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EDITORIAL

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 post-surgical 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

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

Liu S, Tao F. Role of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor regulation in stress-induced pain chronification. *World J Biol Chem* 2017; 8(1): 1-3 Available from: URL: http://www.wjgnet.com/1949-8454/full/v8/i1/1.htm DOI: http://dx.doi.org/10.4331/wjbc.v8.i1.1

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 receptors 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.

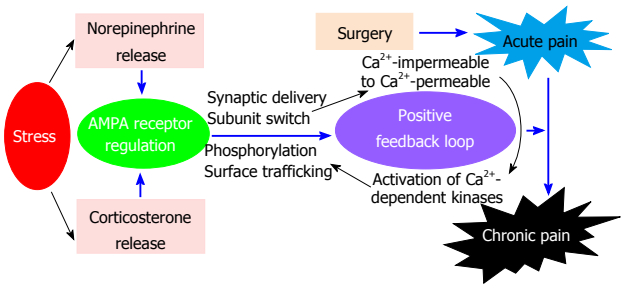

Figure 1 Mechanisms underlying stress-induced pain chronification after surgery. Note that α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor switch from Ca^{2+}-impermeable (GluA2-containing) to Ca^{2+}-permeable (GluA2-lacking) will enhance Ca^{2+} influx and further activate Ca^{2+}-dependent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

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EDITORIAL

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

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

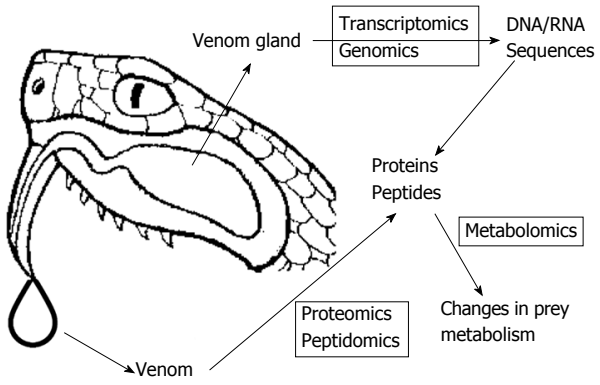

Figure 1 The relationship between "omics" technologies.

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 (rynchops 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, nanosilver 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 *Buthusoccitanus 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 (α7-

nAChR) is among several receptors that are regarded as tumor targets owing to expression specificity and significancy for cancer. The venom isolate alpha-conotoxin ImI from the cone snail *Conus imperialis* possesses high affinity for α7-nAChR and has been used to increase targeted drug delivery to α7-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 α7-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 nanoprobes that have proven useful for tumor targeting and visualization in living animals. Polyethylenimine-coated hexagonal-phase NaYF(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 *Leiurus quinquestriatus* 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 *in vitro*
Streptavidin-conjugated QDs	Biotinylated alpha-bungarotoxin from *Bungarus multicinctus* 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 *Conus imperialis*	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 *Bombina bombina*	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	*Walterinnesia aegypti*a cobra's venom	Significantly increased antitumor effects of the venom
Poly(D,L-Lactide)-based NPs	Toxic fractions obtained from *Androctonus australis hector* and *Buthusoccitanus tunetanus* scorpions' venoms	Enhanced immune response to weakly immunogenic toxins
Chitosan NPs	Cobra *Naja naja oxiana* 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 substantially 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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EDITORIAL

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

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 neurodegenerative 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 acethylcholinesterase activity[61]. Acethylcholinesterases are enzymes belonging to the carboxylesterase family, involved in the regulation of nerve signal transmission at the chemical synapses, by hydrolyzing acethylcholine 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 acethylcholinesterase biosensors are based on the measurement of the residual activity of the enzyme using different substrates, such as acethylcholine and thiocholine, that can be monitored using potentiometric, amperometric and optical devices[62-64]. The LODs that can be obtained using acethylcholinesterase 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 acethylcholinesterases.

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 acethylcholinesterase 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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EDITORIAL

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

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 excitotoxicoty 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 it 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	Astrolglial 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 μL/min)	Clinical implications
Lactate	2.9 ± 0.9 mmol/L	Increased levels seen in ischemia and hyperglycolysis[121-123]
Pyruvate	166 ± 47 μmol/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 ± 4 μmol/L	One of the constituents of the cell membranes
		An increase in levels signifies cell damage[124]
Glutamate	16 ± 16 μmol/L	Marker of excitotoxicity[124]
Glucose	1.7 ± 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 indivisualising 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 perihaemorrhagic 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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REVIEW

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

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, $DX_{35}E$ 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

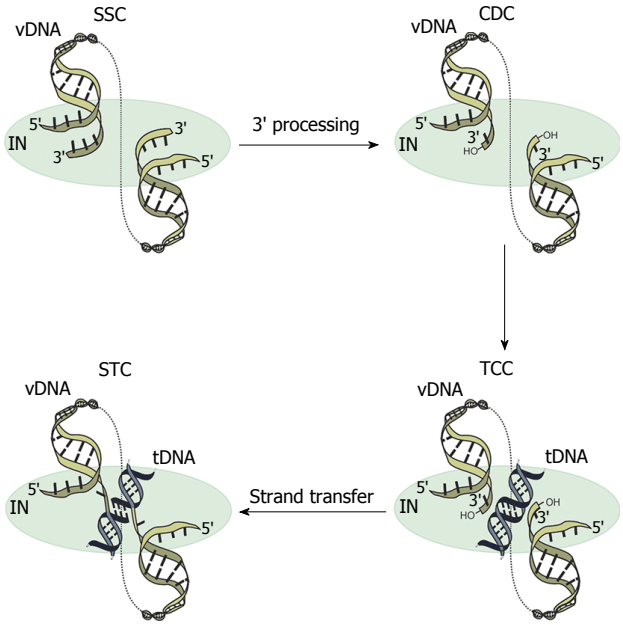

Figure 1 Integrase catalytic functions and intasome complexes. A multimer of integrase (IN) (depicted simply by blue oval) engages the end regions of the linear vDNA molecule (yellow), forming the stable synaptic complex (SSC). During 3'-processing, IN hydrolyzes the vDNA ends adjacent to invariant CA 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).

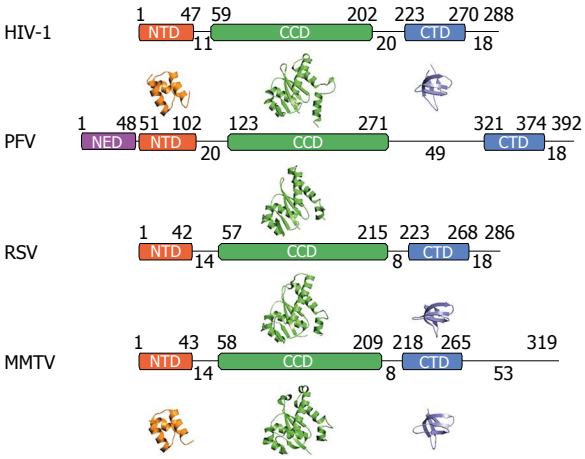

Figure 2 Integrase domain organization and representative secondary structures. Starting and ending residues for integrase (IN) domains are indicated above the boxes, and interdomain linker lengths as well as C-terminal tail lengths are indicated below the lines. Crystal structures of N-terminal domains (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.

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 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 pleotropic 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 IN_4-to-$vDNA_2$ 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

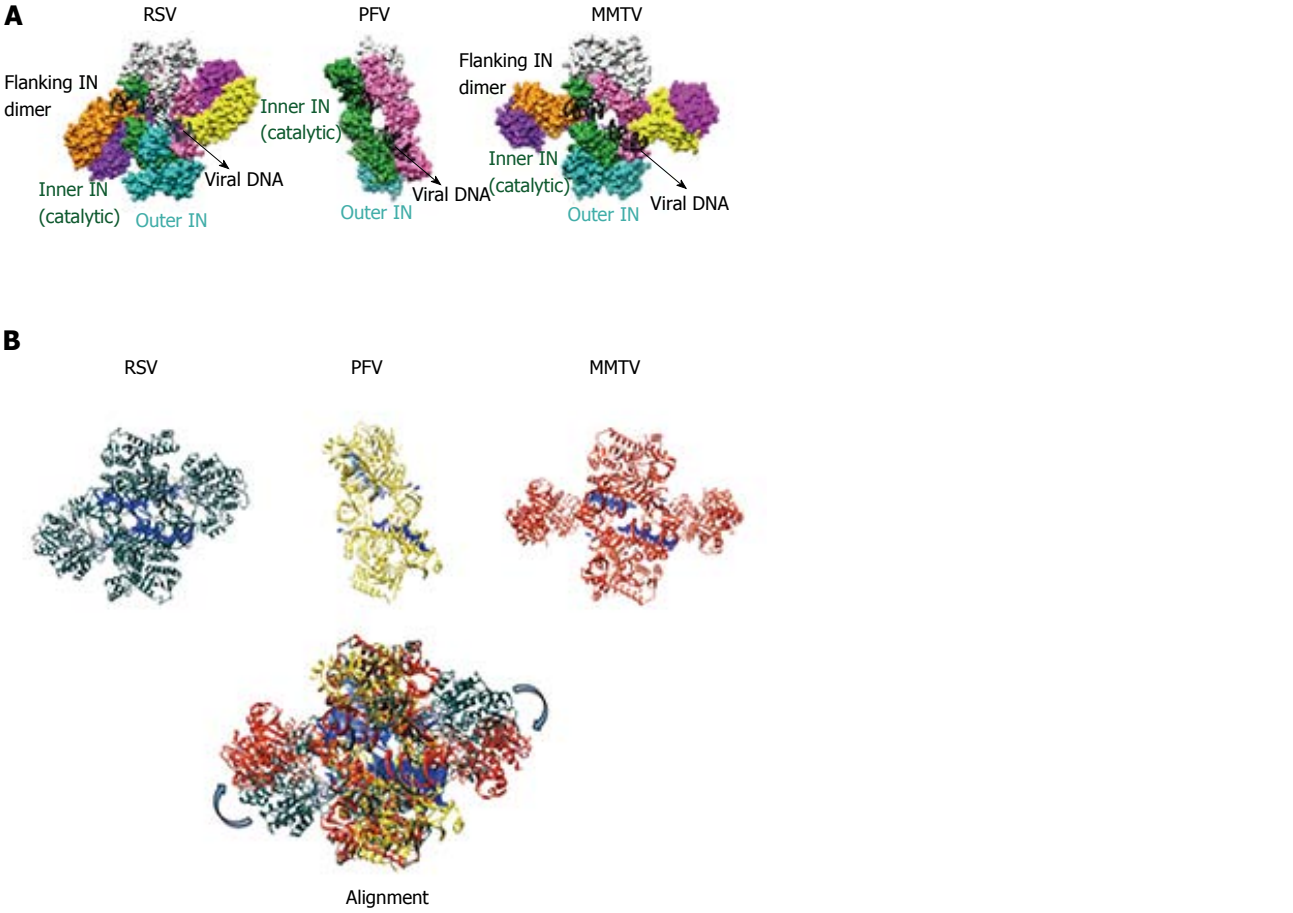

Figure 3 Comparison of prototype foamy virus, Rous sarcoma virus and mouse mammary tumor virus intasomes. A: The PFV intasome comprises two catalytic inner subunits (green and pink) and two outer supportive INs (cyan and light grey). Only the CCDs of the outer subunits are discernable in crystallographic 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 IN 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

