The Chemotherapy of Chagas' Disease: An Overview

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Abstract: The review presents: a) a brief description of the disease; b) a summary of the most important metabolic targets so far identified in *Trypanosome cruzi (T. cruzi)* along with corresponding inhibitor compounds; c) the current state of knowledge on the trypanothione reductase system of trypanosomatids with reference to oxidative stress defenses; d) detailed discussions on *T. cruzi* trypanothione reductase inhibitors such as nitrofuranes, naphthoquinones and phenothiazines. As yet, the chemotherapy of Chagas' disease remains an unsolved problem. Further search for new drugs must continue by means of nucleating existing chemotherapy efforts.

Keywords: Antichagasic agents, chemotherapy targets, trypanosomiasis, trypanothione metabolism, trypanothione reductase inhibitors.

In Memory of Our Beloved Professor A.O.M. Stoppani who Passed Away on March 18th, 2003.

INTRODUCTION

A variety of tropical diseases are produced by eukaryotic protozoa e.g. *plasmodes*, *leishmania* and *trypanosomes.* In particular, trypanosomes are known to be responsible for *Chagas'* disease, *nagana* and *sleeping sickness*, among others [1].

Chagas' disease is one of the most significant parasitic ailments worldwide. In spite of the fact that the enforcement of public health programs, e.g. vector control, has decreased the incidence of new infections, it continues to be endemic in large areas of Latin America; over 40 million people are exposed to the risk, while 18-20 million people (including some 100,000 in the United States) are infected with the causal agent of the disease, namely *Trypanosome (Schizotrypanum) cruzi* (*T. cruzi*) [2,3]. To complete the picture, every year 21,000 people are estimated to die from the parasitosis and over 200,000 new cases arise [4,5].

Trypanosome cruzi is a pleomorphic hemoflagellate protozoan (family *Trypanosomatidae*, order *Kinetoplastida*) [6] with a life cycle, which involves an obligatory passage through vertebrate (mammals, in particular human beings) and invertebrate (hematophagous triatomine bugs, also known as "vinchucas", "barbeiros", "kissing bugs", "assassin bugs", etc.) hosts, spanning a series of phases where different parasite forms exist.

The life cycle initiates when the trypomastigote form is ingested by the insect host where it differentiates into the replicative form, the epimastigote. Thereafter, in the intestine, the epimastigote evolves to the metacyclic trypomastigote form. The latter invades the vertebrate host, either by means of the vector (80-90% of cases), blood transfusion or congenital processes. Once inside the vertebrate (human), the metacyclic form turns into the corresponding replicative form, the amastigote, which goes through several rounds of replication before transforming into the trypomastigote, responsible for the dissemination of the infection throughout the body.

In humans, after infection, the acute phase begins, which, if untreated, lasts for about two months. During this period, the parasite is able to invade and multiply within different host cells, including muscles, fibroblasts and neurons; inflammatory lesions are detected in several organs. The chronic phase of the disease follows, where, typically, patients remain a-symptomatic. However, about 20 to 50% of the cases display symptoms characteristic of this phase, namely cardiac, digestive or neurological malfunctions, accordingly to the endemic area analyzed. Two mechanisms are proposed for pathogenesis in the chronic phase: inflammatory reactivity due to the persistence of the parasite inside the host tissues and induction of auto-immune responses targeted at said tissues [7].

The experience derived from previous extensive malaria eradication programs evidenced the risk of the emergence of insecticide resistance by the insect vectors. In addition, alternate methods of infection (see above) are clearly not taken into account by these programs. In this scenario, one is left with the conviction that the effort should be focused on the causal agent of the infection. Thus, the necessity to develop effective drugs to neutralize the action of the parasite inside the human host is evident. This notion is also supported by the current prevalent opinion that Chagas' disease is to be treated as a parasitic as opposed to an auto-immune condition, an idea previously suggested [8,9]

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The current commercially available drugs used, namely Nifurtimox (Lampit™) and Benznidazol (Radanil™, Rochagan™) have proven efficient only through the acute phase. In addition, both drugs bring along important side-effects such as anorexia, loss of weight, vomit, nausea, diarrhea, etc. [3]. Since the last decade, due to corporate politics, Bayer AG has discontinued commercialization of Nifurtimox (Lampit™) and currently the drug is only produced in El Salvador, being distributed on demand to the rest of Latin America (Galbarini, personal communication).

The interest of the pharmaceutical industry in drug discovery against Chagas' and other tropical diseases has been dwindling for several decades now. Arguably, the main reason for this behavior is economic. In an increasingly global and competitive market economy, one can only understand that the prospect of a reasonable economic return in front of the high developments costs of a successful product (some 300 million dollars) is very low. One reason might be that the vast consumer´s majority would be people from third world countries who, barring some exceptions, will not be able to afford pharmaceutical products at rather steep prices.

This appalling situation has prompted scientists around the globe to attempt to find alternate and cheaper strategies towards drug design. In this respect, two different main approaches have been envisioned, namely *rational drug design* and *combinatorial chemistry based drug design*. The latter consists in the development of therapeutic strategies taking advantage of the chemical combination of existing drugs to produce improved variants in terms of

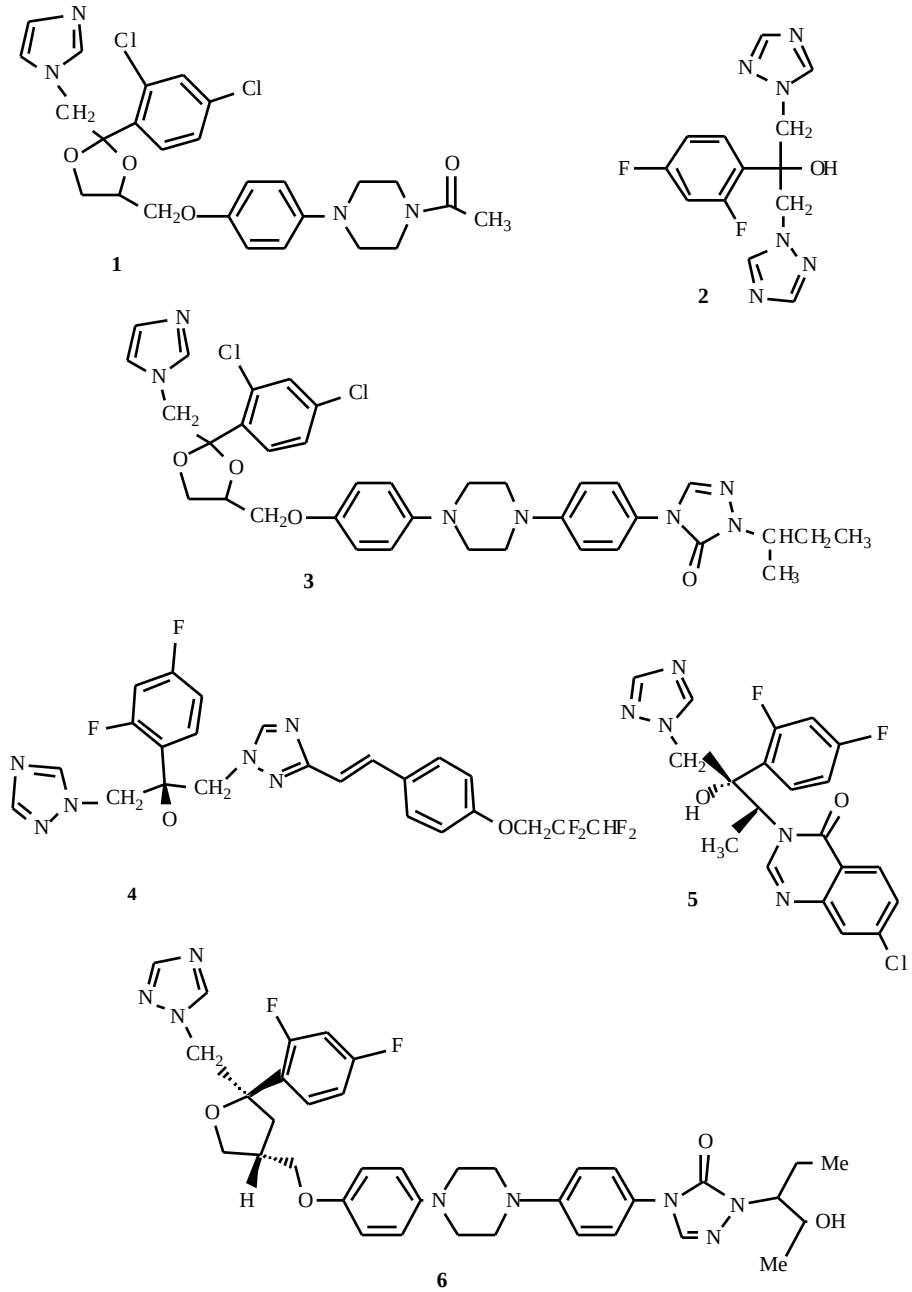

Fig. (1). Azole compounds tested as inhibitors of ergosterol synthesis in *T. cruzi* [14-21]. 1: ketoconazole; 2: fluconazole; 3: itraconazole; 4: posaconazole (SCH 56592); 5: D0870; 6: UR-9825.

chemotherapeutic action. On the other hand, the notion underlying the first approach is that one can design and produce a particular drug based on the structure (and other traits) of its molecular target, i.e. protein, nucleic acid or other types of receptors. The two different approaches are not necessarily applied as alternatives since rational design has been recently enhanced through the use of combinatorial chemistry and HTS. Recently, virtual screening or in silico screening, emerges as a new alternative attracting increasing levels of interest in the pharmaceutical industry as a productive and cost-effective technology in the search for novel leader compounds [10].

Strictly speaking, it is not yet possible to rationally design a new drug [11]. Instead, the correct term to use would be *rational inhibitor design* since this is what generally is achieved. The success of converting an inhibitor into a drug depends on a number of biological factors, which can be classified under the pharmacology or toxicology disciplines, e.g. uptake, metabolic inactivation, excretion, tissue distribution, etc.

The present paper is organized as follows: first, a short description of the most important targets identified in the metabolism of *T. cruzi* is provided. Thereafter, the metabolism and functions of trypanothione, with special reference to trypanothione reductase, are presented. The final section refers to some of the most important *T. cruzi* trypanothione reductase inhibitors known.

TARGETS OF *T. CRUZI* METABOLISM

Trypanosomatids exhibit a fair number of metabolic pathways different or not seen in other eukaryotic microorganism and higher organisms. It has been hypothesized that one reasonable explanation for this is the independent evolution, for around 300 million years, of the *Kinetoplastida*, one of the oldest lineages of protozoa [12].

Over the last couple of decades, new knowledge collected from the study of the biochemistry of *T. cruzi* has permitted the identification of a series of targets for *Chagas´* disease chemotherapy. In this section, the most relevant ones are examined; references to available inhibitors are signaled.

