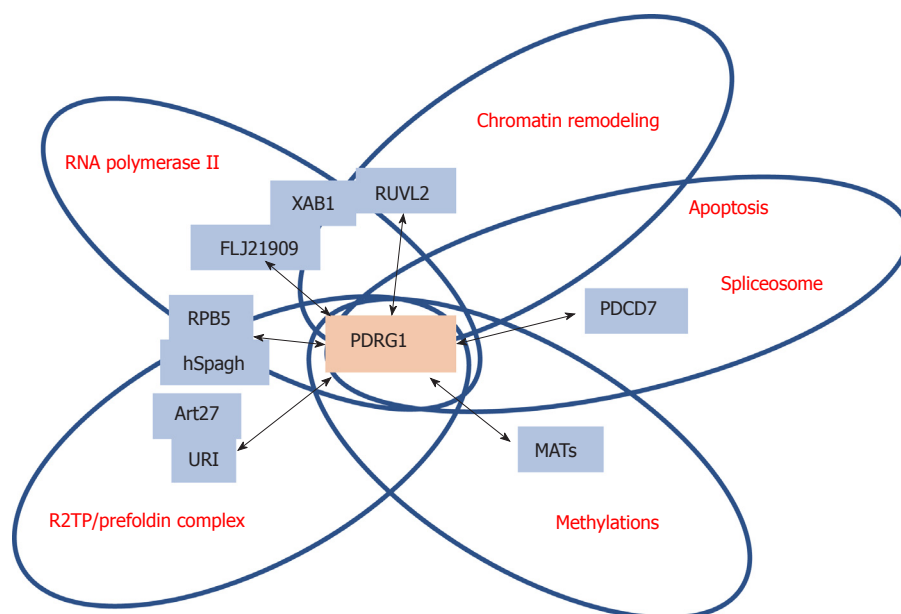


Figure 5 Schematic representation of the main PDRG1 interaction targets. The figure highlights the main targets of interaction with PDRG1 identified using either affinity purification/mass spectrometry or yeast two-hybrid screening. The proteins involved are shown in blue boxes within circles, according to the function or multiprotein complex in which they are involved. PDRG1 appears at the intersection of these circles.

filtration chromatography^[20-22].

Motif searches identified a helix-turn-helix (LNQDEL KALKVILKG) at the C-terminal end of the human and mouse protein sequences^[22]. These motifs are normally involved in interactions with either the DNA or other proteins. Moreover, human and mouse PDRG1 are characterized by the presence of a β -prefoldin-like domain that is also recognized by the Protein Homology/analogy Recognition Engine (PHYRE) in the rat counterpart. PHYRE2 identified prefoldin as the closest homologue to rat PDRG1 during the preparation of a structural model. This model included residues K27-Q106 (Figure 2), thus excluding approximately 25 residues from each protein end. Elimination of the C-terminal end has divergent effects on the interaction of PDRG1 with MAT catalytic subunits MAT α 1 and MAT α 2^[20,21]. Additionally, motif searches carried out with the rat PDRG1 sequence predicted nucleocytoplasmic localization (PSORT II) and a nuclear export signal (NES) involving serine 93; the NetNES score increases between residues E89-L95.

Subcellular localization was initially examined by fluorescence microscopy using PDRG1-GFP and GFP-PDRG1 tagged forms in fixed NIH 3T3 fibroblasts and HCT116 colon cancer cells. In both cases, cytoplasmic aggregates of the protein were described, whereas no localization within subcellular compartments such as the mitochondria, endoplasmic reticulum and nucleus was observed using specific dyes for these organelles^[22]. Moreover, these authors indicated that this distribution was not affected by UV irradiation, and hence, a putative PDRG1-DNA interaction was excluded. In contrast, PDRG1 was found in the nuclear fractions used to isolate protein complexes from LNCaP prostate cancer cells, hence suggesting that the protein may be distributed

between subcellular compartments^[42]. This aspect was reexamined using confocal microscopy and several cell lines in which HA-PDRG1 or PDRG1-EGFP was overexpressed. The results of direct fluorescence and immunofluorescence experiments demonstrated that, in fact, PDRG1 is a nucleocytoplasmic protein in ovary (CHO), kidney (COS-7), neuroblastoma (N2a) and hepatoma (H35) cell lines^[20,21]. Moreover, quantification of the fluorescence signals indicated a stronger protein level in the nuclear compartment, where PDRG1 also colocalized with nuclear speckles containing SC-35.