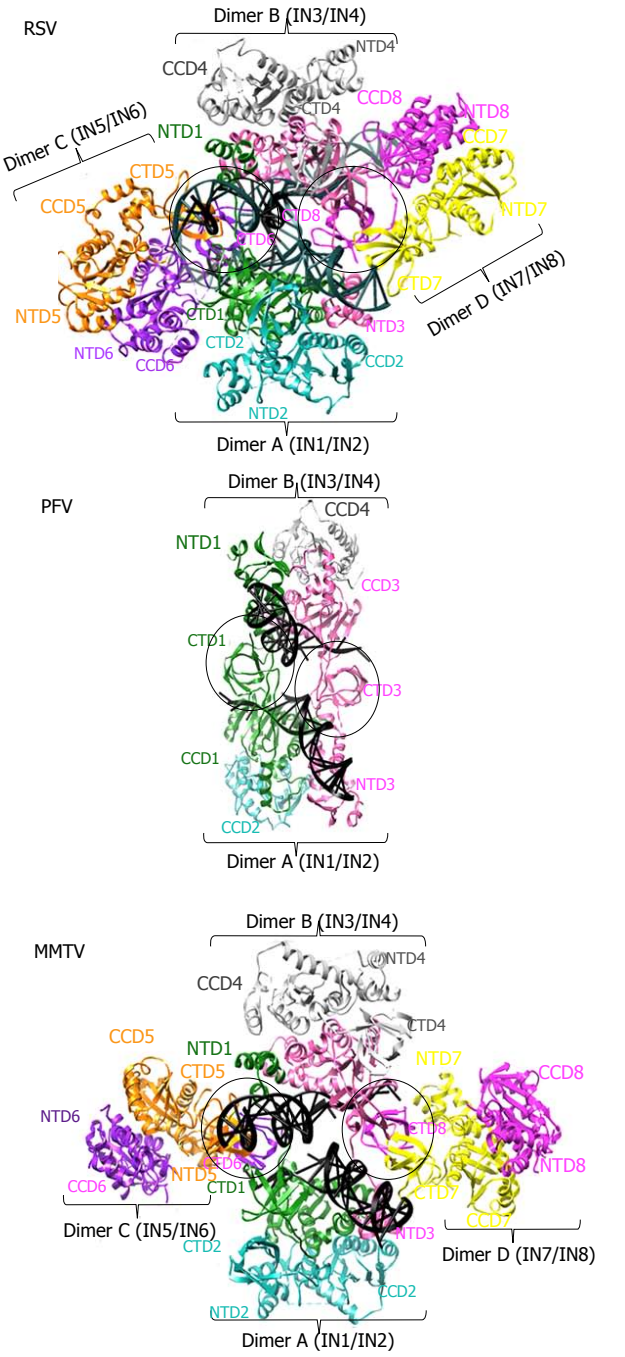

Figure 4 Integrase domain organizations within the prototype foamy virus, Rous sarcoma virus and mouse mammary tumor virus intasome structures. Separate integrase (IN) domains are labeled, with IN monomer coloring code retained from Figure 3. The green IN1 and pink IN3 monomers donate their active sites 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 crystallogenesis, 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 D,DX$_{35}$E catalytic triads to the scissile phosphodiester bonds of tDNA, thus promoting integration[62]. As S_N2 transesterification reactions are isoenergetic, 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

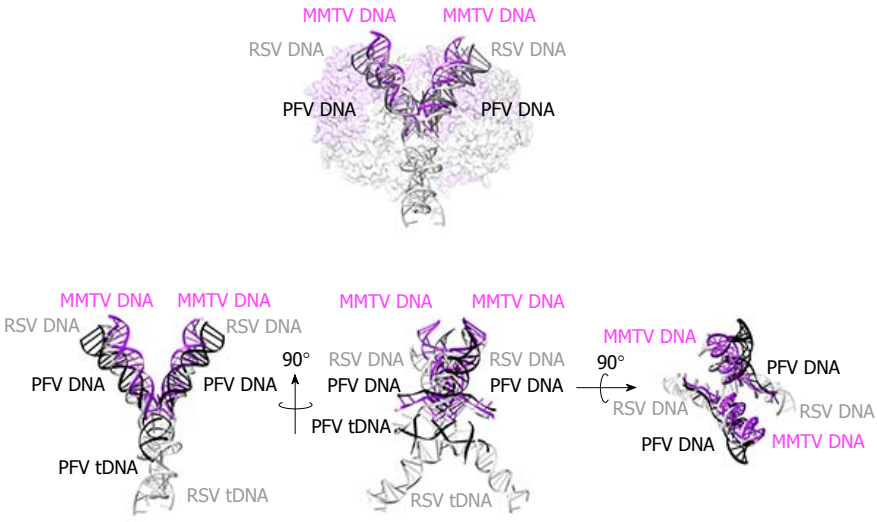

Figure 5 Superposition of prototype foamy virus strand transfer complex, Rous sarcoma virus strand transfer complex, and mouse mammary tumor virus cleaved donor complex structures with respect to vDNA and tDNA. For simplicity, integrase content is either partially transparent or omitted. Color-coding 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 confirmation 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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REVIEW

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

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). Nervertheless, several mature miRNAs are located in the nucleus, like miR-29[38,39], or in the mithocondria, 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

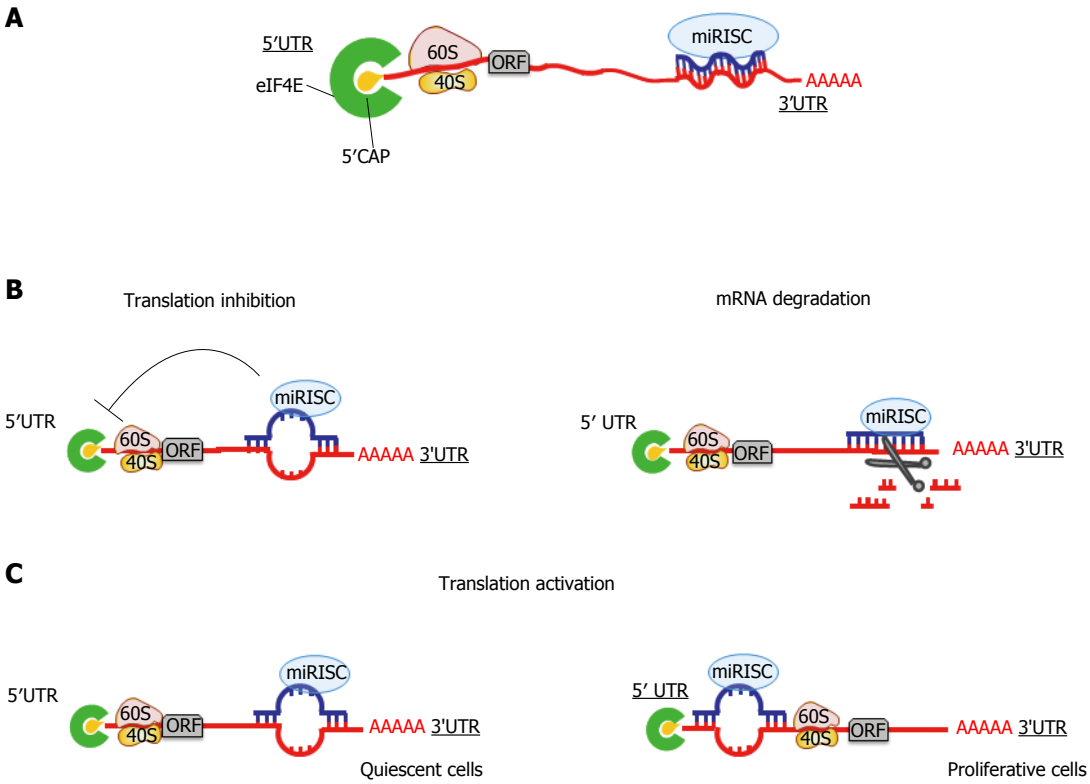

Figure 1 Mechanisms of action of microRNAs. A: miRNAs bind the 3'UTR of a target gene by base pairing. The binding between the miRNA seed sequence (nucleotides 2-8 at the 5'end of the miRNA sequence) and the miRNA regulatory element (MRE) at the 3'UTR of a target gene determines the specific type of 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 m^7G-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 m^7G-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 m^7G-cap and an IRES.

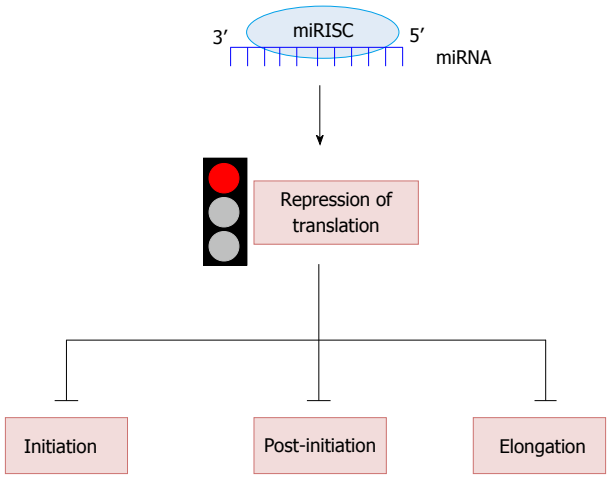

Figure 2 Schematic representation of microRNAs role in translation. miRNAs can inhibit protein synthesis at three different stages of translation: Initiation, the rate limiting step, post initiation and elongation. For details refer to text.

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 m^7G 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 lenght[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 mistery.

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-PKCβ 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 estabilishment of tumor classification, diagnosis and prognosis by miRNA profiling[94-96].

Just like classical protein-coding genes, also miRNA

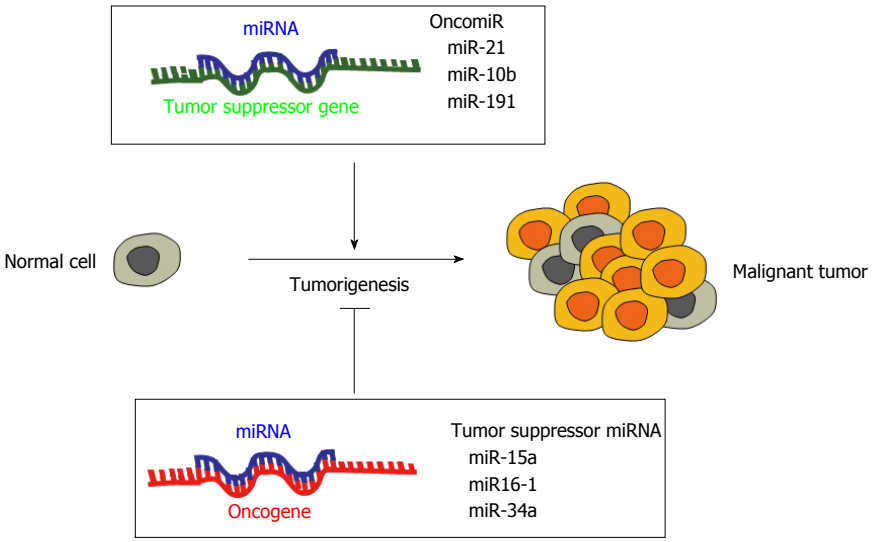

Figure 3 Roles of microRNAs in cancer. miRNAs suppress the expression of their target genes. An oncogenic miRNA, termed oncomiR, can repress the translation of a tumor suppressor gene, stimulating tumorigenesis and leading to tumor formation. Conversely, a tumor suppressor miRNA is able to inhibit the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

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

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.

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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 researcher Cecilio Romaña as one of the most important. Romaña made a great contribution to the classification of vectors as well as knowledge of the transmission and diagnosis of Chagas disease. Romaña 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 Romaña" in accordance with the recommendations of Evandro Chagas (Medical Doctor and son of Carlos Chagas), in recognition of Cecilio Romaña'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 Romaña'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 (× 1000) by cause and by region (excluding the Europe)[1] 2004

Neglected disease	World[2]	Region (OMS criteria)				
		Africa	Americas	East of Mediterran	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
Ascaridiasis[3]	1851	915	60	162	404	308
Trichiuriasis[3]	1012	236	73	61	372	269
Ancylostomiasis[3]	1092	377	20	43	286	364

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

$$DALY = YLL + YLD$$
$$YLL = N \times L$$
$$YLD = I \times DW \times L'$$

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

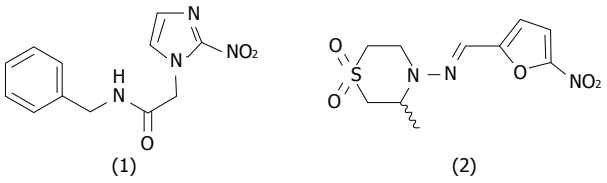


Figure 1 Chemical structures of benznidazole (1) and nifurtimox (2).