Sterol Synthesis

Unlike human hosts, the main sterol in *T. cruzi* metabolism is not cholesterol but ergosterol, a chemical compound also found in fungi. This recent finding triggered an intensive investigation on the identification and potential effect of inhibitors of ergosterol biosynthesis in the survival of the parasite [13,14], some of them previously used as antifungal (see structures in Fig. (**1**)). Compounds such as itraconazole and fluconazole markedly reduced or prevented chronic phase symptoms although could not vanish positive serology [15-18]. The recently discontinued compound D0870, D(+) isomer of fluconazole, displayed a striking inhibitory activity *in vivo*, both in acute and chronic models, leading to unprecedented percentages of parasitological cure [19]. The triazole posaconazole, also known as SCH 56592, inhibited epimastigote proliferation and ergosterol biosynthesis to a higher extent than the classic ketoconazole and led to high cure rates, both in the acute and chronic phase, in animals infected with strains resistant to nifurtimox, benznidazole and ketoconazole [20,21]. Another triazole derivative, UR-9825 was very active against epimastigotes and amastigotes [14]. All these are promising candidates for clinical trials in patients with Chagas' disease. However, resistance induction of *T. cruzi* to azoles, i.e. fluconazole, and the cross-resistance between ketoconazole and miconazole suggest difficulties in the use of these compounds as chemotherapeutic agents.

In other context, it was reported [22] the development of specific non-azole inhibitors for the enzyme oxidosqualene cyclase (c.f. Fig. (**2**)), a key component in sterol

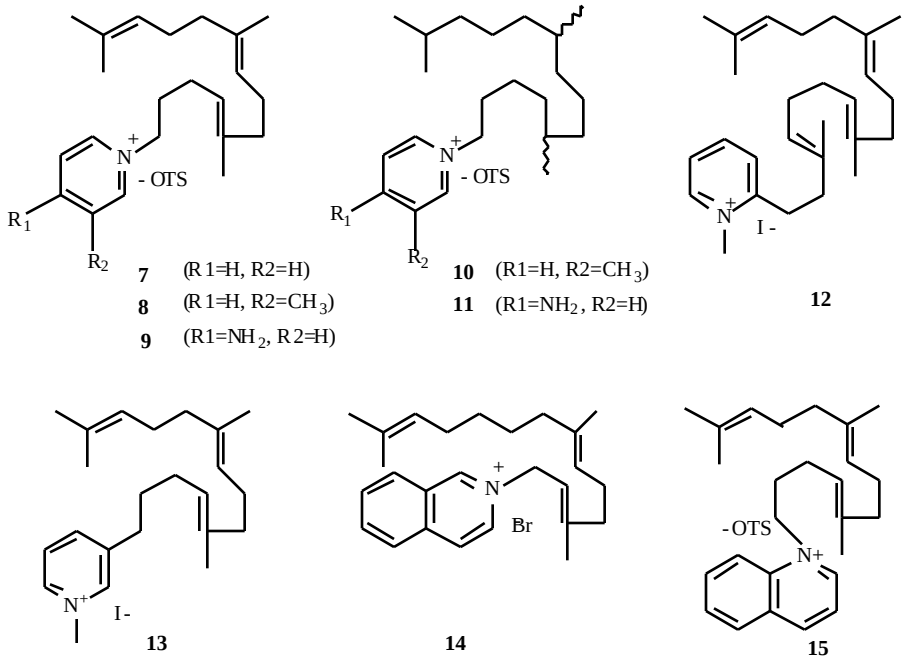

Fig. (2). Inhibitors of oxidosqualene cyclase reported in [22]. Compounds with *T. cruzi* $EC_{50} \leq 20$ nM are shown.

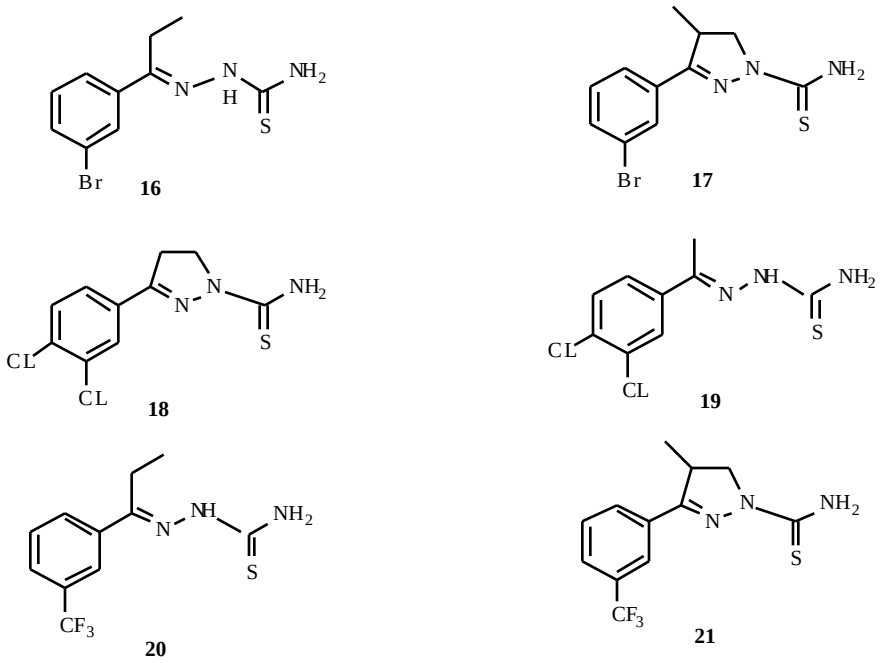

Fig. (3). Thiosemicarbazone inhibitors of *T. cruzi* cruzain reported in [26]. Inhibitors with trypanocidal properties and displaying TR $IC_{50} \leq 100$ nM are shown.

biosynthesis. They were tested and proven highly active (in the nM range) against trypomastigotes.

Cruzipain

Cruzipain (also known as cruzain) is a member of the papain C1 family of cystein proteinases (CPs). The catalytic moiety from *T. cruzi* has high homology to cathepsins S and L, and is absent in all other C1 families described so far [23]. Irreversible inhibitors of cruzipain, such as several peptidyl diazomethylketones, peptidyl fluoromethylketones and peptidyl vinyl sulphones interfered *in vitro* with the *T. cruzi* intracellular cycle, killing the parasite [24]. Vinyl sulphones, in particular, inactivate the parasite by inducing unprocessed cruzipain, and interfering with the secretory pathway [25]. More recently, non-peptidic inhibitors based on the thio-semicarbazone lead (see Fig. (**3**)) were reported as active at the nanomolar range; their small size and low cost make them attractive candidates for drug development [26]. A limitation of CPs as targets for chemotherapy research is the emergence of strains with resistance to inhibitors [27].

Hypoxanthine-Guanine Phosphoribosyltransferase

It is well known that trypanosomatid parasites must rely upon retrieving exogenous purines for nucleotide synthesis. In mammals, these nucleotides are synthesized both *de novo* and salvaged from recycled purine bases. *T. cruzi* converts purine bases to ribonucleotides using the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT). This enzyme is also responsible for the initiation of the metabolism of cytotoxic purine base analogs such as allopurinol. This means that either inhibitors or substrates of HGPRT are potential good candidates for effective chemotherapeutic agents. The hgprt genes from *T. cruzi* and other pathogenic trypanosomatids have been cloned, sequenced and overexpressed in *Escherichia coli*. The recombinant proteins have all been purified and characterized [28]. Purine (3'-azido-3'-deoxyinosine, 3'-deoxyadenosine and allopurinol) and pyrimidine (3'-azido-3'-deoxythymidine) analogs inhibited the replication of amastigotes in culture cell lines [29]. In addition, some purine analogs (see Fig. (**4**)) displayed affinity towards HGPRT from *T. cruzi* [30].

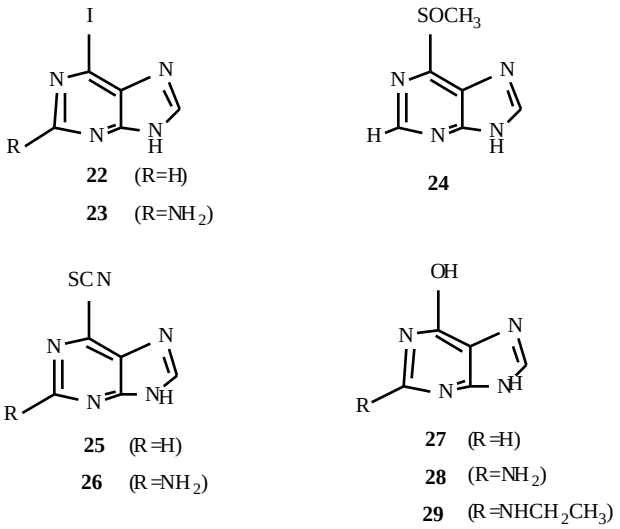

Fig. (4). Purine analogues with inhibition activity against *T. cruzi* HGPRT reported in [30].

DNA Topoisomerases

Class I and II topoisomerases are enzymes whose role is the modification of DNA topology. In particular, topoisomerases II, in kinetoplastids, have been the focus of study from the molecular and cell biology standpoint.

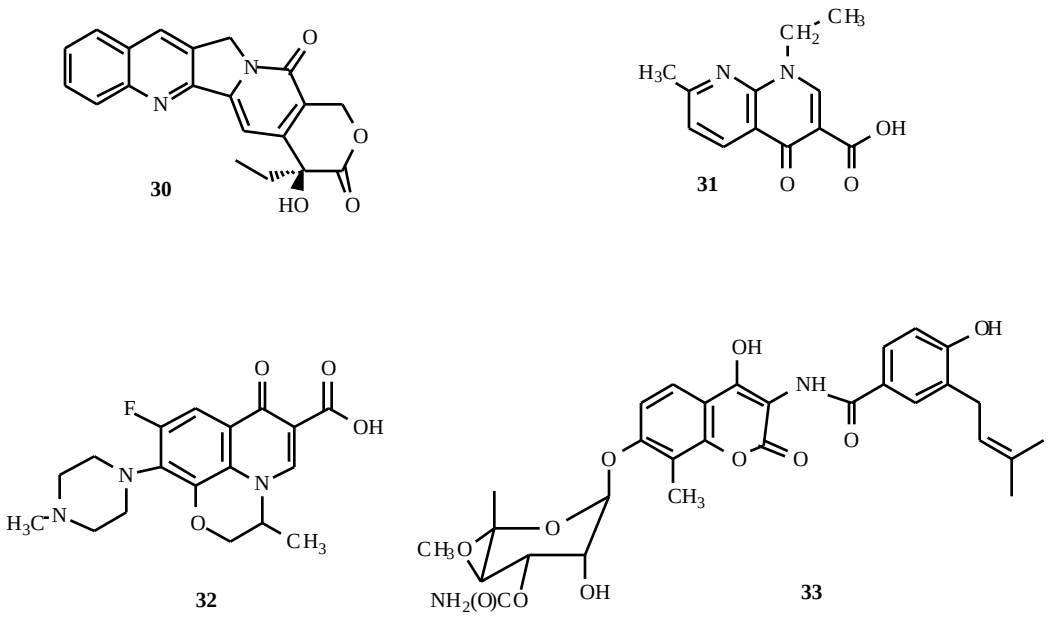

Fig. (5). Inhibitors of *T. cruzi* DNA topoisomerase I (30) and DNA topoisomerase II (31-33) studied in [32] and [33], respectively.