PROTEIN-PROTEIN INTERACTIONS INVOLVING PDRG1

Lately, several high-throughput studies have been carried out with the goal of identifying components of several large protein complexes (Figure 5). For this purpose, the following two main techniques were used: (1) affinity purification of subunits including single (AP) or tandem tags (TAP) followed by mass spectrometry (MS); and (2) yeast two-hybrid (YTH). During some of these studies PDRG1 was identified either as a subunit or interaction target of several complexes, and interestingly, methylation plays a direct or indirect role in many of the processes involving these multimers (e.g., nutrient signaling and mRNA capping).

PDRG1 is indirectly linked to the RNA polymerase II machinery

MS, together with a computer method to minimize the contribution of false positives, was used to identify the interaction network of human RNA polymerase II

using tagged subunits that allowed TAP purification from kidney HEK293 cells^[43]. Some key proteins were selected for additional TAP-MS experiments, and among them are XAB1, which appears placed at the interface with members of chaperone complexes (C19orf2/URI, PFDN6, PFDN2 and UXT), and the AAA+ chaperone-like ATPases RUVBL1/TIP49a and RUVBL2/TIP49b. These experiments showed TAP-XAB1 copurification with FLJ21909 and subsequent TAP-FLJ21909 copurification with PDRG1^[43]. Moreover, this same work also demonstrated copurification of PDRG1 with TAP-RUVBL2, C1orf82/RPAP2 and FLJ21908/RPAP3, although no further validation of the interactions using additional techniques was carried out^[43].

Additional studies to analyze the cytoplasmic assembly of RNA polymerase II were later performed by Boulon *et al.*^[44]. These MS-proteomics studies showed association of unassembled RPB1, the largest subunit of this transcription machinery, with the R2TP/prefoldin-like complex^[44]. Moreover, in this same study the interactions of a central component of the R2TP/prefoldin-like complex, hSpagh that binds HSP90 as a cofactor, were further explored. Independent interactions of hSpagh with RPB1 and RPB5 subcomplexes were then identified, which also involved other members of the R2TP/prefoldin-like complex, such as PDRG1. However, YTH only confirmed the implication of PDRG1 in the interaction with RBP5, although this binding seemed indirect through its association with hSpagh^[44].

PDRG1 may have a role in chromatin remodeling, splicing and apoptosis

AP-MS data also suggested that PDRG1 might be linked to the SWR-C and INO80 chromatin remodeling complexes. Such a relationship could be established by the identification of its interaction targets, RUVBL1 and RUVBL2, in both complexes in experiments carried out in nuclear fractions of human cells^[45,46]. Additionally, YTH screens identified PDRG1 interacting with programmed cell death 7 (PDCD7), Cip1 zinc interacting finger (CIZ1) and microtubule-associated protein 1S (MAP1S) in human testis, although immunoprecipitation only confirmed the PDRG1-PDCD7 interaction^[47]. These results linked PDRG1 with the modulation of apoptosis and the U12-type spliceosome, a process and a complex that involve PDCD7^[48].

PDRG1 is a member of the URI/prefoldin complex involved in nutrient signaling

PDRG1 was identified, together with HKE2, BC014022, POL3A, FLJ21908 and FLJ20643, as a new member of the URI/prefoldin complex with abundance similar to that exhibited by the consensus subunits known at that time^[49]. URI stands for unconventional prefoldin RPB5 interactor, also known as RMP, and some of the interaction studies described in this section showed this interaction. Additional data from this AP-MS study identified PDRG1 interacting with TIP49a/b in HEK293/FRT and HeLa cells, as well as bound to BC014022,

DPCD, FLJ20643, NUFIP, RPB5MP and UXT1^[49]. Reciprocal studies using FLAG-tagged PDRG1 identified interactors with moderate (POL3A, PPP1CA, PPP1CC, PFDN2, HKE2, RPB5, PPP1CB, NPM1, UXT1, H2BS, H2BR, RPB5MP, FLJ21908, BC014022, H2AZ, NOP5/NOP58, FLJ20643) or low probability scores (TUB6, HSP70/1, HSP70/1B, BAF53, TIP49a, TIP49b)^[49]. However, none of these PDRG1 interactions were confirmed by immunoprecipitation. More recently, MS analysis of immunoprecipitates from nuclear fractions of LNCaP prostate cells stably transfected with FLAG-URI also showed the presence of PDRG1 in the R2TP/prefoldin-like complex, and the interaction was confirmed by detection of URI and PDRG1 in anti-Art27 immunoprecipitates^[42]. Both studies considered the PDRG1-URI interaction abundant, according to the number of peptides retrieved^[42,49].