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. H_2O_2 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

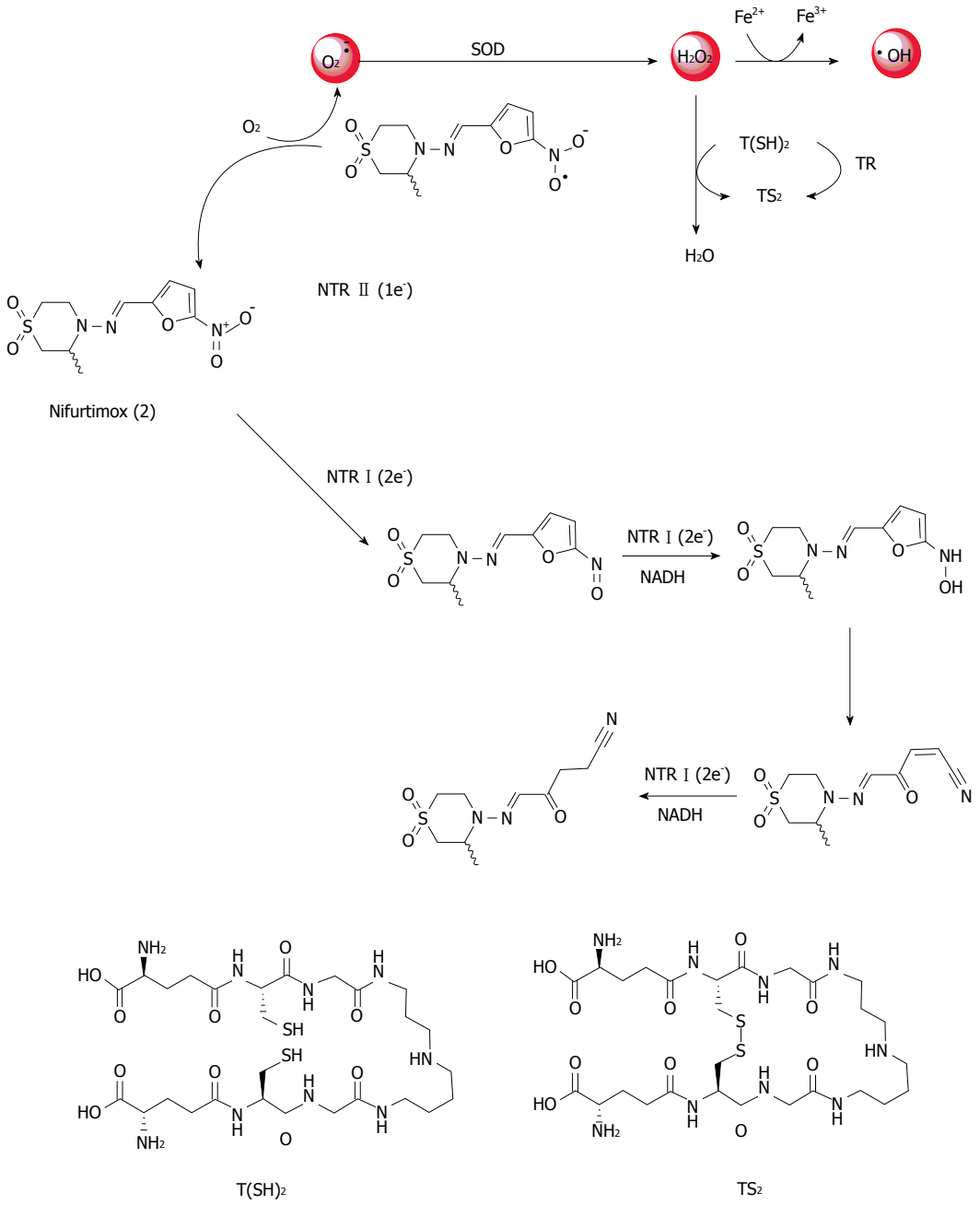


Figure 2 Nifurtimox (2) as a generator of reactive oxygen species. SOD: Superoxide dismutase; TR: Trypanothione reductase; NTR: Nitroreductases; $T(SH)_2$: Reduced trypanothione; TS_2: Oxidized trypanothione.

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

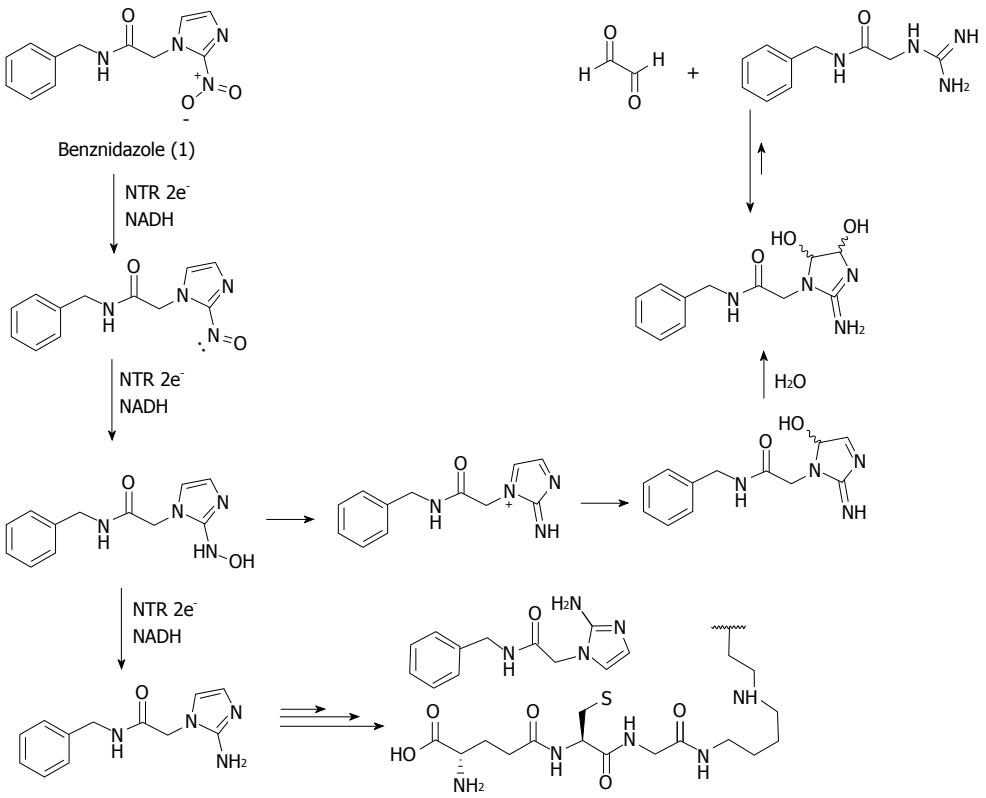


Figure 3 Benznidazole (1) is converted to nitroso metabolites and N-Aryl hydroxylamine, which can be enzymatically reduced to the corresponding 2-aminoimidazole derivative or nitrenio ion, which then is spontaneously di-hydroxylated generating a glyoxal molecule and guanidine derived.

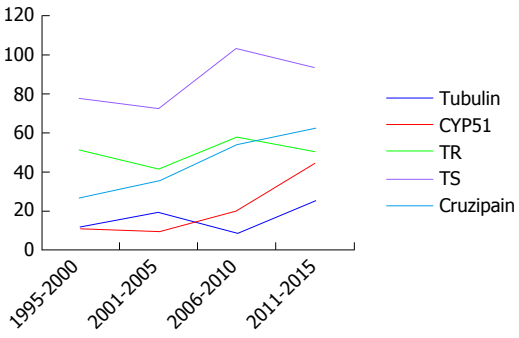


Figure 4 Search results of the terms "Trans-sialidase", "Trypanothione reductase", "Cruzipain and/or Cruzain", "CYP 51 and/or sterol 14α -demethylase" and "Tubulin" with "Trypanosoma cruzi". The results show the evolution of the number of papers published since 1995, grouped in 5-year intervals. TS: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

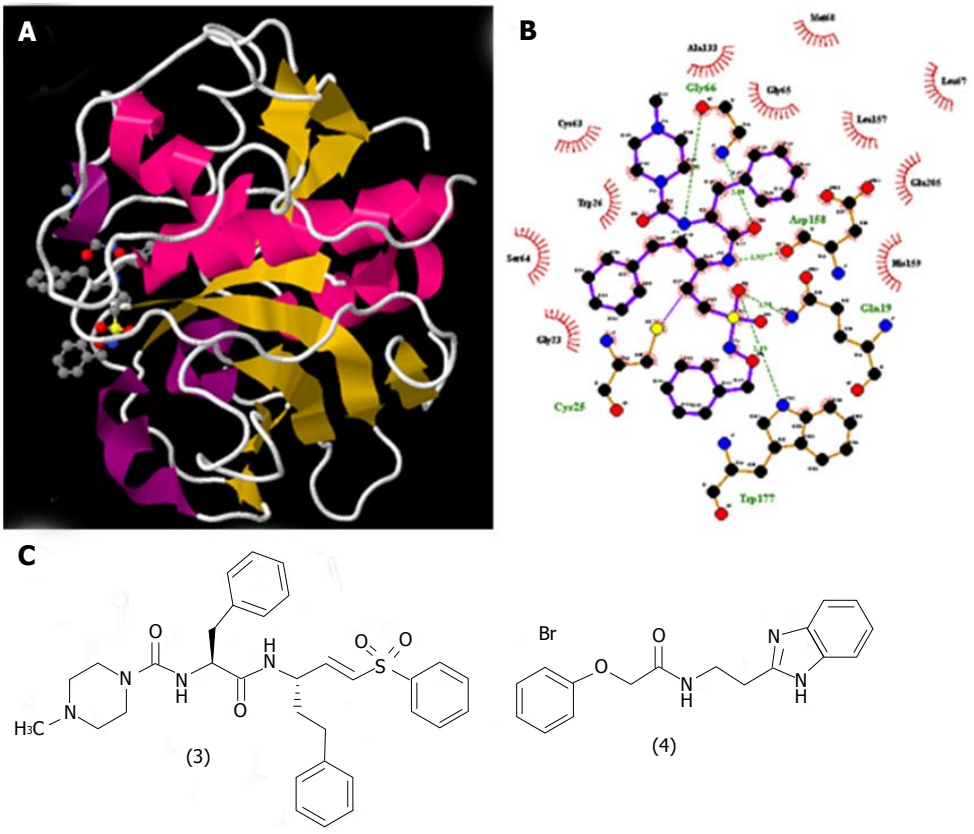


Figure 5 Structures of cruzain and its ligands. A: Three-dimensional structural representation of cruzain crystallized with vinyl sulfone derivative (3). Structure deposited in the Protein Data Bank under the code 1F2C[45]; B: Schematic representation in two dimensions of the binding mode of (3) to cruzain 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 Ki = 6.53 ± 0.59 μmol/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

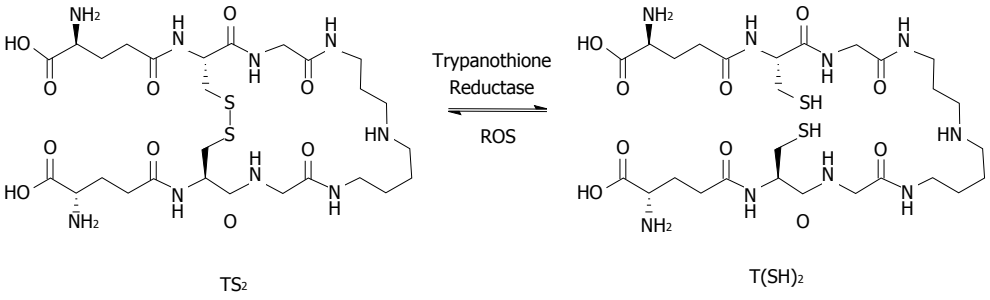


Figure 6 The equilibrium between the oxidized (TS_2) and reduced [$T(SH)_2$] forms of trypanothione. The reduction process takes place with catalysis of trypanothione reductase and the oxidation process occurs spontaneoulsy by oxidative action of reactive oxygen species (ROS).