Among trypanosomatids, the gene encoding topoisomerase II is highly conserved. The enzyme is expressed in epimastigotes but not in trypomastigotes; the encoding mRNA is present in both forms. Several inhibitors of bacterial topoisomerase II (compounds **31-33** in Fig. (**5**)) presented activity against *T. cruzi*, inhibiting both proliferation and differentiation processes, disrupting the kinetoplastid and nucleus [31,32]. Similarly, an inhibitor of topoisomerase I (compound **30** in Fig. (**5**)) disrupted the nuclear and mitochondrial DNA in *T. cruzi* [33].

Dihydrofolate Reductase and Pteridine Reductase

Dihydrofolate reductase (DHFR) and thymidylate synthetase, two widespread enzymes involved in nucleotide synthesis, constitute a bifunctional protein in different species of protozoa. The *T. cruzi* gene coding for the DHFR domain was cloned and expressed [34]. Available inhibitors include several derivatives of methotrexate (drug inhibitor of the human enzyme), some of them with a greater selectivity for the parasite enzyme [35] Pteridine reductase (PTR1) is an enzyme, only found in trypanosomatids and plant pathogens,

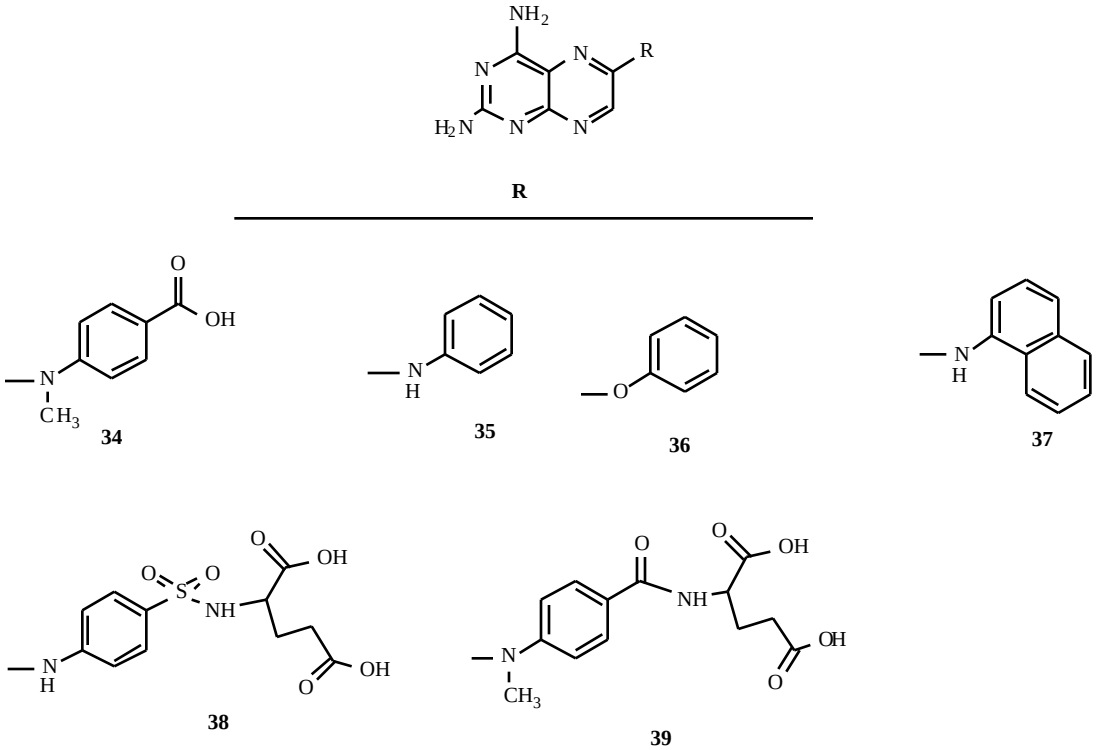

Fig. (6). Pteridine analogues with inhibition activity against *T. cruzi* pteridine reductase II, studied by theoretical docking in [41].

primary related to salvage and reduction of unconjugated pterins [36], but that also catalyzes the reduction of folate to dihydrofolate and tetrahydrofolate. Since the enzyme is 200 times less sensitive to methotrexate, it compromises the effectiveness of antifolate drugs targeting DHFR [37,38]. In addition, a second pteridine reductase (PTR2) was identified and expressed in *T. cruzi* [39,40], which can only reduce dihydropterin and dihydrofolate substrates but not pterin and folate. A docking study was recently performed on a set of pteridine analogues (shown in Fig. (**6**)) at the active site of PTR2 and binding energies, better than that of methotrexate, were obtained for the assayed compounds [41].

Glyceraldehyde-3-Phosphate Dehydrogenase

The inhibition of glycolitic related enzymes is a somewhat novel approach for the development of anti *T. cruzi* drugs. The rationale is that intracellular amastigotes are thought to obtain its energy entirely from glycolysis [42]. In particular, one attractive target is the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Structure comparison with the mammalian counterpart led to considering development of specific inhibitors for the parasite enzyme [43]. In subsequent studies, it was found that highly oxygenated natural flavonoids from *Neoraputia magnifica* [44] and adenosine derivatives [45] showed significant activity against *T. cruzi* GAPDH. Refer to Fig. (**7**) for structural details.

Farnesylpyrophosphate Synthase

The synthesis of a variety of sterols and polyisoprenoids in pathogenic protozoa involves the enzyme farnesylpyrophosphate synthase (FPPS). This enzyme

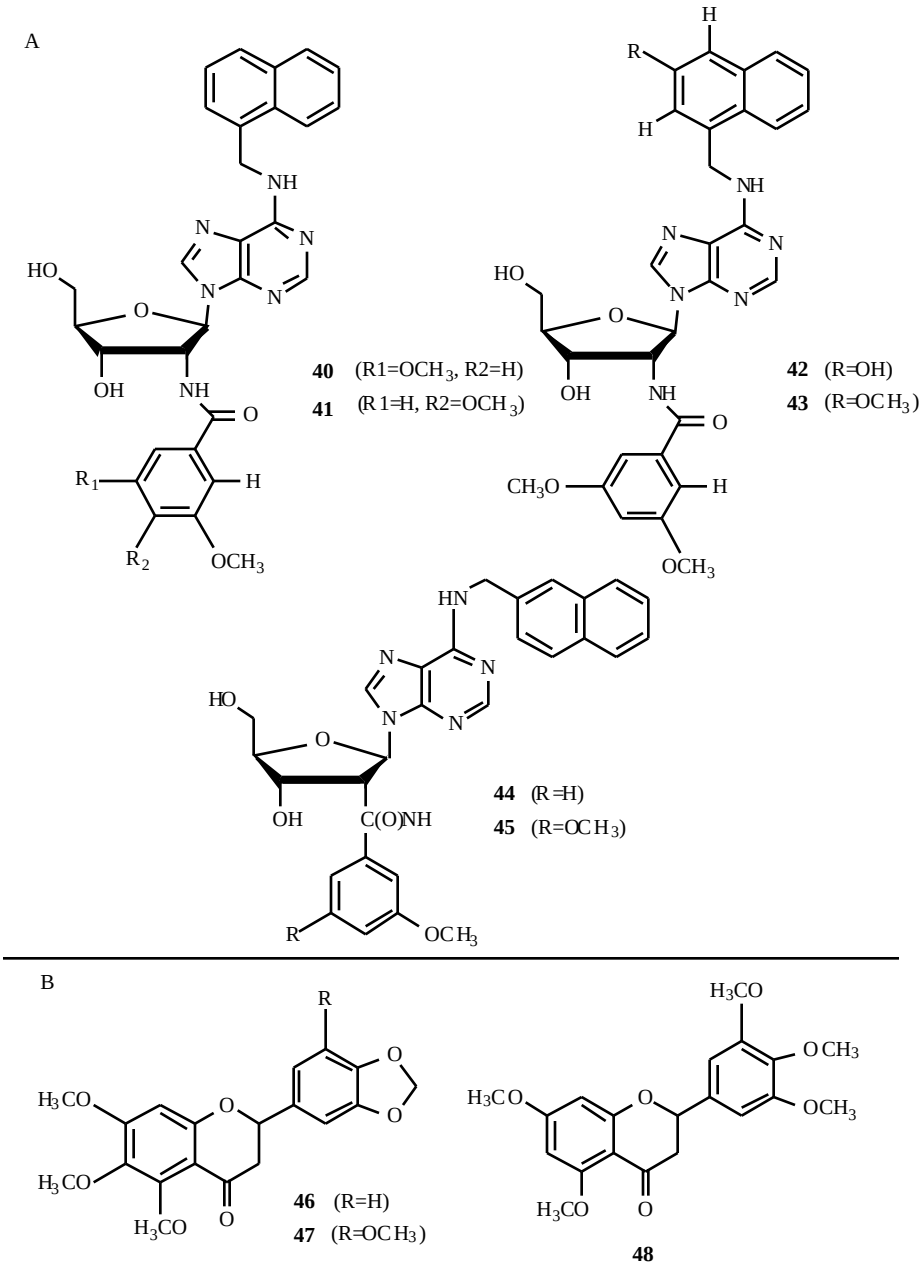

Fig. (7). Inhibitors of *T. cruzi* GAPDH. A: flavones assayed in [44]; B: adenosine analogues studied in [45] with $IC_{50} \leq 10\mu M$.

catalyzes the production of farnesylpyrophosphate and marks the branching point of the two synthetic pathways. The farnesylpyrophosphate synthase gene from *T. cruzi* was cloned and sequenced. The derived enzyme was inhibited by nitrogen containing biphosphonates [46], shown in Fig. (**8**). In particular, pamidronate caused a decrease of parasitemia in infected mice, also inhibiting the *in vitro* intracellular replication of amastigotes [47]. These compounds display a selective inhibition, a fact that has been ascribed to the drug accumulation in the so called acidocalcisomes in *T. cruzi*, which are acidic organelles rich in calcium, magnesium, sodium, zinc, etc. [48].