PDRG1 overexpression did not modify URI or Art27 levels, whereas overexpression of URI upregulates PDRG1 protein levels, an effect that was ascribed to protein stabilization^[42]. The PDRG1-URI interaction seems to involve the hook-shaped β -strands of the URI N-terminal prefoldin-like domain, through which interaction with Art27 is also established. In fact, these authors suggested that all prefoldin-like proteins of the R2TP/prefoldin-like complex (URI, Art27, PDRG1, PFD2, PFD6) use the hook to interact with each other^[42].

AP-MS experiments carried out in HeLa cells identified URI, TIP49/RUVL, RMP and RPB5 in association with STAP-1, and hence potentially linking PDRG1 with signal transduction and the TOR complex^[50]. These interactions were further confirmed by immunoprecipitation, where anti-URI binds RPB5 and STAP-1 and anti-TIP48 rendered TIP49, URI, STAP1 and SKP2. Similarly, PDRG1 could be indirectly implicated in the roles of URI as a transcriptional repressor and as a regulator of apoptosis that involve interactions with the RPB5/POLR2E subunit of the three RNA polymerases and PPI γ ^[51,52]. In the latter case, phosphorylation of mitochondrial URI by S6K1 allows dissociation of the complex. Moreover, the URI complex is targeted by nutrient signaling and, in turn, participates in the control of gene expression downstream of the TOR kinase, a process that is regulated by methylation of the protein phosphatase PP2A^[53].

PDRG1 controls production of S-adenosylmethionine

In yeast, URI deletion alters expression of genes involved mainly in amino acid metabolism, although none of the key genes in the methionine cycle seems affected^[50]. On the other hand, YTH screening of a rat liver library identified PDRG1 interacting with MAT α 1, a result that was further confirmed by immunoprecipitation and pull-down^[20]. These results linked PDRG1 with methionine metabolism and AdoMet-dependent methylations^[20], which are altered in tumor cells.

MAT α 1 is the catalytic subunit of homotetrameric MAT I and homodimeric MAT III, the isoenzymes synthesizing AdoMet in normal liver, and accumulates

in the cell nucleus in liver disease and extrahepatic cells^[12,14]. Despite the nucleocytoplasmic localization of both MAT α 1 and PDRG1, their interaction was restricted to the nuclear compartment, where large association states (approximately 360 kDa) could be detected^[20,21]. Taking into account the size of each protein and their individual oligomeric states, this means binding of two PDRG1 hexamers per nuclear MAT I homotetramer. Moreover, pull-down assays also showed the ability of PDRG1 to interact with the MAT α 2 catalytic subunit of heterotrimeric MAT II, the main isoenzyme found in extrahepatic tissues, fetal liver and hepatic diseases^[20,21]. This fact could be expected from the high percentage of identity (84%) between the MAT α 1 and MAT α 2 subunits^[54]. Additionally, the PDRG1-MAT α 2 interaction interface seems to overlap with the binding site for the regulatory MAT β subunit that is displaced from the oligomer upon PDRG1 attachment^[20]. Further insights into the putative areas of interaction were obtained through the use of truncated PDRG1 forms. These protein variants were designed according to information obtained from the available structural model and lack the N-terminus, the C-terminus or both protein ends^[20]. Analysis of their interaction with MAT α 1 and MAT α 2 indicated a role for the C-terminal end of PDRG1 in the interaction, an area that is not involved in the interaction with other members of the R2TP/prefoldin-like complex, which seem to use the hook for this purpose^[42].

Tissue *Pdrg1* expression mimics the expression profile of *Mat2a* (the MAT α 2 gene), while following the opposite pattern from that exhibited by *Mat1a* (the MAT α 1 gene)^[20]. This fact coincides with the preferential nuclear localization of MAT α 1 in those cell types/tissues where *Mat1a* expression is low, thus increasing the probability of interaction with PDRG1 in those settings. This interaction impairs AdoMet production by approximately 50%, as demonstrated *in vitro* using purified MAT α 1 or MAT α 2 homo-oligomers or MAT II hetero-oligomers and PDRG1. Similarly, decreased global DNA methylation levels were detected in cultured cells upon overexpression of PDRG1 or coexpression of PDRG1 and MAT α 1^[20,21]. Altogether, these results provide the first evidence for PDRG1 function as a modulator of methyl donor production.