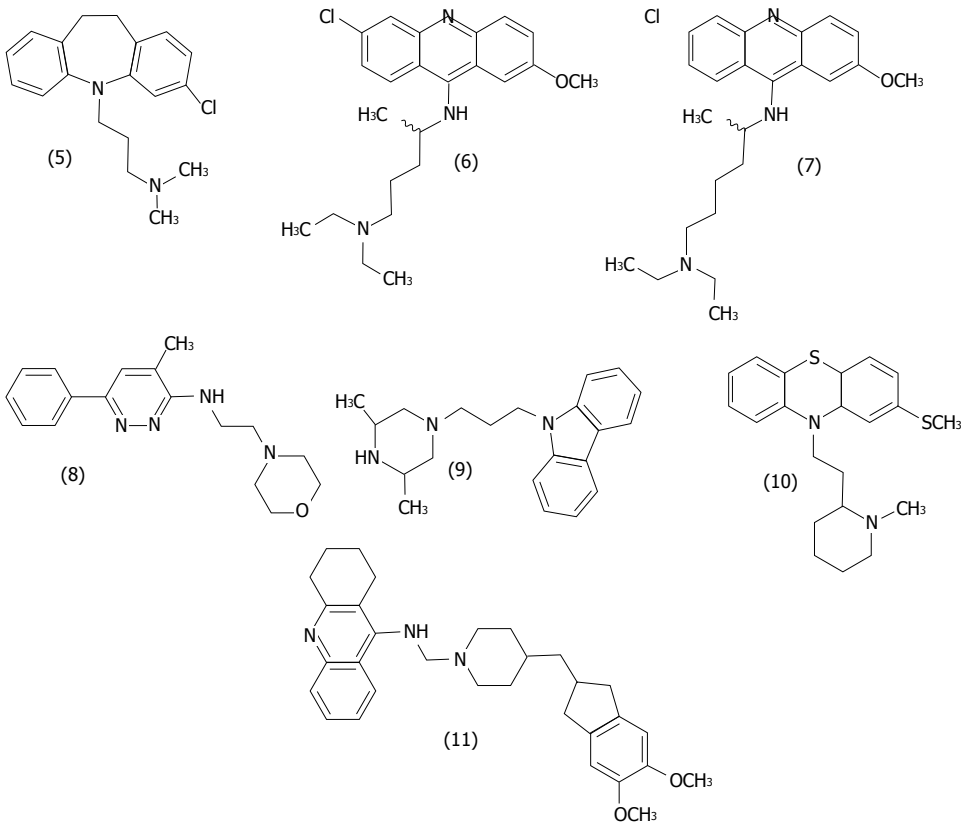


Figure 7 Structure of some non-peptidic *T. cruzi* trypanothione reductase inhibitors.

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 Ki values of 2.4 μmol/L and 13.8 μmol/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

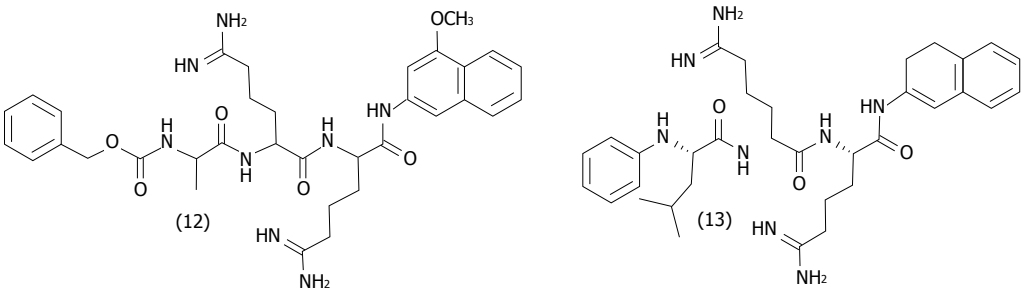


Figure 8 Structure of peptidic inhibitors of *T. cruzi* trypanothione reductase.

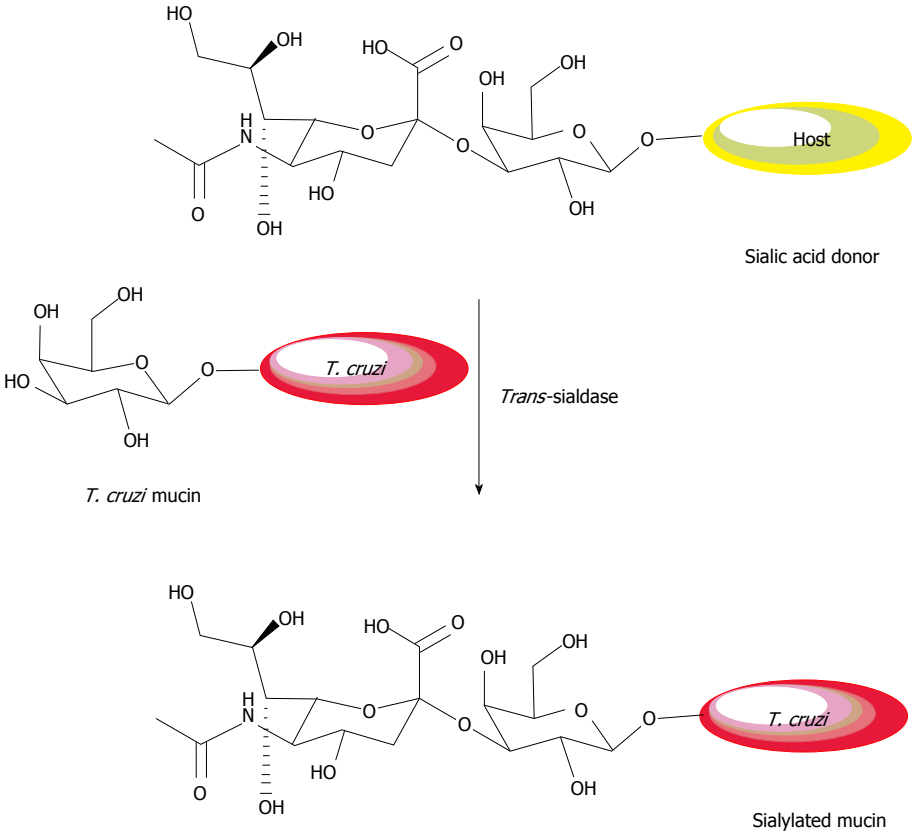


Figure 9 Transfer of sialic acid from a host glycoprotein to a β-galactopyranose from the parasite. This transfer occurs at the position 3 of the terminal sugar of the oligosaccharide which it is attached. Adapted from GIORGI and LEDERKREMER[60].

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*-acetyllactosamine 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 (Ki) being at the millimolar order, and some of them are shown in

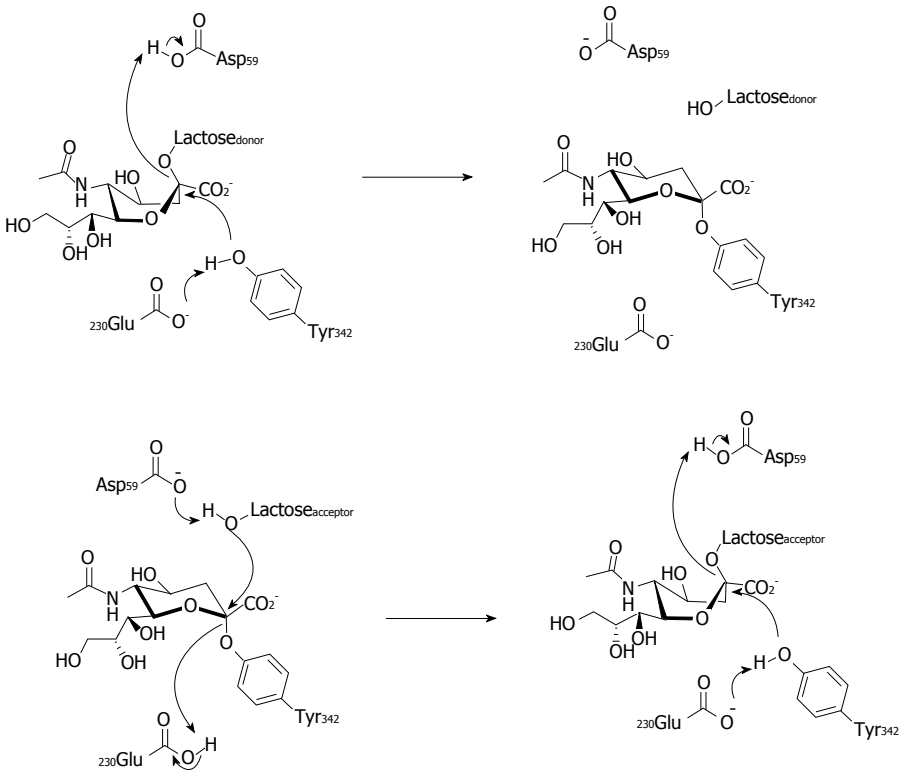


Figure 10 Proposed mechanism for the sialic acid transfer on the TcTS, showing Tyrosine 342 as the nucleophilic residue[62].

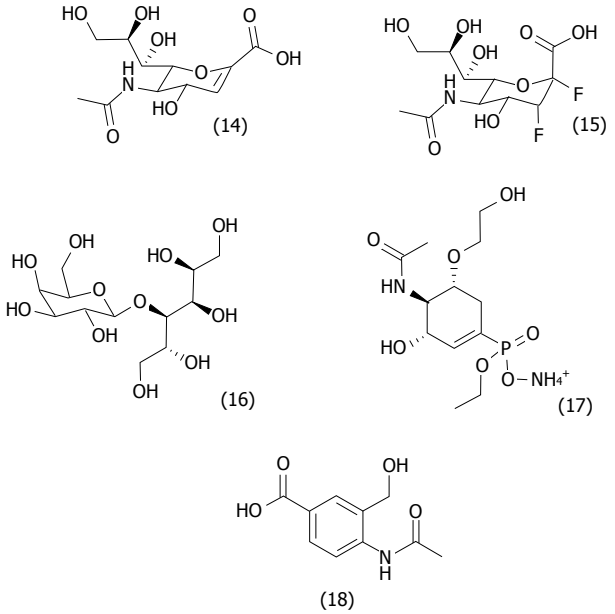


Figure 11 Chemical structures of the first *T. cruzi trans*-sialidase inhibitors (14-18). The more potent is (18), with a Ki = 300 μmol/L.

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 (Ki = 1.5 μmol/L), DANA was reported to inhibit TcTS at a very high concentration, at Ki = 12.3 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+3OHSial. 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 IC_{50} = 4.7 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 Ki = 7.3 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.

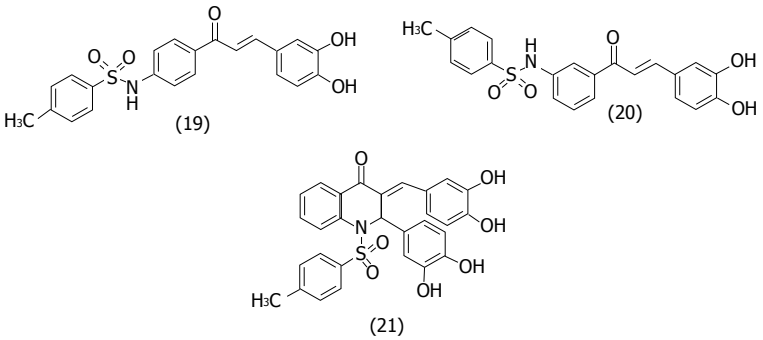


Figure 12 Chemical structures of the sulfonamide-chalcone derivatives designed by Kim *et al*[68] (19, IC_{50} = 0.9 μmol/L; 20, IC_{50} = 2.5 μmol/L; 21, IC_{50} = 0.6 μmol/L).

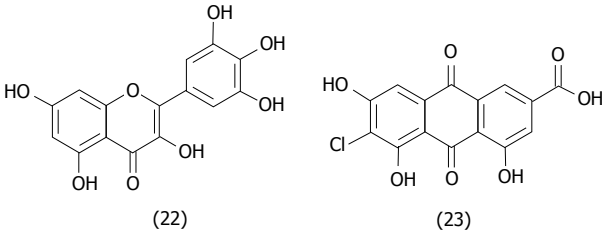


Figure 13 Chemical structures of myricetin (22, IC_{50} = 17 μmol/L) and the anthraquinone (23, IC_{50} = 0.58 μmol/L), TcTS inhibitors found by Arioka *et al*[69] from a natural products library.