Arginine Quinase

Vertebrates, including human, use creatine kinase for the storage of ATP in the form of phosphocreatine, a phosphagen that is able to maintain ATP homeostasis during muscle contraction. It was recently reported [49,50] that trypanosomatid parasites, *T. cruzi* and *T. brucei*, possess an alternative pathway, which uses arginine kinase as the catalyst for arginine phosphorilation to produce the analogous phosphagen, phosphoarginine. This pathway is also widespread through the invertebrate phylum, including a great variety of phosphagens other than arginine, but not creatine.

Creatine quinase and arginine quinase are homologous proteins belonging to a family of conserved proteins with phosphotransferase activity, namely guanidino kinases. There is an evident relationship between the activity of guanidino kinases and the energy requirements within the cell. In *T. cruzi*, in particular, it has been suggested that the action of arginine quinase acquires primary importance during the invertebrate phase of the life cycle where, due to variations in insect feeding status, the nutrient supply is not as constant as in the human host [51,52]. Thus, phosphoarginine is a rapid source of energy either during bursts of cellular activity or under starvation stress conditions.

The finding of an alternative phosphagen and its biosynthetic pathway in parasites, points to arginine quinase as a possible chemotherapeutic target. Some experiments have been reported [53] showing that arginine quinase inhibition resulted in parasite growth inhibition in culture. Canavanine turned out to be a potent inhibitor of the enzyme. Additional research is needed to determine if this promising target is indeed crucial for parasite survival.

Polyamine Synthesis

Polyamines are essential requirements for cell growth and differentiation, which explains why polyamine metabolism has drawn considerable attention as a chemotherapeutic target in parasite infections. Polyamines serve numerous functions inside the cell; e.g. promoting chromatin condensation, stabilizing tRNA's structure and conformational transitions in DNA, modulating neuro-transmission and helping post-translational modification of proteins [54]. In addition, in trypanosomatids (*T. cruzi* included), the polyamine spermidine is found within the structure of trypanothione, a metabolite that makes a central contribution to the maintenance of an intracellular reducing environment (see below).

In contrast to other trypanosomatid organisms, *T. cruzi* is not affected by difluormethylornithine (DFMO), a rationally designed drug that inhibits the enzyme ornithine decarboxylase (ODC), a key component of the polyamine biosynthesis pathway. This is not surprising since ODC has not been detected in any stage of *T. cruzi´*s life cycle. On the other hand, *T. cruzi* was found to be susceptible to difluoromethylarginine (DFMA), a compound related to DMFO. DFMA is supposed to inhibit arginine decarboxylase (ADC) but ADC activity in *T. cruzi* was only found in the trypomastigote form albeit at almost negligible levels [55].

Further investigations are required to both determining the essential functions of polyamines for cell survival and elucidating the synthetic pathway in *T. cruzi*.

Enzymes of the Trypanothione Metabolism

Dithiol trypanothione (N_1,N_8-bis(glutathionyl)-spermidine, $T(SH)_2$), henceforth referred to as trypanothione,

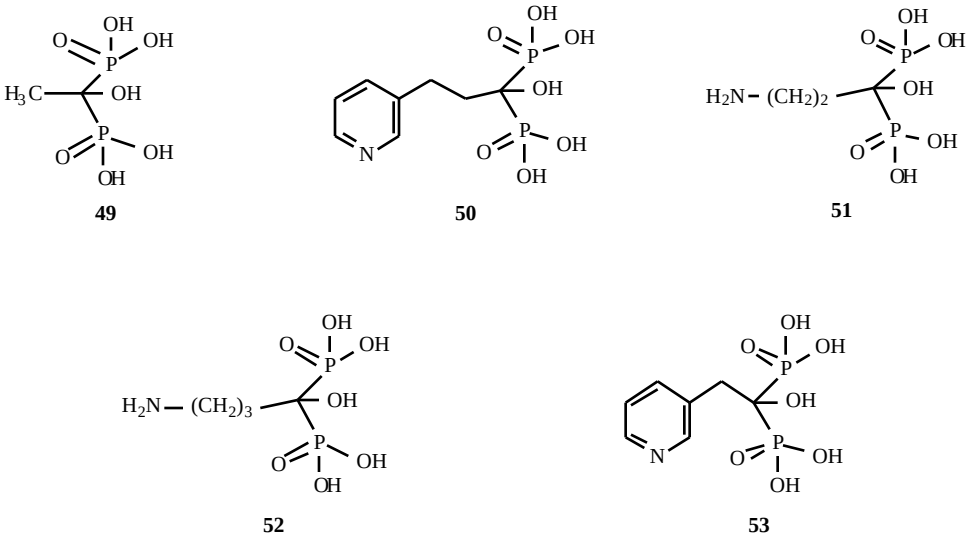

Fig. (8). Biphosphonates with inhibition activity against *T. cruzi* FPPS reported in [46].

is a low molecular weight thiol exclusively found in parasitic protozoa of the order Kinetoplastida. The enzymes of *T cruzi´*s thiol metabolism constitute very attractive target molecules due to the absence of trypanothione from the mammalian host and trypanosomatids' sensitivity against oxidative stress.

In the next sections, the trypanothione metabolism of trypanosomatids is presented, with emphasis on the trypanothione reductase system.

TRYPANOTHIONE

Metabolism

Dithiol tripeptide glutathione (GSH) and the polyamine spermidine are precursors for the biosynthesis of trypanothione. The first step in the synthesis of glutathione (consequently trypanothione), is the ATP-dependent ligation of L-Cys and L-Glu to yield γ-glutamylcysteine, a reaction catalyzed by γ-glutamylcysteine synthetase (GCS) [56,57]. This is the rate limiting step for the whole process [58]. The second enzyme in the synthetic pathway, glutathione synthetase, catalyzes the ATP-dependent ligation of L-Gly and γ-glutamylcysteine. In contrast with GCS, this enzyme has not yet been characterized in any trypanosomatid parasite though the genome sequencing project suggests a location for the glutathione synthetase gene in *Leishmania major* [59].

The other precursor of trypanothione, namely spermidine is not supplied by a single mechanism among the different trypanosomatid species. While *T. cruzi* takes up polyamines, which are converted into spermidine, from the surrounding medium (salvage), in African trypanosomes and *Leishmania*, spermidine is synthesized in a completely endogenous way starting from ornithine. Ornithine, by means of ODC (see above), is decarboxylated to putrescine, which in turn, is converted into spermidine by a reaction catalyzed by spermidine synthase (SPDSYN). At first, it was thought that a single enzyme was responsible for the synthesis of trypanothione from glutathione and spermidine [60]. In fact, trypanothione synthesis involves two different enzymes; glutathionylspermidine synthetase (GspS) that yields N_1 and N_8-glutathionylspermidine from glutathione and spermidine, and trypanothione synthetase (TS), which catalyzes addition of a second glutathione molecule [61,62].

Functions

Trypanothione, much like its vertebrate homologous counterpart, glutathione, participates in numerous cellular processes. The most important ones are briefly described next. For a thorough review the reader is referred to [59,63].

Ascorbate Homeostasis

$T(SH)_2$ is likely to be the main responsible for the regeneration of ascorbate in *T. cruzi* [64], indicating its close relationship with the homeostasis of ascorbate in the parasite. In addition to trypanothione spontaneous reduction, it has been suggested that there exists a vitamin C redox cycle based on the presence of ascorbate and dehydroascorbate reductase within the parasite cell [65]. In this sense, the so called p52 protein with $T(SH)_2$-GSSG (glutathione disulfide) thioltransferase activity has been described to reduce dehydroascorbate in a glutathione dependent fashion [66]. Yet, the very high K_M values for both substrates (dehydroascorbate and glutathione disulfide) could not account for the observed rate of dehydroascorbate metabolism. Additional research is necessary to get a deeper insight into the pathways that link trypanothione with ascorbate metabolism.

Scavenging of Metals and Drugs

It is well known that trypanosomes display resistance against arsenical and antimonial drugs. Melarsoprol (the only drug available against the late stage East African sleeping sickness caused by *Trypanosoma brucei rhodesiense*) or the organic pentavalent antimonials used against *Leishmania* are examples [67,68]. There are several mechanisms by which these parasites develop this type of resistance, namely loss of drug uptake, failure to reduce pentavalent antimonial drugs to the toxic trivalent form, and extrusion and sequestration of drug-thiol conjugates. Trypanothione is thought to be involved in the latter mechanism.

Leishmania cells resistant to trivalent arsenite or antimony display an amplification of the *pgpa* gene [69,70], which codes for an ATP-binding cassette (ABC) transporter, PGPA, homolog to MRP-1 (Multidrug Resistance-Associated Protein 1) [71]. PGPA transports arsenite conjugated to glutathione in leishmania [72]. Specifically, in *L. tarantolae*, trypanothione forms adducts with arsenite and antimonite [73] suggesting its involvement in conjugate transport by PGPA. There is an additional metal-thiol transporter system identified as an ATP dependent As(III)-glutathione pump, which transports As(III) and Sb(III) in complex with trypanothione as well as $As(GS)_3$. This pump is also thought to contribute to metal resistance by extrusion of As(III)- or Sb(III)-trypanothione conjugates outside the cell [73].

It was previously shown an increase in the levels of trypanothione concomitantly to the amplification of the PGPA gene in resistant *Leishmania* species [74,75]. This is caused by the amplification of the genes encoding for the heavy subunit of γ-glutamylcysteine synthetase, key enzyme in the synthesis of glutathione, and the overexpression of ODC, key enzyme in spermidine generation (see above).

In *T. brucei*, overexpression of MRP-A (Multidrug Resistance-Associated Protein A), closely related to PGPA of *L. tarentolae*, enhanced resistance to melarsopol [76]. In contrast, less than half of resistance was achieved when trypanothione biosynthetic enzymes (ODC, GCS) were overexpressed. Although there is no conclusive evidence to assert that *T. brucei* MRP-A does indeed transport arsenite-thiol conjugates, all the accumulative evidence suggests so [72].