ROLE OF PDRG1 IN DISEASE

To date, there is no disease directly linked to alterations in *PDRG1*, although the OMIM 610789 code has been associated with this gene. In fact, the NCBI database already includes descriptions of more than 700 SNPs in this gene, including point mutations in the ORF, frameshifts and early stop codons (Table 1). However, the impact of these alterations remains to be studied.

PDRG1 and cancer

The chromosomal location of *PDRG1* coincides with a region with high frequency of sequence gains in several types of human cancers, including breast, liver, lung, colon and rectum tumors^[25,55-59]. Moreover,

p53 is downregulated in several human cancers, and hence, its regulation of the *PDRG1* gene is expected to be impaired. The same is true for miR-214, which is also downregulated in cervical, breast, HCC and bladder tumors^[33,60-62]. Using commercial cancer profiling arrays, 60%-85% upregulation of *PDRG1* was detected in a variety of tumor types with impaired p53 expression, confirming this hypothesis^[47]. This same study also showed that the percentage of patients with increased *PDRG1* levels was dependent on the type of tumor analyzed. In bladder cancer, miR-214 levels were associated with the tumor stage and recurrence, whereas an inverse correlation with *PDRG1* expression was detected^[33]. Moreover, this tumor suppressor role was found to be exerted through miR-214 binding to the 3'-UTR of the *PDRG1* mRNA, in turn leading to a reduction in its levels.

Correlation of elevated *PDRG1* and *GLUT1* expression with poor pathological response has been reported in residual rectal cancer cells after pre-operative chemotherapy^[63]. However, *PDRG1* expression levels were not associated with the overall survival of the patients despite its proposed roles in DNA damage regulation and tumor growth. Furthermore, a role for PDRG1 in cancer can also be inferred from the reports linking its interaction target URI to the development of ovarian cancer and HCC^[64,65]. In this same line, the reduced miR-214 levels found in breast cancer cells lead to the accumulation of the enhancer of Zeste homologue 2 (EZH2) methyltransferase. EZH2 is a component of the polycomb repressive complex 2 (PRC2) and catalyzes the inclusion of the repressive epigenetic mark H3K27me3^[61]. Levels of this repression mark are enhanced in HCC^[66], where nuclear accumulation of MAT α 1 is induced, putatively favoring its interaction with PDRG1. This apparent contradictory effect could be explained by the high affinity of EZH2 for AdoMet, which putatively makes this particular methyltransferase insensitive to the decreased methyl donor concentrations resulting from the nuclear MAT α 1/PDRG1 interaction.

The upregulated expression of *PDRG1* correlates with enhanced PDRG1 protein levels, for example in colon cancer samples (mainly in epithelial cells) and in some lung cancer cell lines (A549 and H446)^[47,67]. Tumor cell lines have been used for stable or transient overexpression and downregulation of PDRG1 to study effects on proliferation, apoptosis or the response to UV irradiation. PDRG1 overexpression in A549 and H446 lung cancer cells enhanced proliferation, whereas no morphological changes in NIH 3T3 fibroblasts, HCT116 colon cancer or H35 hepatoma cells were observed^[20,22,67]. Regarding apoptosis, no signs of this process were detected in NIH 3T3 or HCT116 cells after overexpression^[22]. In contrast, downregulation of *PDRG1* in T24 and 5637 bladder cancer cell lines by overexpression of miR-214 led to decreased proliferation, enhanced apoptosis and reduced invasiveness^[33]. *PDRG1* knockdown in RKO colon and A549 and H446 lung cancer cells also caused reduction in cell growth^[47,67].