The compound (18) was the most potent among the synthesized compounds, with a Ki = 300 μmol/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 μmol/L concentration, resulting in 103 compounds. Then, the second screening selected those compounds with IC_{50} < 100 μmol/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 14α-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

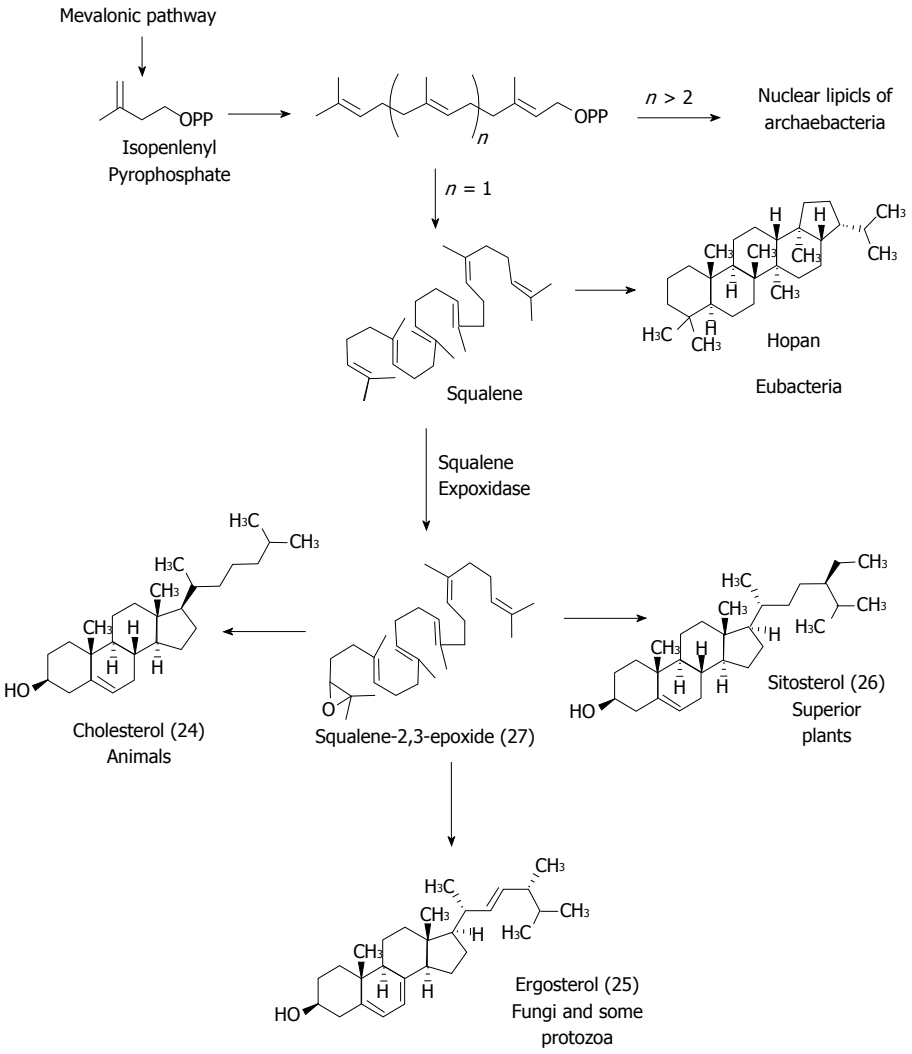


Figure 14 Comparative biosynthesis of natural membrane steroids of different organisms.

sterol 14α-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-14α-demethylases families; for example, lanosterol (29), eburicol (31) and obtusifoliol (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

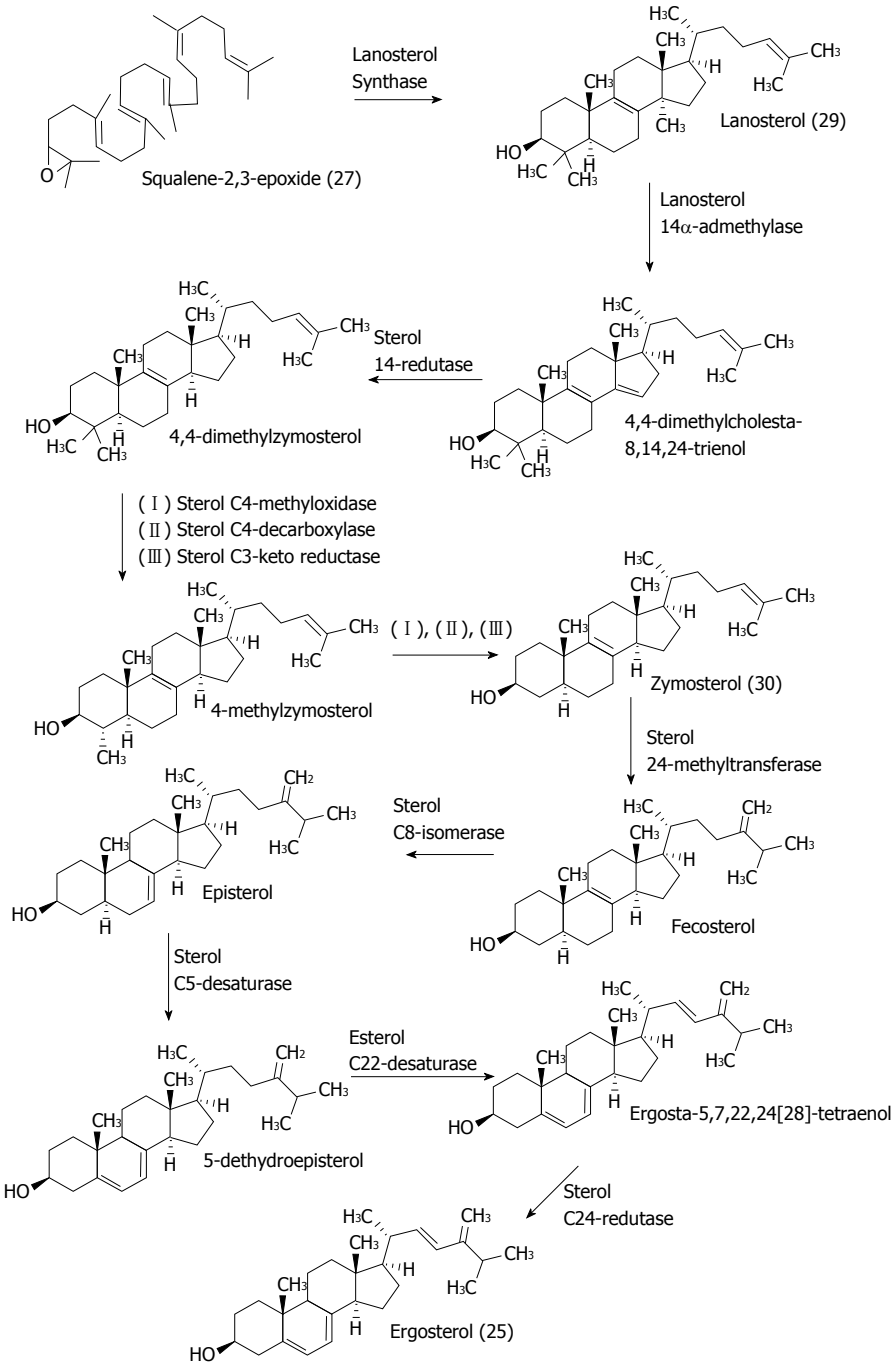


Figure 15 Ergosterol (25) biosynthesis in yeast (Saccharomyces cerevisiae), from squalene oxide.

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

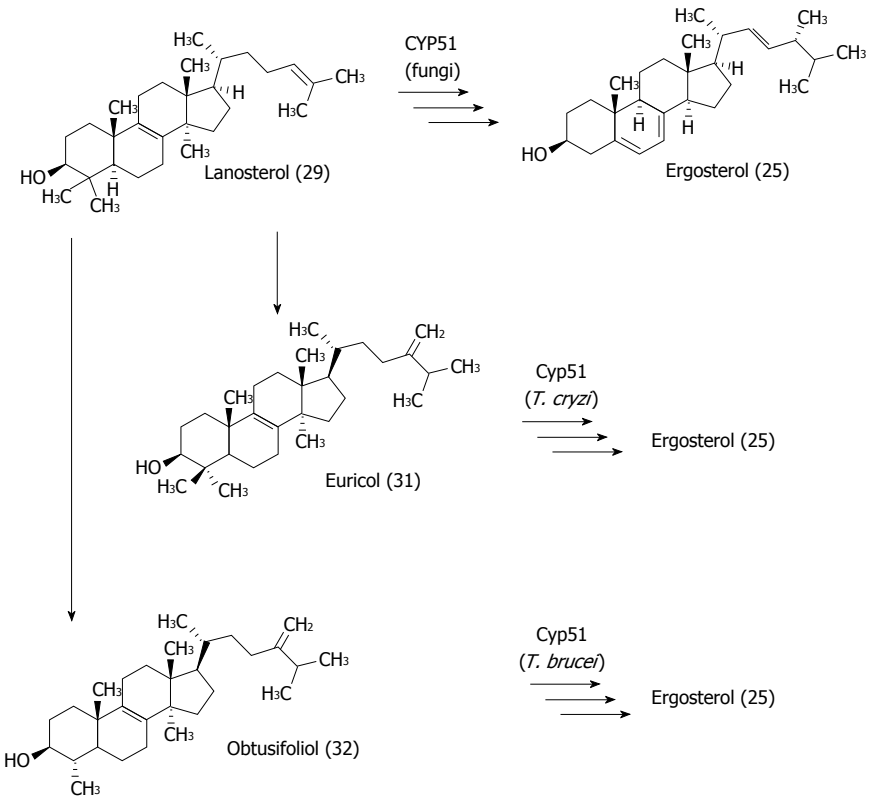


Figure 16 Structure of CYP51 preferential substrates in fungi (29), *T. cruzi* (31) and *T. brucei* (32).

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

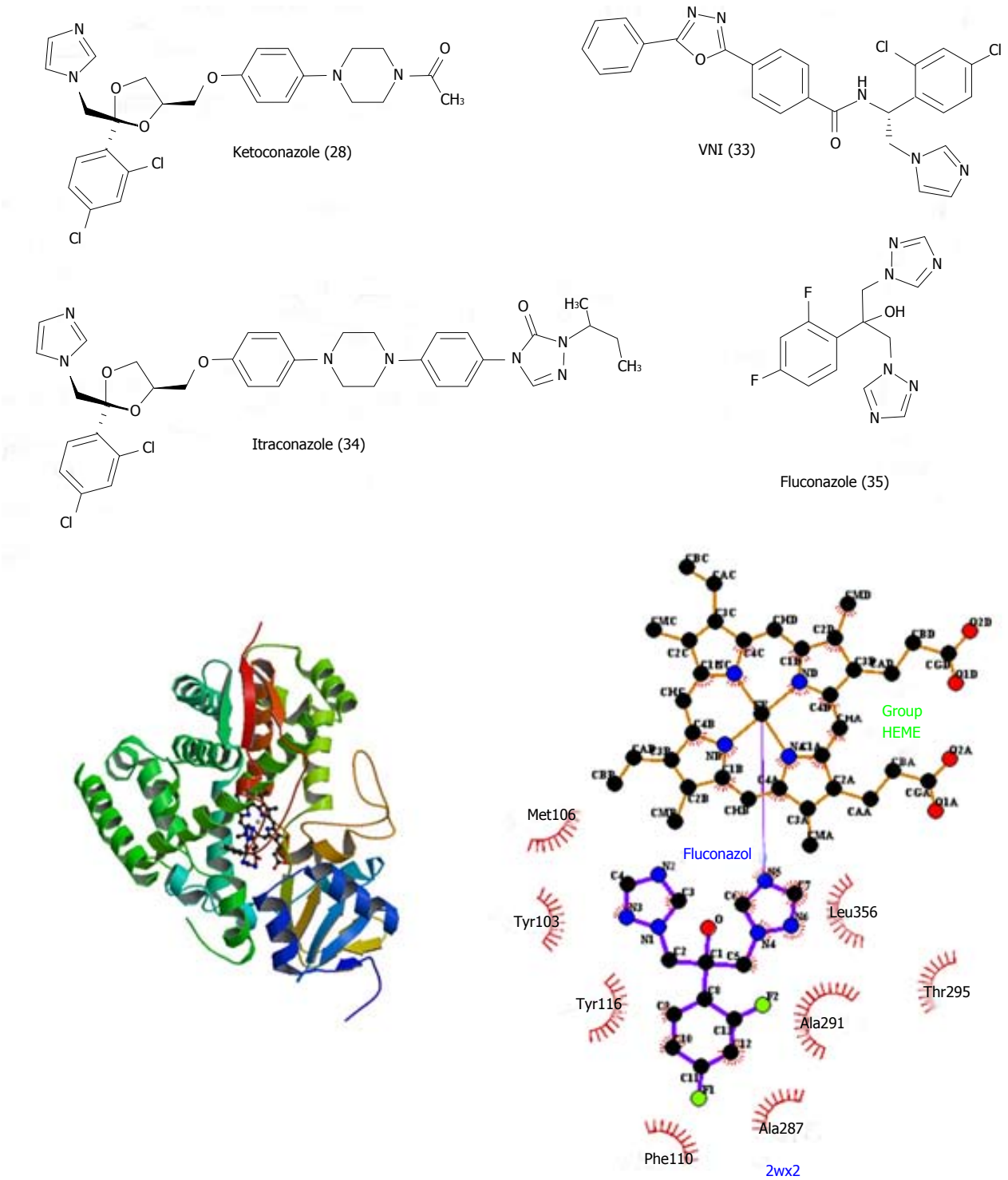


Figure 17 Structures of azole derivatives active in *T. cruzi* infection model: Ketoconazole (28), VNI (33), itraconazole (34) and fluconazole (35). Crystallographic structure of *T. cruzi* CYP51 with fluconazole (35) linked to the catalytic site of the enzyme [available at Protein Data Bank under the 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