Synthesis of Deoxyribonucleotides

The reduction of ribonucleotides to the respective deoxyribonucleotides by means of ribonucleotide reductase is an essential reaction for *de novo* synthesis of DNA precursors [77]. The enzyme requires external electron donors to function, which are commonly supplied by small dithiol proteins such as thioredoxin and glutaredoxin [78], as seen in Fig. (**9**), D and C, respectively. In turn, oxidized thioredoxin is reduced by thioredoxin reductase taking redox

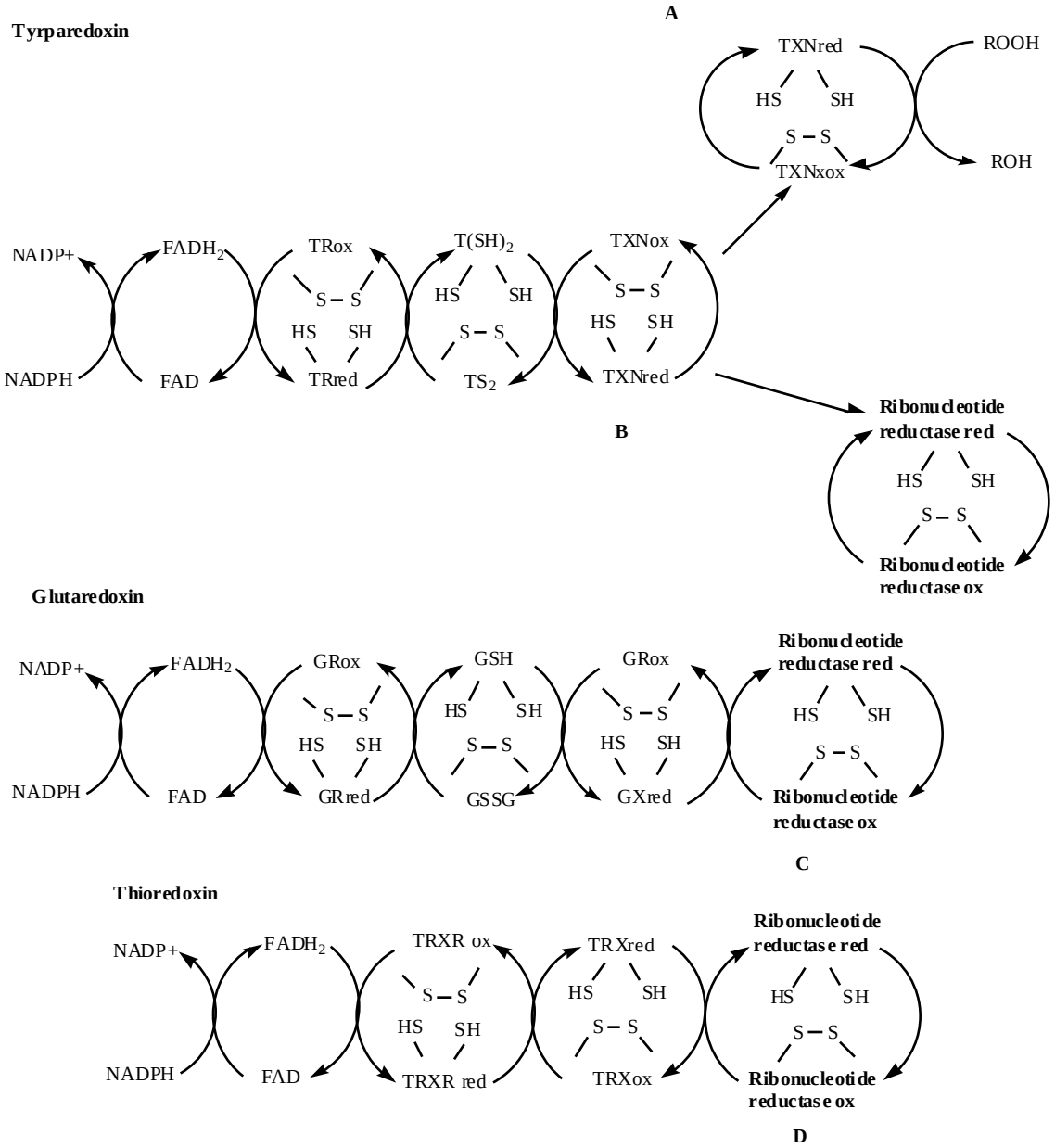

Fig. (9). Metabolic functions of tryparedoxin, glutaredoxin and thioredoxin related to deoxyribonucleotide synthesis and hydroperoxide neutralization. *TR:* trypanothione reductase; *$T(S)_2$:* trypanothione disulfide; *$T(SH)_2$:* trypanothione; *TXN:* tryparedoxin; *TXNPx:* tryparedoxin peroxidase; *GR:* glutathione reductase; *GSSG:* glutathione disulfide; *GSH:* glutathione; *GRX:* glutaredoxin; *TRXR:* thioredoxin reductase; *TRX:* thioredoxin; *ROOH:* hydroperoxide; ox: oxidized enzyme; red: reduced enzyme.

equivalents from NADPH [79]. Glutaredoxin is regenerated by glutathione in a non-catalytic reaction. The cycle is closed by the reduction of glutathione disulfide by glutathione reductase [80].

Unlike glutathione, trypanothione acts as a direct donor for ribonucleotide reductase (*T. brucei*) [81,82]. This finding makes trypanothione unique in that it is the first case of a non-protein dithiol working as a spontaneous reductant of ribonucleotide reductase. At lower trypanothione concentrations than the enzyme's K_M, however, the reaction is not spontaneous anymore but catalyzed by the enzyme tryparedoxin. This time, the electrons flow first from trypanothione reductase to trypanothione and tryparedoxin and then to the final acceptor, ribonucleotide reductase (c.f. Fig. (**9**), path B). Given that tryparedoxin concentration within the cell is very large (5% of the total soluble protein), the trypanothione/tryparedoxin couple should maintain ribonucleotide reductase in the reduced state. In a similar fashion to the action exerted by glutathione to glutaredoxin, trypanothione is a very powerful inhibitor of tryparedoxin [81]; these effects suggest that DNA synthesis is somewhat related to the cell redox state.

As mentioned above, thioredoxin is a small dithiol protein which reduces class I ribonucleotide reductases. In *T. brucei*, the enzyme reduces ribonucleotide reductase in presence of human thioredoxin reductase [83]. However, in contrast to tryparedoxin, the cell concentration of thioredoxin is unusually low thus it is speculated that it may be playing a minor role as a physiological electron donor of the trypanosomatid ribonucleotide reductase.

In a recent study, a *T. cruzi* enzyme of sequence similar to human glutaredoxin has been expressed [84]. Several action mechanisms were considered, based on the fact that the protein structure seems to correspond to a typical glutaredoxin, in particular displaying the thioredoxin folding [85,86]. The different pathways between the mammalian and parasite metabolism (path B and C in Fig. (**9**)) were also taken into account. The existence of a glutaredoxin activity in *T. cruzi* renders the metabolic pathway even more complex than the one presented in Fig. (**9**); it appears that trypanosomatids would have as much as three different electron donors for the reaction catalyzed by ribonucleotide reductase. Furthermore, if the activity of glutaredoxin in the parasite is coupled to TR (instead of GR in the human host), it may well be a substitute for tryparedoxin in the redox cascade to neutralize hydroperoxides and other deleterious agents (see next section). Considering that the protein could be a target for the design of specific inhibitors with antiparasitic properties, its essentiality and specificity should be studied in detail.

Oxidative Stress and Reduction of Hydroperoxides

Trypanosomatids, and other organisms dwelling in aerobic environments, are exposed to reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). These intermediates are spawned from a number of sources such as cellular respiration, cofactors (reduced flavins, thiols, etc.), drug metabolism, and externally by the host's immune defense system [63]. The most reactive species is hydroxyl radical, thought to be generated from $O_2^{\cdot -}$ and H_2O_2 following the classical Haber-Weiss reaction:

$$O_2^{\cdot -} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \quad (1)$$

$$H_2O_2 + Fe^{2+} \longrightarrow OH^{\cdot} + OH^- + Fe^{3+} \quad (2)$$

The hydroxyl radical can cause lethal damage by reacting with various cellular components such as DNA and membrane lipids. Enzyme removal is not possible after the radical forms. Instead, the $OH^{\cdot}$ radical can be trapped non-enzymatically by low-molecular weight scavengers such as vitamins A, C, E (β-carotene, ascorbate and tocopherol, respectively) and thiols, notably trypanothione (in the parasite) and glutathione (in the mammalian host). This trapping constitutes the last resort the cell has at hand when the general strategy to minimize $OH^{\cdot}$ formation by enzymatic means fails.

In most organisms, the enzymatic defense against superoxide anion is achieved by means of superoxide dismutase (SOD) [87], which turns superoxide anion into hydrogen peroxide. Subsequently, hydrogen peroxide is transformed by catalase and various peroxidases (glutathione peroxidase, cytochrome c peroxidase, ascorbate peroxidase, etc.) [88,89].

Due to the lack of catalase and glutathione peroxidase [90,91], trypanosomes appear to have an impaired enzymatic defense against oxygen derivatives thus, they have long been deemed specially sensitive to oxidative stress [91]. In fact, for *T. cruzi*, the available drugs are redox cyclers giving rise to formation of ROS [92,93]. Yet, *T. cruzi* is able to tolerate rather low concentrations of hydrogen peroxide thanks to spontaneous reduction by trypanothione [94]. In addition, a unique trypanothione-dependent enzyme cascade neutralizes hydroperoxides, which was shown to comprise the enzymes trypanothione reductase, the previously mentioned tryparedoxin (c.f. synthesis of deoxyribonucleotides section) and tryparedoxin peroxidase [95]. In the overall redox reaction, electrons formally flow from NADPH onto tryparedoxin peroxidase *via* trypanothione and tryparedoxin (c. f. Fig. (**9**), path A).

Tryparedoxin peroxidase has an activity similar to human glutathione peroxidase although it functions at a much slower rate [96]. This is compensated by the high intracellular concentration (ca. 6 % of the total soluble protein in *C. fasciculata*). The parasite's tryparedoxin peroxidase is a member of the widespread family of peroxiredoxins [97], and a probable alternate defense against oxidative stress to the glutathione peroxidase system given it was found both in trypanosomes and leishmanias [98-101]. In addition to hydrogen peroxide, it can reduce t-butyl hydroperoxide, phospahtidylcholine hydroperoxide and linoleic acid hydroperoxide.

A second peroxidase system relying on the trypanothione/tryparedoxin couple has been recently discovered in *T. cruzi* and *T. brucei;* this is structurally familiar to typical glutathione peroxidases except for the presence of cysteine instead of selenocysteine [102,103]. The enzyme does not appear to function with glutathione as electron donor but also accepts thioredoxin [103].

Despite the oxidative stress defenses in trypanosomes seem to be somewhat more robust than originally thought, the fact still remains that these parasites are susceptible to the effect of free radicals, the action of which being dependent on the balance between the formation of ROS and the anti-oxidant mechanisms in the parasite and the host.

The Trypanothione Reductase System of Trypanosomatids

All trypanosomatids have a unique thiol metabolism where the ubiquitous glutathione reductase (GR) is replaced by a trypanothione reductase (TR). So far, the only organism where both glutathione reductase and trypanothione reductase coexist is *Euglena gracilis* [104]. TR, though being a relative newly discovered enzyme, represents one of the most widely studied aspects of the trypanosomes metabolism. It belongs to the well known family of NADPH dependent FAD-disulfide oxidoreductases, which also includes glutathione reductase, lipoamide dehydrogenase and thioredoxin reductase [105]. The enzyme has been purified, characterized and crystallographic data from *C. fasciculata* and *T. cruzi*, both in free form and in complex with substrates and inhibitors, are available [106-112].