Table 1 SNPs for the human *PDRG1* gene listed in the NCBI database leading to mutated protein forms

SNP code ¹	Base change	Mutated residue	Type of mutation
rs777897331	1A>C	M1L	Missense
rs748585638	19G>A	E7K	Missense
rs867545839	23G>T	R8L	Missense
rs779425406	29T>C	L10P	Missense
rs755286962	32G>A	R11Q	Missense
rs150958567	34T>C	Y12H	Missense
rs142496377	35A>G	Y12C	Missense
rs375829085	46G>A	V16M	Missense
rs760411451	59C>T	A20V	Missense
rs763777690	57_68delCGCCG- AGGAGGT	A20_V23del	Indel
rs765247648	67G>T	V23L	Missense
rs759432900	83G>A	R28Q	Missense
rs772260316	91G>A	V31M	Missense
rs139473332	92T>C	V31A	Missense
rs147359616	110G>T	R37M	Missense
rs201481003	113A>C	N38T	Missense
rs749744129	119A>C	N40T	Missense
rs142890852	121C>G, 121C>T	R41G, R41stop	Missense, stop gained
rs539323269	122G>A	R41Q	Missense
rs746198655	134G>A	R45K	Missense
rs370045763	155G>C	S52T	Missense
rs758120112	157C>G	L53V	Missense
rs748723095	175G>T	V59F	Missense
rs775880591	184G>A	G62R	Missense
rs200644098	188A>G	N63S	Missense
rs767238840	190A>G	M64V	Missense
rs764202244	208C>T	H70Y	Missense
rs777531663	210_211insAA	P71Nfs	Frameshift
rs566583428	211C>T	P71S	Missense
rs768828941	223_224ins ²	E75Vfs	Frameshift
rs750997250	251_252delTG	L84Rfs	Frameshift
rs745401995	264A>G	I88M	Missense
rs780722451	265G>C	E89Q	Missense
rs201754951	274C>T	R92W	Missense
rs186921219	275G>A	R92Q	Missense
rs140650422	295G>A	V99I	Missense
rs200378363	299A>T	N100I	Missense
rs565384763	301C>T	R101C	Missense
rs779220725	302G>A	R101H	Missense
rs753937019	317A>G	Q106R	Missense
rs143995288	319G>A	G107S	Missense
rs768088765	323A>G	K108R	Missense
rs200900452	326C>T	P109L	Missense
rs759134978	328G>A	E110K	Missense
rs776274160	332T>C	L111P	Missense
rs370586022	340T>C	F114L	Missense
rs764544630	344_345insA	N115Kfs	Frameshift
rs760255766	359A>G	N120S	Missense
rs772012403	361C>T	Q121stop	Stop gained
rs149510387	365A>G	D122G	Missense
rs774273098	366T>G	D122E	Missense
rs548757558	377C>T	A126V	Missense

¹Information collected in January 2017; ²Inserted sequence TGAGCT TTTCTTTTACATGCCCTGCAATAGAGACTGGGTGA.

However, stable silencing in H35 hepatoma cells did not induce significant changes in growth of any of the clones analyzed or in their morphology^[20]. This downregulation was never above 70%, suggesting that further suppression of *PdrG1* expression in hepatoma cells could be lethal. Additionally, the differences observed may arise

not only from the downregulation extent achieved in each tumor cell but also from the diverse procedures used for silencing and the distinct basal levels in the corresponding normal cells. Altogether, these results suggest a putative role for PDRG1 in apoptosis and proliferation, which might be exerted through its interaction with different targets. Among those identified to date, PDCD7 contributes to the modulation of apoptosis, MAP1S participates in cell cycle progression through its interaction with RASSF1A, and CIZ interacts with p21 for their shuttling from the nucleus to the cytoplasm.

Focusing our attention on stably silenced H35 hepatoma cells, the maximum downregulation achieved is still well above the levels detected in normal liver^[20,21]. Nevertheless, important changes in the expression profile were found using microarrays, these alterations affecting a large variety of pathways^[20,21]. A total of 114 genes consistently changed their pattern in different clones, of which only 80% could be ascribed to GO pathways. Among upregulated genes, *Lpin1* was found. This is an essential gene in fat metabolism that is regulated by p53 and induced by DNA damage and glucose deprivation^[68]. *Lpin1* codes for a bifunctional protein that catalyzes diacylglycerol synthesis and acts as a transcriptional coactivator in hepatocytes to regulate fatty acid oxidation^[68]. In contrast, expression of neither the *TP53* nor the *Mat* genes were significantly altered in the silenced cells, but many of the routes affected are implicated in pathological processes in which the pattern of *Mat* gene expression is modified^[20,21].