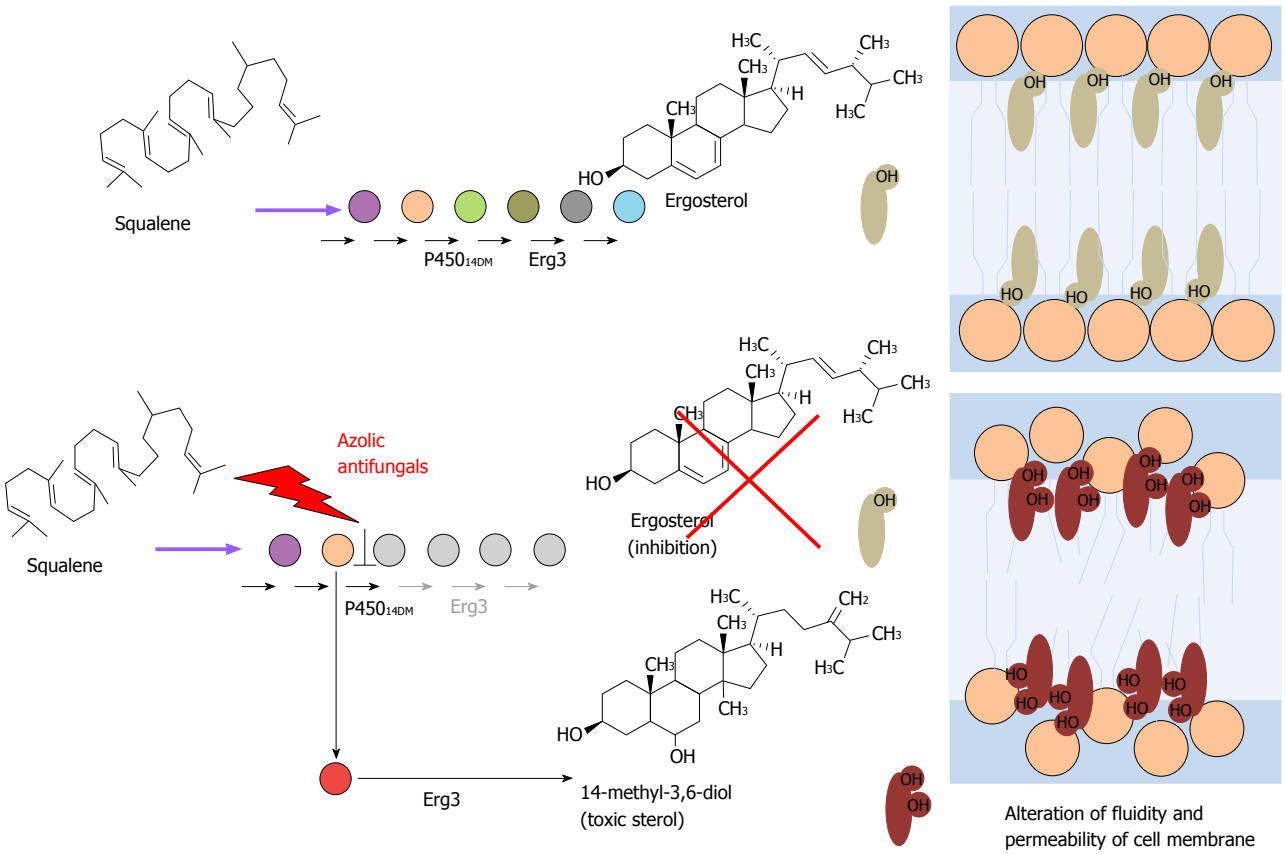


Figure 18 Schematic representation of the mechanism of actions of azolic compounds upon ergosterol (25) synthesis and subsequente alteration of composition and organizarion of cell membrane.

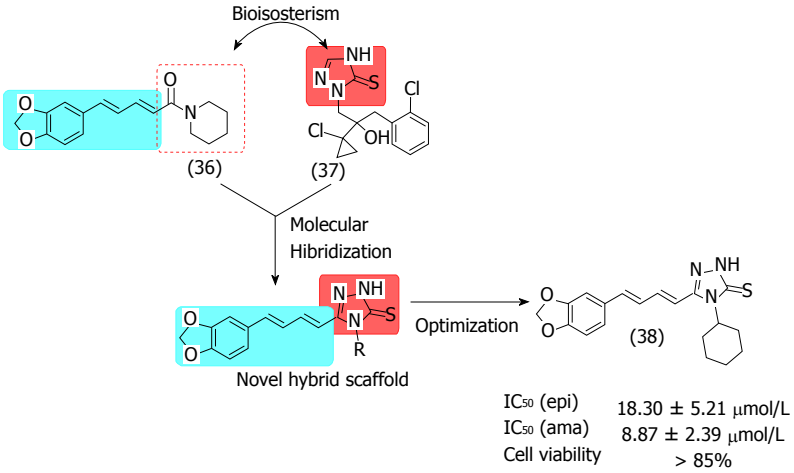


Figure 19 Structures of piperine (36), prothioconazole (37) and cycloexyltriazole (38), and its IC_{50} values against epi- and amastigotes of *T. cruzi* and the toxic profile against murine macrophages.

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 derivatives 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.

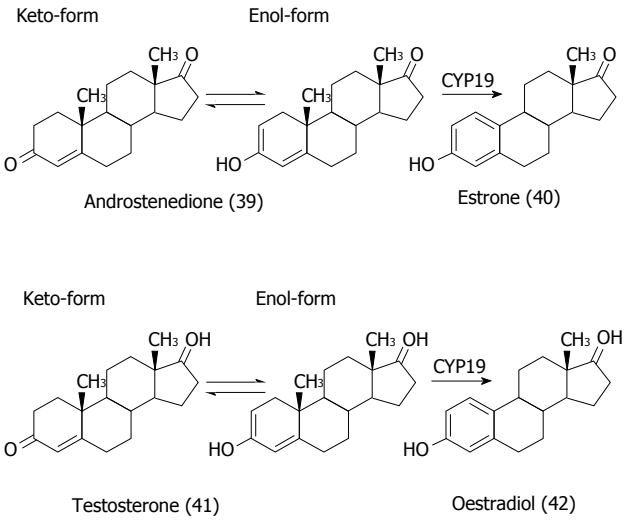


Figure 20 Reactions catalyzed by aromatase (CYP19): Conversion of androstenedione (39) to estrone (40) and testosterone (41) to oestradiol (42).

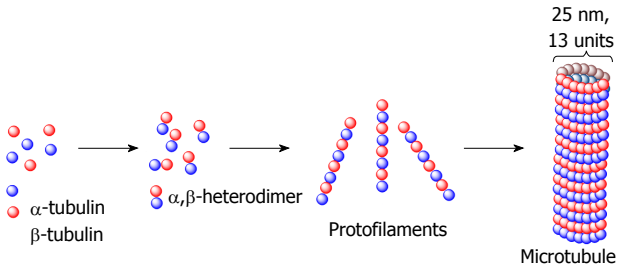


Figure 21 Schematic representation of protofilaments formed by polymerization of the heterodimers of alpha and beta tubulin.

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

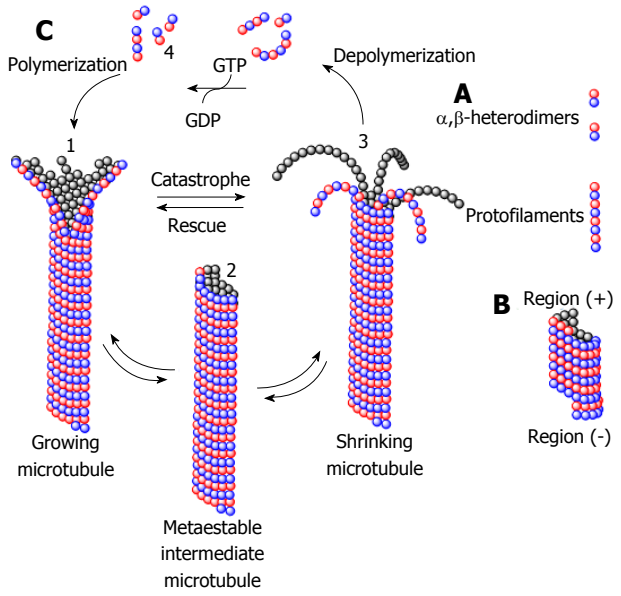


Figure 22 Schematic representation of polymerization/depolymerization dynamics of microtubules. A: Formation of protofilament with alpha e beta-tubulin heterodimers; B: Formation of microtubule with regions (+) and (-); C: Relationship between polymerization and GTP hydrolysis equilibrium, where is perceived: (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 hydrolisys rate is superior; and (4) the microtubule salvation mediated by GTP consumption.

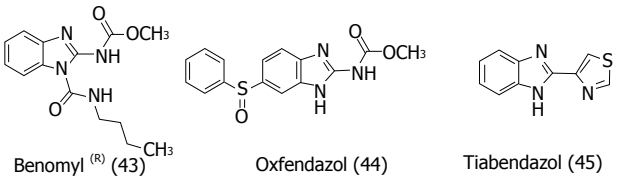
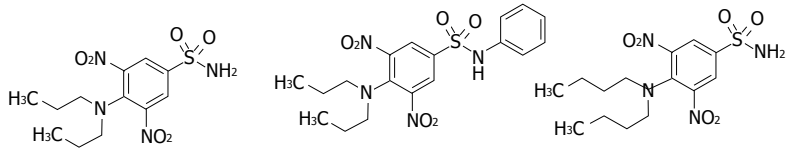


Figure 23 Structure of benzimidazolic compounds active on yeast (43) and nematoda (44, 45) but inactive against mammalian tubulin.

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 monoterpenoid 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	*L. donovani* (amastigotes)	*T. b. brucei* (variant 221)	*T. b. brucei* (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 IC_{50} values (micromolar) of oryzalin (46) and the sulfonamide-dinitroanilin derivatives (47 and 48) against kinetoplastide and mamallian cells[98].

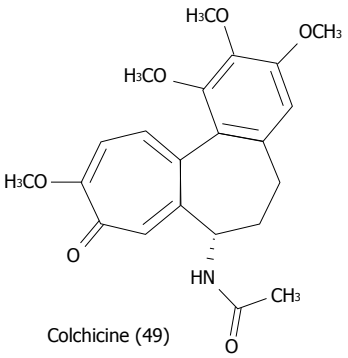


Figure 25 Chemical structure of colchicine (49).

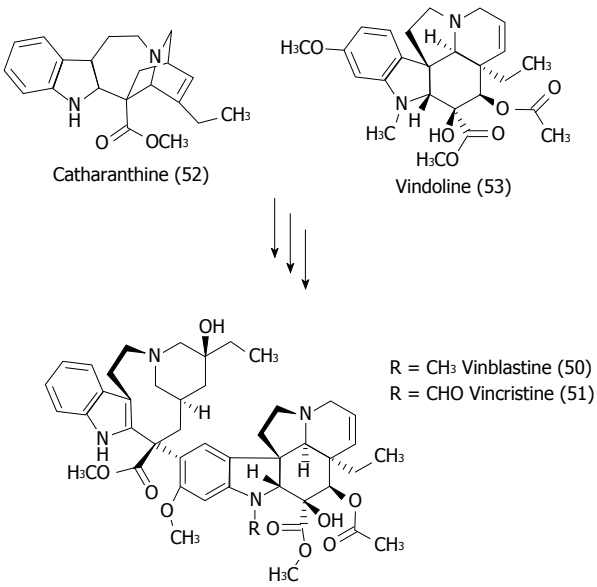


Figure 26 Structures of the vinca alcaloids (50-51) and its biochemical precursors (52-53).