The catalytic function of TR is the reduction of its cognate substrate trypanothione disulfide ($T(S)_2$) to the above mentioned dithiol form, $T(SH)_2$. Trypanothione exerts all its physiological functions in the reduced state hence the relevance of the TR catalyzed reaction. TR is active as a homodimer, subunit mass of about 52 KD. In the active site of each monomer, the cofactor FAD is found together with a dithiol/disulfide bridge essential for catalysis. The catalytic site is rather complex, comprising two regions (subsites), namely the NADPH site (N-site) and the substrate site (G-site), which are separated in space. During the chemical interconversion, reducing equivalents flow from NADPH to the disulfide substrate (e.g.; trypanothione) with

FAD and the disulfide bridge as intermediates. Two proton relays, one at each site, modulate the transfer [113,114].

As stated before, TR is the homolog counterpart of GR from the mammalian host. A comparison with the crystallographic available structures of GR [115,116] reveals that the overall structure of the two enzymes is highly conserved. There is an overall 40% sequence similarity and the structural catalytic features are alike. TR may also replace thioredoxin reductase, although trypanosomatids do possess a conventional thioredoxin [83].

From its discovering and due to the fact that TR was not present in the mammalian host, it was evident that it constituted an attractive target for knowledge based drug design against trypanosomatids. The key feature about TR is that it displays mutually exclusive specificity in relation to GR [64,117,118]; each enzyme is specific for its cognate substrate thus, in principle, it would be possible to selectively inhibit the parasite enzyme without affecting the mammalian one.

TR accepts trypanothione and glutathionylspermidine, as physiological substrates. They carry a net charge of +1 and +2, respectively, whereas glutathione disulfide (GSSG), GR´s substrate, possesses a net charge of -2. This behavior is explained by the different nature of the binding sites in the two enzymes; in particular, there is an exchange of five residues: Ala34, Arg37, Ile113, Asn117 and Arg347 in human GR are substituted by Glu18, Trp21, Ser109, Met113 and Ala343, respectively, in TR from *T. cruzi* (residue number as in corresponding crystal structures) [112,116]. These replacements render the active site of TR negatively charged and sort of hydrophobic, that is, more suited to host positively charged compounds while GR cavity, being a positively charged environment, makes the entrance of polar and negative charged species easier. Furthermore, the binding site of TR is wider than that of GR [119].

The most important prerequisite for any chemotherapeutic target is that it is essential for the survival of the organism to be fought. This is the case of TR as shown by numerous genetic studies performed in several species of leishmania and trypanosome where it was attempted to modulate the levels of the enzyme [120-124]. A conclusion from these studies is that residual levels of trypanothione reductase and *de novo* synthesis of trypanothione can maintain basal thiol concentration. Yet, these two factors are not enough to cope with oxidative stress scenarios (see below). This suggests that the key feature in trypanosomatids thiol metabolism is not the concentration of thiols but the regeneration of trypanothione by TR, which becomes rate limiting at low concentrations of the latter [59]. In the absence of trypanothione regeneration, the reductive power is impaired hence deleterious species derived from the oxidative metabolism are not properly inactivated.

INHIBITORS OF *T. CRUZI* TRYPANOTHIONE REDUCTASE

Over the last couple of decades, a great number of inhibitors of *T. cruzi* growth, either *in vitro* or *in vivo*, have been reported. Several previous reviews include a thorough discussion on the different aspects of inhibitors of specific enzymes acting as trypanocidals, in particular the possible use of drug leads [3,125-131]. For a comprehensive list of *in vitro* active molecules against *T. cruzi* see [132]. Surprisingly, a great proportion of the agents with activity against trypanosomatids are involved in the trypanothione metabolism.

Here we will focus specifically on some compounds that are inhibitors of *T. cruzi* TR, which we have studied from the biochemical and molecular modeling standpoint over the last few years.

Nitrofuranes

Nitroaromatic compounds have widespread actual or potential use in Medicine and cancer therapy. Their biological responses are controlled by their redox properties. Since the introduction of nifurtimox in the 1965-1975 period nitrofuran compounds have been widely studied as trypanocidal agents.

Nitrocompounds and nitrofuranes, in particular, are one class of drugs for which direct proof of radical production in intact target organisms has been demonstrated [133]. The production of nitroanion radicals is catalyzed by cellular nitroreductases [134]:

$$R\text{-}NO_2 + \text{“nitroreductases”} \longrightarrow R\text{-}NO_2^{\cdot -} \qquad (3)$$

The nitroanion radical can undergo a futile reaction in aerobic conditions where back-oxidation occurs:

$$R\text{-}NO_2\text{-} + O_2 \longrightarrow R\text{-}NO_2 + O_2^{\cdot -} \qquad (4)$$

Under hypoxia, however, the following disproportionation prevails:

$$2\ R\text{-}NO_2^{\cdot -} \longrightarrow R\text{-}NO_2 + R\text{-}NO_2^{2-} (= R\text{-}NO) \qquad (5)$$

Reaction (5) leads to the nitroso derivatives, thus commencing a reaction chain, which may involve the hydroxylamine and amine derivatives, potentially carcinogenetic species. Although the coupling of the first and second reaction would mean that little or no net nitroreduction takes place in aerobic conditions [133], there still may be a stimulation of respiration, a feature of significance for the production of oxidative stress.

Nitrofuranes were previously found to be “subversive” (turncoat/sabotage inhibitors) substrates of TR [135]. In this seminal work, a series of synthesized nitroderivatives were assayed as inhibitors of *C. fasciculata* TR. The term subversive denotes the fact that a substrate is reduced in a unique electron step by the flavoenzyme to yield the corresponding radical, which in turn, reacts with molecular oxygen to produce superoxide anion radicals. The normal reduction of trypanothione is being prevented thus the thiol/disulfide ratio inside the cell is lowered. Then, the very same function that normally would provide defense against free oxygen radicals is in fact enhancing the effect of oxidative stress. Since the reaction of the radical species with oxygen regenerates the subversive substrate, these compounds constitute catalysts for the oxidative stress. Note that the nitrofuran radicals can be generated in different manners. From reactions comprising (mainly microsomal) cell oxidoreductases (the nitroreductases previously mentioned)

such as NADPH cytochrome P450 reductase and lipoamide dehydrogenase (LipDH) as well as from the trypanothione reductase catalyzed reaction [93,136]. The strongest impact is attained with the reaction in TR since perhaps the most important cell defense mechanism against free radicals would be endangered.

The chemical reactions following the introduction of a nitrofuran derivative (e.g. nifurtimox) into epimastigotes are thought to be the following [137]:

$$NAD(P)H + H^+ + 2R\text{-}NO_2 \longrightarrow 2R\text{-}NO_2^{\cdot -} + NAD(P)^+ + 2H^+ \quad (6)$$

$$R\text{-}NO_2^{\cdot -} + O_2 \longrightarrow R\text{-}NO_2 + O_2^{\cdot -} \quad (7)$$

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (8)$$

$$O_2^{\cdot -} + H_2O_2 \longrightarrow O_2 + OH^{\cdot} + OH^- \quad (9)$$

Reaction (8) being catalyzed by SOD and reaction (9) is the abbreviated form of the Fe (or Cu) dependent Haber-Weiss reaction previously presented. Similar reactions have been observed in *C. fasciculata* [138].

Because nitrofuran compounds inhibit structurally related antioxidant enzymes other than TR, including the host GR [139-141], it is desirable to design nitrofuran inhibitors which selectively interact with the parasite flavoenzyme. Several research groups were devoted to that task and as a result, some promising nitrocompounds were reported. One of these is chinifur (compound **57**, Fig. (**10**)), a former bactericidal nitrofuran with an aminoalkyl side chain that acts as subversive substrate of TR but binds weakly to GR and to the related flavoenzyme LipDH [142]. The study of these derivatives also pointed to the importance of hydrophobic interactions in the design of selective inhibitors for TR.

In a comparative study, a series of nitrofuran drugs were surveyed for their ability to inhibit *T. cruzi* TR and LipDH and the human GR [143]. The compounds were moderate inhibitors of TR and GR and some of them, namely nifuroxime, nifuroxazide and nifurprazine (c.f. Fig. (**10**)) were no substrates for GR. Nifurprazine and nifuroxazide proved to be better inhibitors of *T. cruzi* in culture as compared to nifurtimox.

In another study, a series of nitrofurazones and nitrothienyl analogs, thought to interact with TR, were designed and synthesized in which the semicarbazide moiety consisted of different alkyl and aromatic chains aiming at mimicking the spermidine part of trypanothione [144]. These nitroderivatives were later shown to reduce to yield the free radical species [145]. In fact, by means of theoretical docking studies, the importance of the spermidine arm of trypanothione was evidenced as it proved to be that moiety contributing the most to the substrate binding to TR [119]. Some of the compounds side chains had also the ability to acquire a positive charge at physiologic pH (see Fig. (**11**) for examples). Theoretical docking procedures suggested that the charged derivatives had higher binding energies to TR as compared to the uncharged ones [Iribarne *et al*., unpublished results]. These derivatives were not found to be significantly better inhibitors of *T. cruzi in vitro* growth. Kinetic inhibition assays for these inhibitors are currently lacking in order to complete the picture and to assess the validity of the docking results.

A related work applied the CoMFA-SIMCA methodology to the previous series of nitrofurazones and nitrothiophenes to correlate the *in vitro* inhibition activity with the physicochemical properties of the molecules [146]. The derived models pointed to some geometric and chemical parameters that should be taken into account to get a potential *T. cruzi* growth inhibitor; in particular, there should not be a positive charged center in the region 15-16 Å from the nitro group. This result is not in line with the outcome of the above mentioned docking studies. Therefore, inhibition of TR may not be the main mechanism by which nitro-compounds exert their growth inhibition effect.