As mentioned in the initial sections of this mini-review, PDRG1 was identified when the effects of UV irradiation were analyzed. Therefore, it was not surprising that stable HCT116 clones overexpressing HA-PDRG1 displayed increased sensitivity to UV irradiation or that this agent upregulated *PDRG1* in several human cell lines, including some lacking p53 or carrying mutant forms of this protein^[22]. In fact, higher *PDRG1* mRNA levels were detected after irradiation upon *TP53* repression by doxycycline in Tet-inducible DLD1 colon cancer cells as well as in *TP53*^{-/-} cells. These data suggested an inverse correlation of *PDRG1* and *TP53* expression but also with that of *Cip1* (the p21 gene, also named *CDKN1*), in turn indicating involvement of a p53-independent mechanism^[22]. Additional studies carried out by Jiang *et al*^[47] demonstrated that only agents inducing genotoxic stress (adriamycin, etoposide, camptothecin and UV) upregulated *PDRG1* expression.

The link between PDRG1 and radioresistance was further supported when an inverse correlation between PDRG1 and ATM levels was detected in lung cancer cells after irradiation^[67]. In fact, *PDRG1*-silenced cells exhibited high ATM levels together with increased p53 phosphorylation on serine 15, and tumors generated in nude mice using these silenced clones were more susceptible to irradiation^[67]. Oleuropein treatment of nasopharyngeal cancer cell lines reduced *PDRG1* and *HIF1α* expression and protein levels, while inducing miR-519d levels^[34]. Further examination of the effects

of the treatment revealed that oleuropein avoids HIF1 α downregulation of miR-519, in turn decreasing PDRG1 levels in the cells and favoring sensitivity to irradiation both in the cells and in tumor xenografts.

PDRG1 in liver disease

Pdrg1 expression has also been analyzed in models of liver disease, where the *Mat1a* to *Mat2a* expression switch was detected together with oxidative stress. The results reported to date indicate no significant alteration of hepatic *Pdrg1* levels in early stages of a model of Wilson disease, the Long Evans Cinnamon rats, despite the evident accumulation of copper^[20]. However, acute liver injury induced by D-galactosamine intoxication induced the *Mat* expression switch, nuclear accumulation of MAT α 1 and the upregulation of *Pdrg1* expression and its nuclear levels^[20]. Of note, the upregulation of *Pdrg1* reported in this model was more modest than that measured in hepatoma cells.

PDRG1 in T cell selection

Autoimmune diseases present altered T cell selection mechanisms that have been explored using microarrays and animal models such as the non-obese diabetic (NOD) mice^[69]. Thymocytes obtained from this model of human autoimmune diabetes have a reduced number of genes allowing distinction between positive and negative T cell selection compared to cells from a control mouse strain. This fact was especially important in the negative selection gene set, which included *Pdgr1*. Basal *Pdrg1* expression was reduced in NOD thymocytes, and this gene was also identified among the high-quality candidates for *D2mit490*-linked defective thymic deletion, *D2mit490* being a marker for the loci contributing to the defective negative selection detected in NOD mice^[69].

CONCLUSION

PDRG1 is a member of the R2TP/prefoldin-like complex with nucleocytoplasmic distribution. In acute liver injury and a variety of tumors, high levels of *PDRG1* expression have been detected. Several protein-protein interactions involving PDRG1 have been found while exploring the components of multiprotein complexes, putatively linking this protein to processes such as splicing, apoptosis and nutrient signaling. However, the role of these interactions and how and when do they occur remains unclear. Increased *PDRG1* expression correlates with decreased levels of AdoMet, the main cellular methyl donor. Moreover, this increased expression also correlates with nuclear accumulation of MAT α 1 and with changes in epigenetic methylations that may be of a different sign depending on the AdoMet affinity of each specific methyltransferase. Such behavior can be explained by the decreased capacity for AdoMet synthesis exhibited by the MAT isoenzymes upon their interaction with PDRG1. This modulatory role of AdoMet levels is the first function ascribed to PDRG1. The fact that this interaction is

restricted to the nucleus may exert a counteracting effect on liver pathology, precluding the objectives pursued by the nuclear accumulation of MAT α 1. Moreover, the interest and impact of this nuclear interaction could be of major importance for most cells, where PDRG1 expression is higher and MAT α 1 localization is restricted to this compartment. Altogether, the aspects that deserve further attention include the identification of the interaction surfaces in complexes involving PDRG1, how the interactions are regulated, and the structure of the oncogene or its regulation. Knowledge of structural features may be of use for the design of new therapeutic options that, for example, interfere with pathological protein-protein interactions involving PDRG1.

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