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 (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

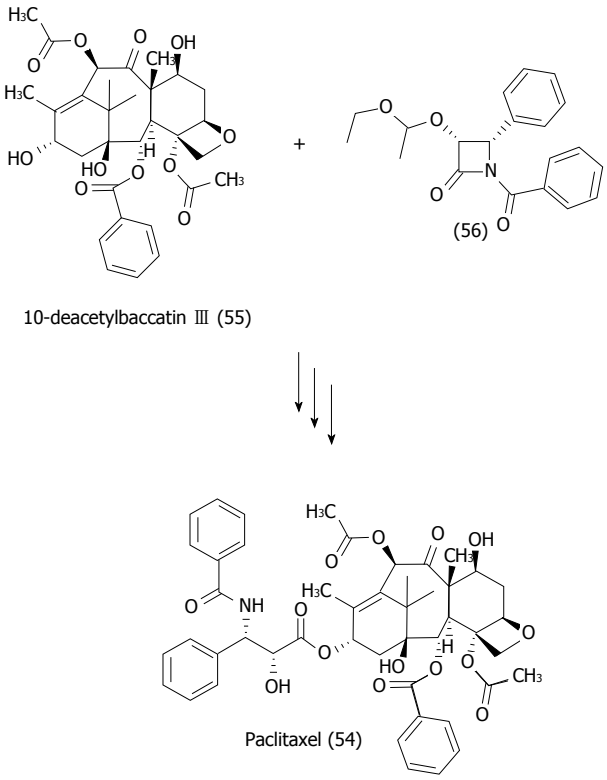


Figure 27 Synthetic strategy developed by Ojima *et al*[110] on the synthesis of paclitaxel (54) from the natural precursor 10-deacetylbaccatin Ⅲ (55).

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 kinetoplastids. 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

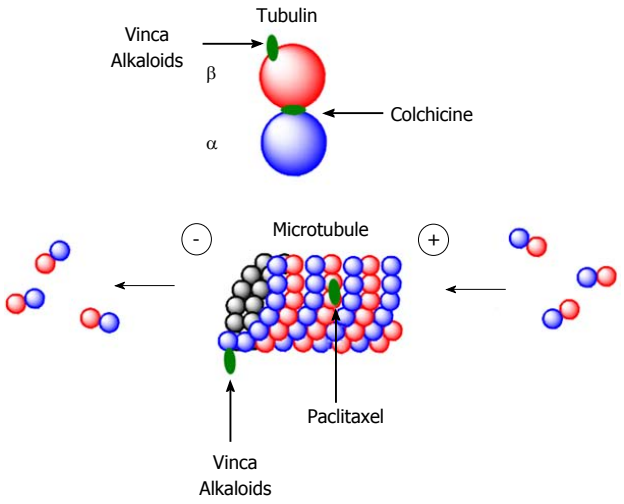


Figure 28 Representation of the binding site locations for the main tubulin ligands.

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

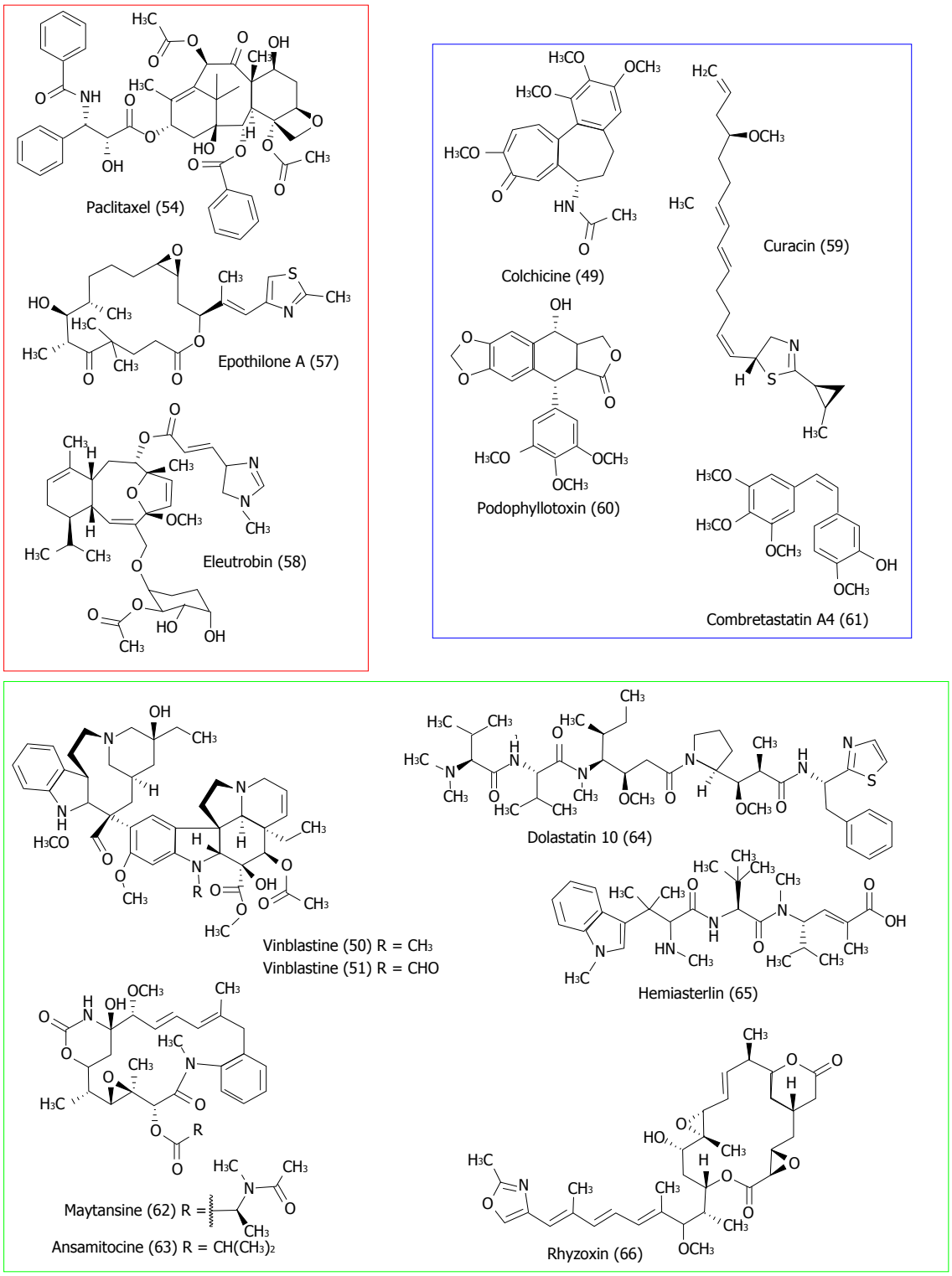


Figure 29 Structures of some ligands of the three main binding sites of tubulin: Taxol site (red), vinca alkaloids site (green) and colchicine site (blue).

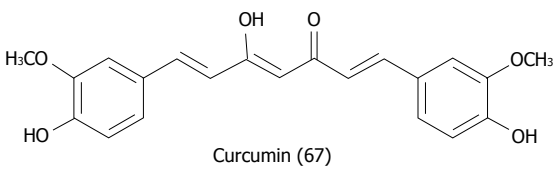


Figure 30 Chemical structure of curcumin (67), a natural product that stabilizes microtubules by binding in a unique binding site of tubulin.

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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MINIREVIEWS

Use of thyroglobulin as a tumour marker

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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, 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

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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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

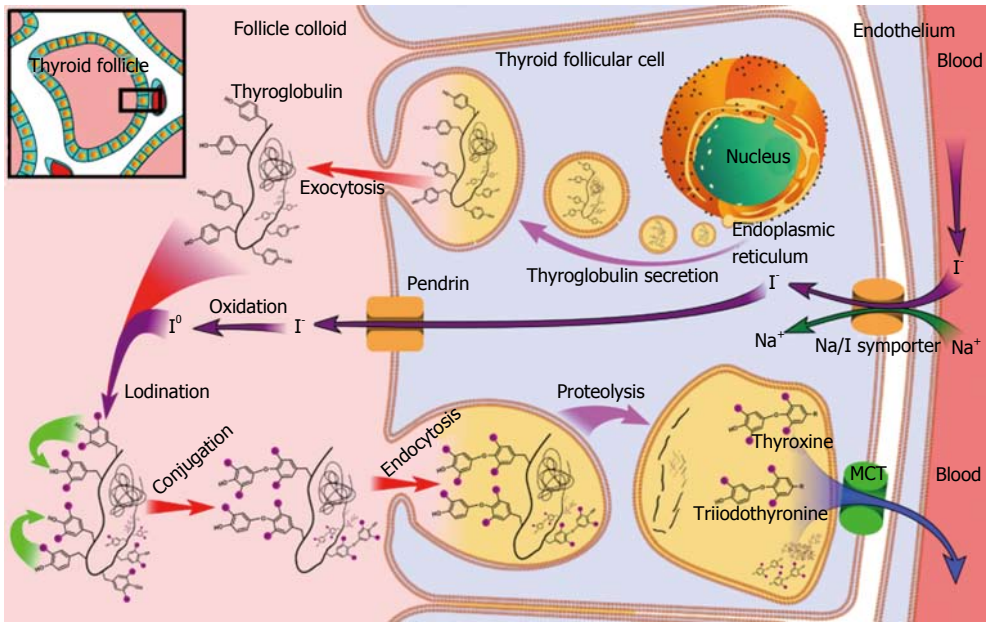

Figure 1 Metabolism of thyroglobulin[20].

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 thyroxin 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, thyroxin 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 thyroxin 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
			Benign or malignant thyroid disease	TG can be used subsequently to monitor disease activity
3-4 wk after surgery	Anti-TGAb, successful local surgery or no metastatic disease			Do 125-Iodine scan to screen for residual local disease and distant metastases
		Incomplete local surgery, metastatic disease or recurrence		Remnant ablation therapy (131-Iodine or further surgery) is indicated
Long term follow-up	Anti-TGAb			Do other tests to monitor disease activity
	Cure or stable disease			Disease activity can be monitored by periodic TG measurements
			Recurrence or progression	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 thyroxin 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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ORIGINAL ARTICLE

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

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 over-production 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 μmol/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 ℃ in a humidified atmosphere with 5% CO_2. 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 μmol/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 μmol/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 μmol/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 Facscan 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 3methyadenine (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 Na_2EDTA; 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 ℃. 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 (10^6 cells) were harvested and homogeneized 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 recommended 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 ℃ 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 Facscan 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 [$DiOC_6(3)$] labeling was carried out according to Molecular Probes recommendations; briefly cells were incubated 20 min at 37 ℃ with 20 nmol/L of $DiOC_6(3)$, cells were then centrifuged at 600 × g for 3 min then resuspended in DPBS. The analysis was performed using a Facscan 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 $DiOC_6(3)$. 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%

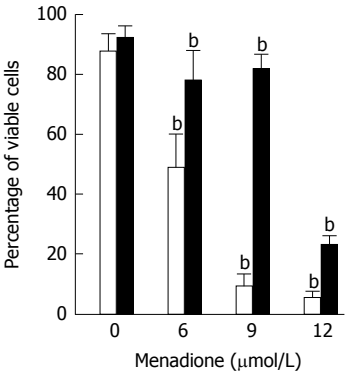

Figure 1 Protection against menadione-induced mitochondrial depolarization with L-carnitine. C2C12 cells were either untreated (white histogram) or pretreated with 500 μmol/L of L-carnitine (dark histogram) and incubated for 24 h with desired concentration of menadione (from 0 to 12 μmol/L). Mitochondria integrity was evaluated using DiOC6(3) probe staining; fluorescent cells were counted using a cytometer. Results are presented as the mean value ± 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 μmol/L of menadione and the percentage decreased to respectively 8% and 6% at concentrations of menadione of 9 and 12 μmol/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 μmol/L of menadione and in the presence of L-carnitine, 70% of the cells exhibited fluorescence. At 12 μmol/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 μmol/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 μmol/L L-carnitine and with 9 μmol/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 μmol/L: Figure 3A; 6 μmol/L: Figure 3B; 9 μmol/L: Figure 3C and 12 μmol/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 μmol/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 μmol/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 μmol/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 μmol/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 μmol/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 μmol/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 μmol/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 μmol/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.