From the theoretical results, one can hypothesize that the lack of selectivity towards TR usually shown by (neutral) nitrofuran compounds may be due to the fact that the nitro group is able to establish contacts with the charged residues at GR binding site, namely Arg37 and Arg347. Alternatively, strong interactions would take place with the enzyme disulfide bridge both in GR and TR active sites. This is apparent in Fig. (**12**) where the output of a Molecular Dynamics trajectory is presented for a nitrofuran derivative (compound **67** in Fig. (**11**)) bound at TR's active site. As a result, the binding affinity for the GR would be augmented thus decreasing the selectivity for the parasite flavoenzyme. On the other hand, nitrocompounds carrying side chains capable of protonating at physiological pH (e.g. chinifur)

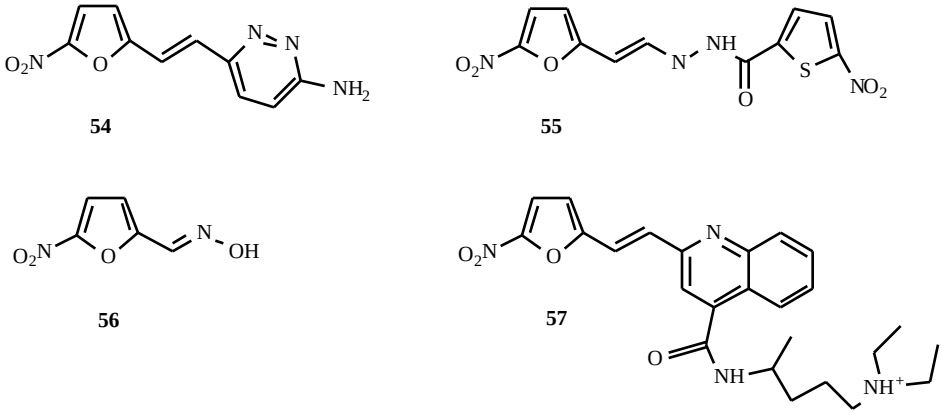

Fig. (10). Nitrofuran derivatives with selective inhibition activity against *T. cruzi* TR reported in [142,143]. 54: nifurprazide; 55: nifurzide; 56: nifuroxazide; 57: chinifur.

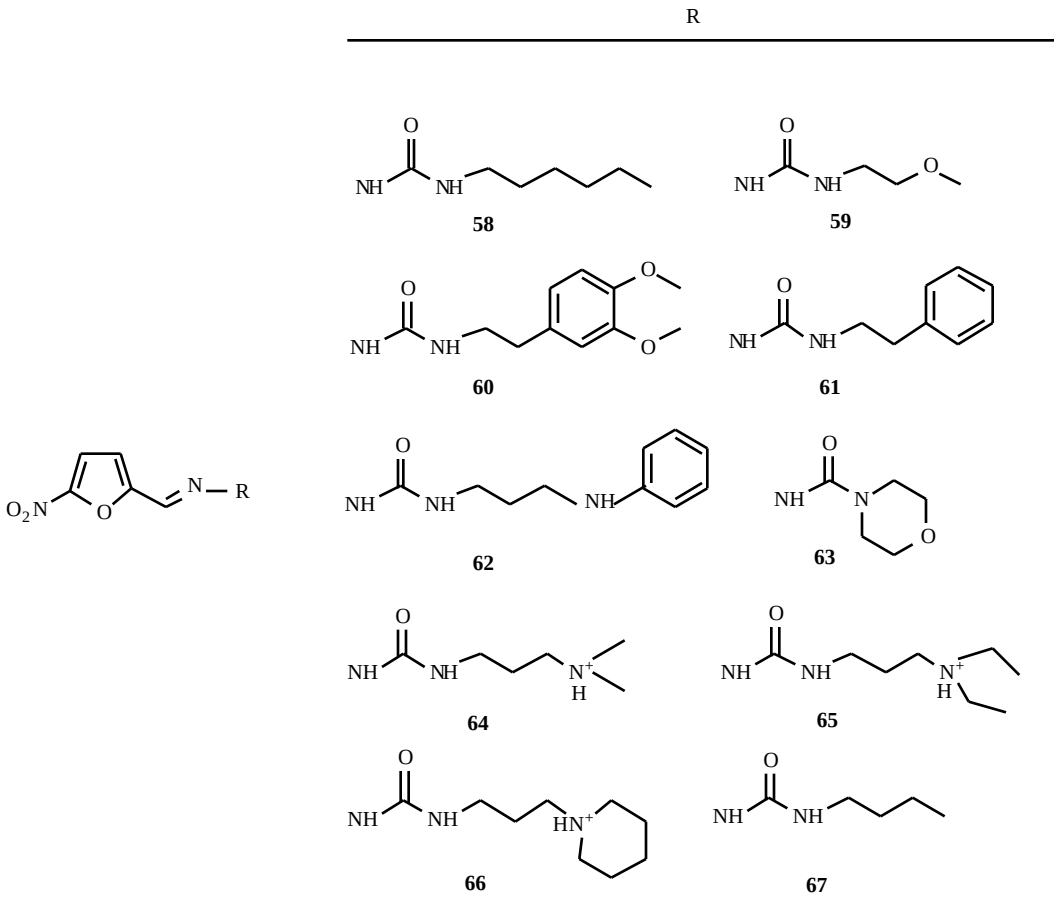

Fig. (11). Trypanocidal nitrofuran structures with spermidine-like subtituents reported in [144].

would benefit from the previously discussed differences in electrostatic features between TR and GR hence selectivity would increase. The role of charge in ligand selectivity towards TR is revisited in the next sections.

It then appears that the requirements to attain selective inhibition of TR - chiefly, a positive center in the structure of the nitro-derivatives - collide with the desirable traits the same compounds should have in order to get at their potential target. That is, a rather lipophilic (neutral) nature and not too bulky a size to be able to get across cell and organelle membranes.

Naphthoquinones

Naphthoquinones embody a group of substances present in all aerobic cells and with multiple applications in Medicine such as cytotoxicity and therapeutic utility against cancer [147]. They are also known as effective cytostatic, antivirals, antifungal and antibacterial agents. The capacity to undergo redox cycling makes naphthoquinones a group of very reactive molecules. *p*-Naphthoquinones have been thoroughly studied and their properties, activities and applications are well known. In contrast, much less is known about *o*-naphthoquinones.

Among *o*-naphthoquinones, β-lapachone (3,4-dihydro-2,2-dimethyl-2H-napthol[1,2b]pyran-5,6-dione), isolated from the lapacho tree (*Tabebuia avellanedae*) has proven to be a potent cytostatic agent in different human tumor cells, such as human and murine leukemia, melanoma, hepatoma, colon carcinoma, lymphoma and glioma, as well as epidermoid laryngeal, lung, prostate, ovarian and breast cancer [148]. Depending on target, time and drug dose, the cytostatic effect has been ascribed to apoptosis or necrosis. On these grounds, β-lapachone has been suggested for clinical use.

With respect to parasite infections, a fair number of naphthoquinone derivatives, both synthetic and from natural sources, have been assayed as trypanocidal agents. Among them, some *o*-naphthoquinones and *p*-naphthoquinones drugs including menadione, plumbagin, juglone, lapachol and the previously mentioned β-lapachone are unusually active [149-154].

Quinones, in general, accept electrons, either one or two depending on the nature of donor and the mechanism of the redox reaction. The enzymatic reduction *via* the transfer of a single electron yields the semiquinone radical. Numerous mammalian enzymes are able to catalyze this reaction, namely NADPH-cytochrome P450 reductase, NADH cytochrome b5 reductase, NADH-ubiquinone oxidoreductase and thioredoxin reductase. Provided that the semiquinone radical remains attached to the active center of the enzyme, it may be reduced to the hydroquinone species by means of an hydride coming from the surrounding environment (i.e. NAD(P)H). Two-electron transfer reactions are catalyzed by the flavoenzymes known as diaphorases such as the cytosolic enzyme from rat liver and the cytosol flavoenzyme DTD

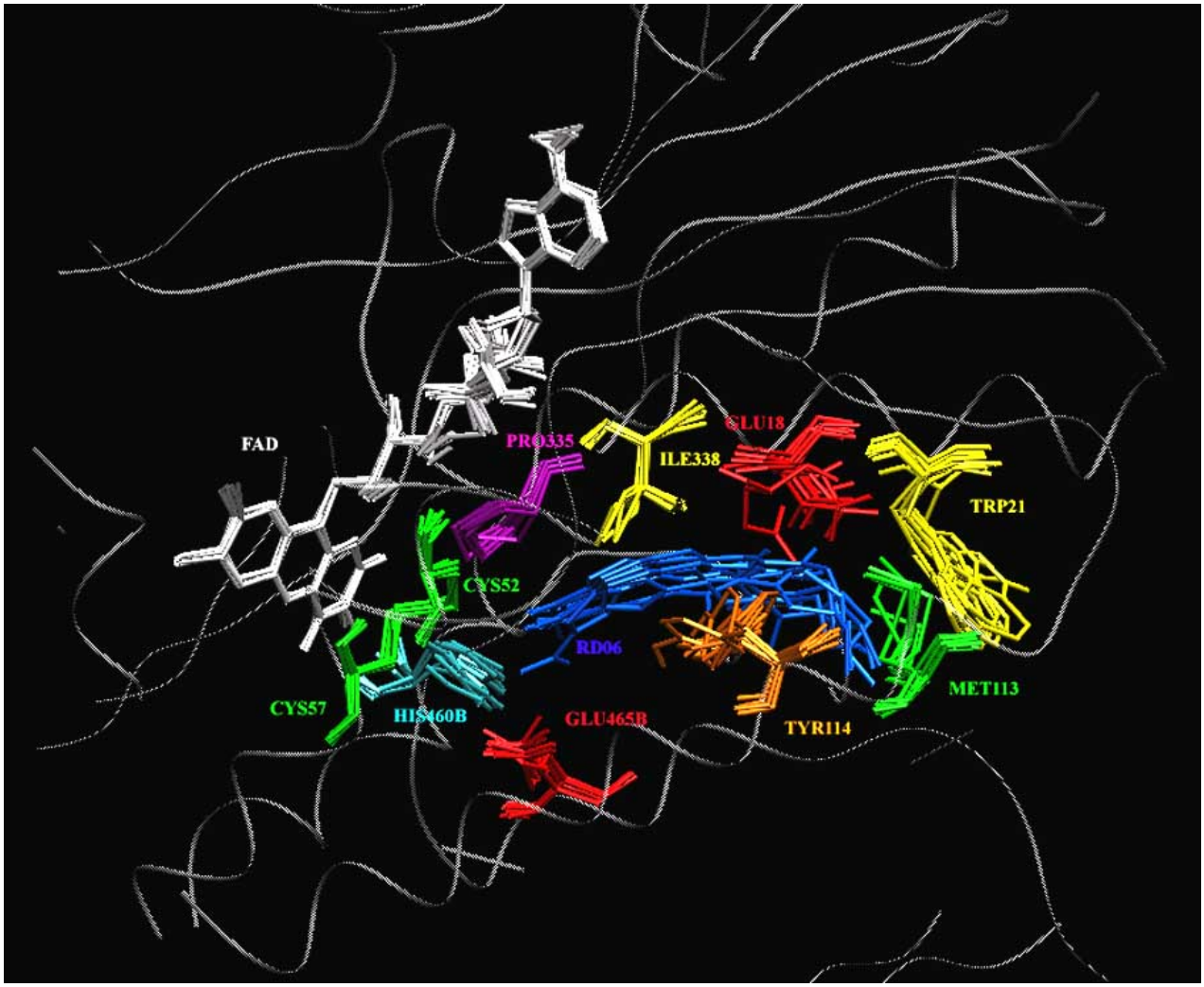

Fig. (12). Molecular dynamics simulation of a modeled structure of nitrofuran compound **67** in Fig. (**11**) at the active site of *T. cruzi* trypanothione reductase. The crystallographic coordinates [112] were used for the enzyme structure. The ligand and side chains of the relevant residues - numbering as in the crystal structure - at the active sites are represented as stick models; conformations were selected along 250 ps trajectory simulation. The protein backbone is shown in ribbons style. The distance between the nitro group and the enzyme disulfide bridge (SG from CYS52) is highlighted suggesting the possibility for the generation of the nitro-radical. Similar binding modes for nitrocompounds are found in GR.