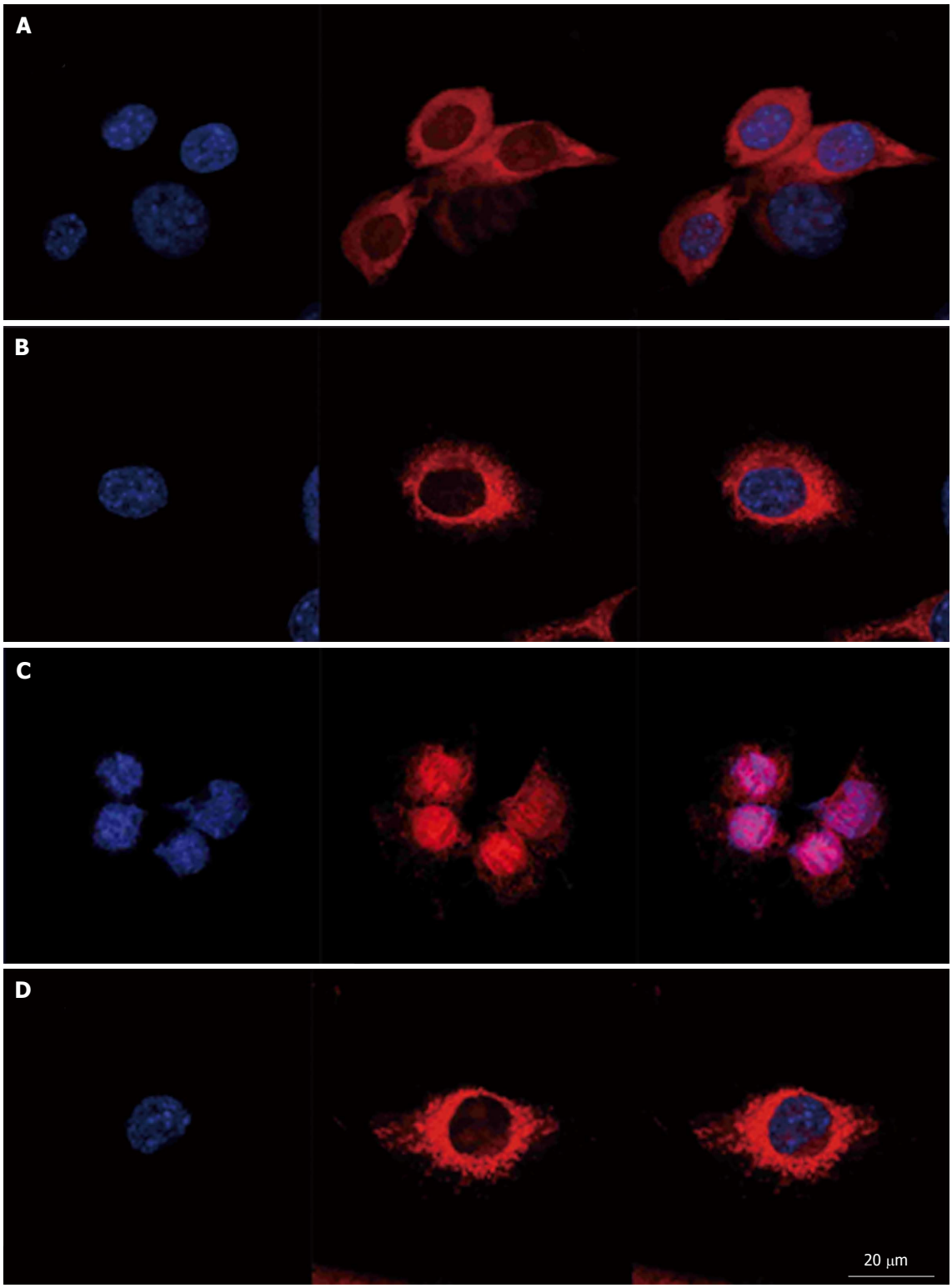

Figure 2 Prevention of menadione-induced mitochondrial distribution with L-carnitine. C2C12 cells were either untreated or pretreated with 500 μmol/L of L-carnitine and incubated for 24 h with menadione (0 and 9 μmol/L). Nucleus and mitochondria morphology was evaluated after staining with Hoechst 33342 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 μmol/L of menadione; D: C2C12 pretreated with L-carnitine and treated with 9 μmol/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 μmol/L of menadione, only 51% of the cells remain unstained by PI. The cell survival decreased as the menadione con-

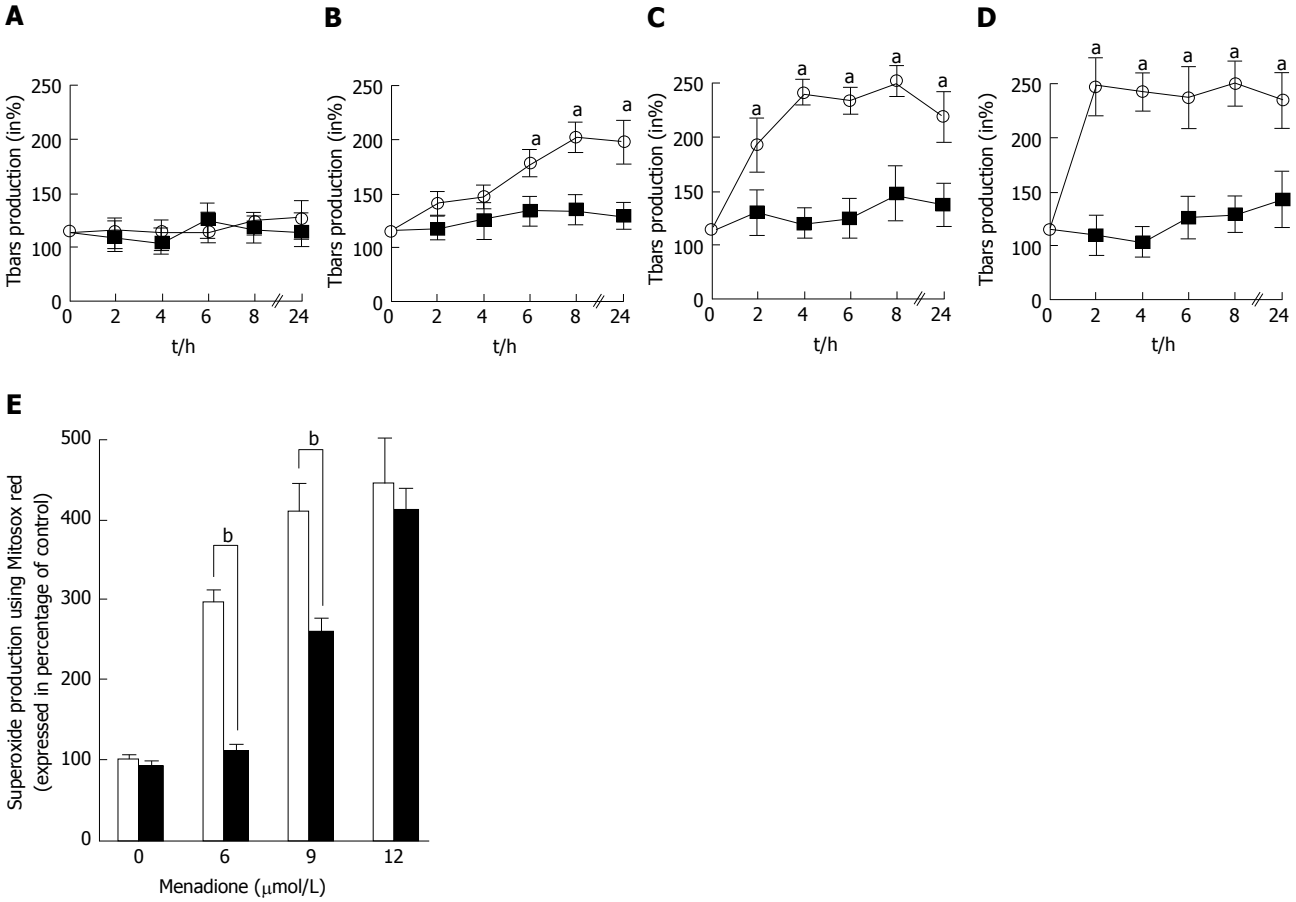

Figure 3 Characterization of reactive oxygen species production. A-D: Tbars production was determined in C2C12 cells in the presence of various amounts of menadione from 1 to 24 h. Results were expressed in percentage of the control cell Tbars production. Tbars production was analyzed in the presence of 0 (A), 6 (B), 9 (C) and 12 μmol/L (D) of menadione in control cells (empty circles and dashed line) and in cells pre-treated with 500 μmol/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 μmol/L of L-carnitine (black histogram) and incubated for 24 h with desired concentration of menadione (from 0 to 12 μmol/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 ± 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 μmol/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 μmol/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 K_3) 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, $O_2^{-}\bullet$ and, in the presence of metal ions, to $OH\bullet$[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 μmol/L. In addition, menadione induced a significant increase in ROS generation in the mitochondria; at a dose of 6 μmol/L of menadione, more than 67% of the cells were over producing ROS. Menadione was also able to

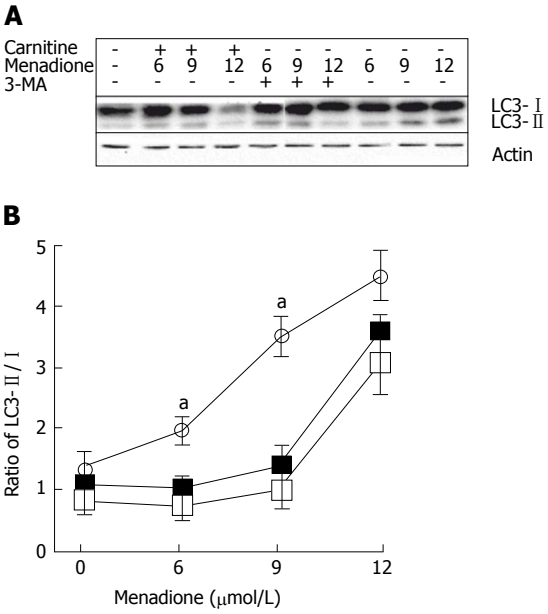

Figure 4 Menadione induces autophagy in C2C12 cells. A: Western blot for the determination of LC3 Ⅰ and Ⅱ forms. Initial cells were either treated with L-carnitine, menadione or 3-MA; B: LC3- Ⅰ and Ⅱ isoforms were detected by western blotting and quantified. The ratio between Ⅰ and Ⅱ forms was calculated 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 (± 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

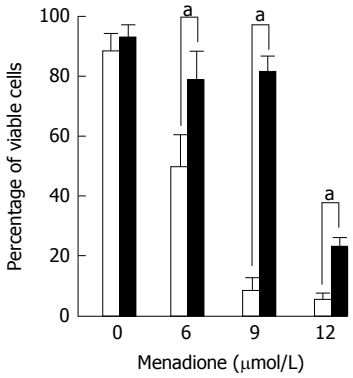

Figure 5 Protection of menadione-induced C2C12 cell death by L-carnitine. C2C12 cells were either untreated (white histogram) or pretreated with 500 μmol/L of L-carnitine (dark histogram) and incubated for 24 h with desired concentration of menadione (from 0 to 12 μmol/L). Cell survival was evaluated 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 ± 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.

ACKNOWLEDGMENTS

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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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ORIGINAL ARTICLE

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/NSHN'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/NSHN 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

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 Yepez 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, *n*	Deaths, *n*	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) CLASBIs 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 baumanii* 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 NSHN 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
Staphylococcus aureus			
Oxacillin	60% (5)	61.20%	54.60%
Pseudomonas aeruginosa			
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%
Klebsiella pneumoniae			
Ceftriaxone or ceftazidime	60% (5)	71.20%	28.80%
Imipenem or meropenem	20% (5)	19.60%	12.80%
Acinetobacter baumanii			
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/NSHN: 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 document 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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