(DT-diaphorase) [155]. Three types of hydroquinones have been proposed: (a) redox stable hydroquinones; (b) redox labile hydroquinones which subsequently reoxidize with formation of semiquinone and ROS and (c) redox-labile semiquinones that immediately rearrange to potent electrophiles undergoing biological alkylating reactions. Besides, semiquinone radicals may be generated through non-enzymatic reactions including quinone and quinol dismutation, hydroquinone oxidation by oxygen, quinone reduction by superoxide anion or radiolysis and reaction of the triplet-quinone with adequate reductants. All these reactions are analyzed in [156].

The production of oxygen radicals by the oxidation of semiquinones and hydroquinones, such as superoxide anion, precursor of hydrogen peroxide and the highly toxic hydroxyl radical, renders quinones very cytotoxic species. The following reactions, where R stands for "reductant" i.e. the dithiols trypanothione or glutathione, and Q stands for "quinone", illustrate the mechanism [157,158]:

$$R\text{-}H_2 + Q \longrightarrow R + QH_2 \quad (10)$$

$$QH_2 + O_2 \longrightarrow Q^{\cdot} + O_2^{\cdot -} + 2H^+ \quad (11)$$

$$QH_2 + Q \longrightarrow 2Q^{\cdot} + 2H^+ \quad (12)$$

$$QH_2 + O_2^{\cdot -} \longrightarrow Q^{\cdot} + H_2O_2 \quad (13)$$

$$Q^{\cdot} + O_2 \longrightarrow Q + O_2^{\cdot -} \quad (14)$$

$$Q^{\cdot} + Q^{\cdot} + 4H^+ \longrightarrow 2QH_2 \quad (15)$$

$$Q^{\cdot} + O_2^{\cdot -} + 2H^+ \longrightarrow Q + H_2O_2 \quad (16)$$

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (17)$$

In this way, quinones are able to cause the depletion of free cellular thiol species (i.e. trypanothione and glutathione).

The redox cycles of some *o*-naphthoquinones, in particular a group of mansonones and β-lapachone structural analogs (shown in Fig. (**13**)), have been evidenced in

presence of oxygen consuming cells [147]. In this study, hydrogen peroxide and superoxide anion were produced when the *o*-naphthoquinones were incubated with *L. seymouri* and *C. fasciculata* extracts. It was also concluded that *p*-naphthoquinones were less active as oxygen radicals producing species as compared with *o*-naphthoquinones. This behavior was attributed to the differences in electronic configuration between the two groups [159]. In the case of β-lapachone and α-lapachone, the notably difference in biological activity correlates positively with the capacity to generate oxygen radicals since the more active compound (β-lapachone) is also the one displaying the stronger redox cycling. This is suggesting that the oxygen active species have an important role in the biological effects of naphthoquinones.

Naphthoquinones establish interactions with all three enzymes TR, GR and LipDH. For TR, in a similar fashion to nitrofuran compounds, menadione, plumbagin, β-lapachone and other 1-4 naphthoquinones are subversive substrates [135,160-162]. Meanwhile, 1,4-naphthoquinones are mainly reversible inhibitors of GR and barely subversive substrates [163,164].

Both nitrofuranes and naphthoquinones can be reduced by a variety of cellular reductases triggering the production of oxygen radicals, a process that brings about the consumption of thiol species. When the acting reductase is TR, the subversive process may take place preventing the regeneration of $T(SH)_2$. The combination of these two effects may affect significantly the intracellular levels of the dithiol rendering the parasite more susceptible to the deleterious effects of free radical species. This hypothesis is supported by the thorough study reported in [162] aiming at obtaining 1,4 naphthoquinones with selectivity towards *T. cruzi* TR. There, it was concluded that the inhibition of TR alone is not sufficient for a significant trypanocidal activity but that the coupling with redox cycling is the crucial parameter.

Other quinone compounds isolated from natural products were assayed against *T. cruzi* and showed trypanocidal activity [165-167]. They include the trihydroxylated anthraquinone purpurin, the polyprenylated benzoquinone 7-epiclusianone and the 1,4-naphthoquinone 2,3,3-trimethyl-2,3-dihydronaphtho[2,3-b]furan-4,9-quinone.

Phenothiazines and Other Tricyclic Compounds

Among the many compounds assayed as potential trypanocidal agents phenothiazines stand out.

Phenothiazines constitute a group of tricyclic neuroleptic compounds traditionally employed as antidepressants in clinical cases of psicosis, scitzophrenia and related disorders. The first phenothiazine to be administered as neuroleptic was chlorpromazine.

Only a couple of decades ago, it was realized that phenothiazine compounds were active as anti-trypanosomal and anti-leishmanial agents. Soon afterwards, a number of works on the inhibition effect of phenothiazines were reported [168-177]. More recently, molecular modeling techniques showed that tricyclic antidepressants, in particular phenothiazines, contained some of the best-fitting probes at the active site of TR and could indeed inhibit the parasite

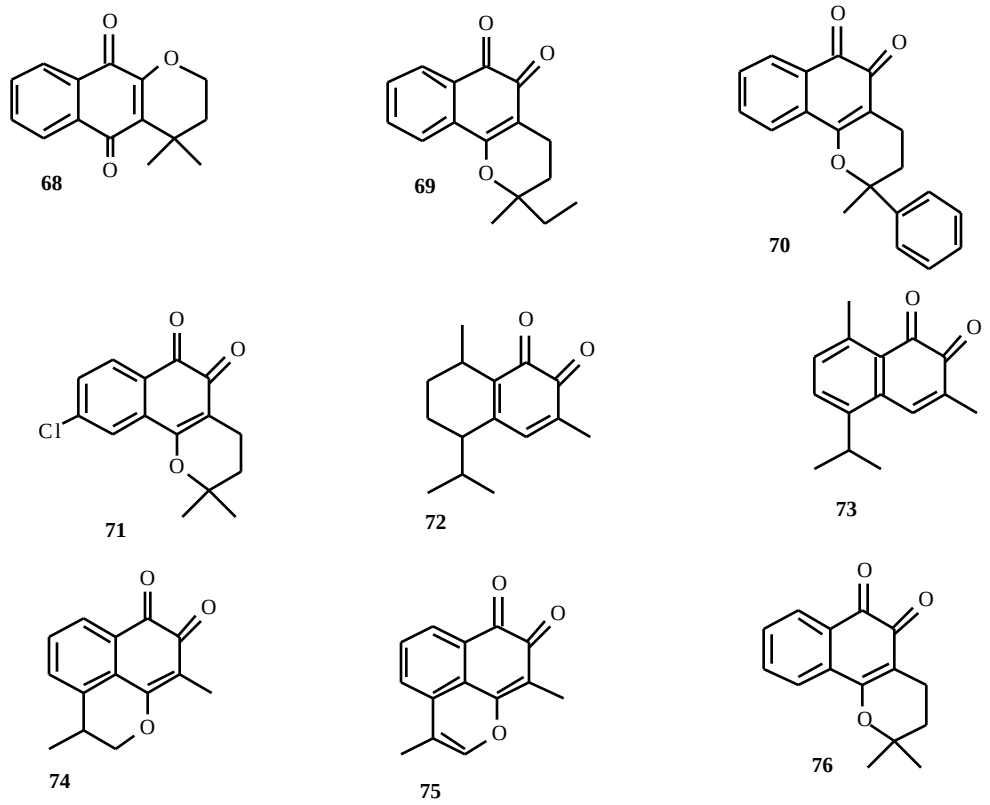

Fig. (13). o-naphthoquinones assayed in [147]. 68: α-lapachone; 69: 2-ethyl-β-lapachone; 70: 2-phenyl-β-lapachone; 71: 9-chloro-2-methyl-β-lapachone; 72: mansonone A (5,6,7,8-tetrahydro-3,8-dimethyl-5-isopropyl-1,2-naphthoquinone); 73: mansonone C; 74: mansonone E; 75: mansonone F; 76: 2-methyl-β-lapachone (β-lapachone).

flavoenzyme [178]. The most active compound studied in the initial screenings was the non-phenothiazine tricyclic clomipramine. In another study [179], an extensive series of synthetic phenothiazines acted as reversible inhibitors of TR; among them, there were commercial drugs such as chlorpromazine, trifluopromazine, thioridazine, promethazine, and N-substituted and polysubstituted derivatives. Chlorpromazine (compound **79** in Fig. (**14**)) remained as the best phenothiazine inhibitor of the enzyme *in vitro* and virtually any change to the (dimethylamino) propyl pendant diminished activity. More important, a few of the most active derivatives were not human GR inhibitors (see structures in Fig. (**14**)).

In a related research, phenothiazine cationic radicals were generated by means of H_2O_2/peroxidase reactions [180]. The radical derivatives, which included promazine, chlorpromazine and trifluoropromazine among others, were potent irreversible inhibitors of TR, in particular, some of them achieved 100% of enzyme inhibition at concentrations where reversible inhibition by non-radical species would not take place [179]. In this case, the inhibition may be achieved through chemical modification of enzyme groups at the active site by interaction with the highly reactive radical derivatives. The same radicals were subsequently found to be inhibitors of *T. cruzi* LipDH [181].

Theoretical docking studies were performed on the whole phenothiazine series reported in [179,180]. The results strongly suggest that phenothiazine derivatives are able to form more stable complexes with *T. cruzi* TR as compared to nitrofuranes and nitrothiophenes [Iribarne *et al.*, unpublished results]. Here again, as it was the case with previous docking studies on nitrofuranes, the importance of a charged side chain in the structure is evidenced. Overall, the positively charged derivatives displayed higher interactions energy with the parasite enzyme and, at the same time, corresponding complexes with human GR were less stable (lower interaction energies than the neutral counterparts).

A Principal Components Analysis (PCA) was also attempted on the above mentioned phenothiazine compounds along with a number of nitrofuran derivatives, some of which were presented in Fig. (**11**), and several aminodiphenylsulfides (see below). The generic structures are depicted in Fig. (**15**) where three different substructures are discriminated for each compound family. Electronic (molecular charges and frontier orbital energies), hydrophobic (logP), steric (surface area) and binding affinity (docking interaction energies at TR and GR active sites) properties were used as independent variables while *in vitro* parasite growth inhibition and enzyme (TR and GR) inhibition data were considered as dependent variables for the study. As a

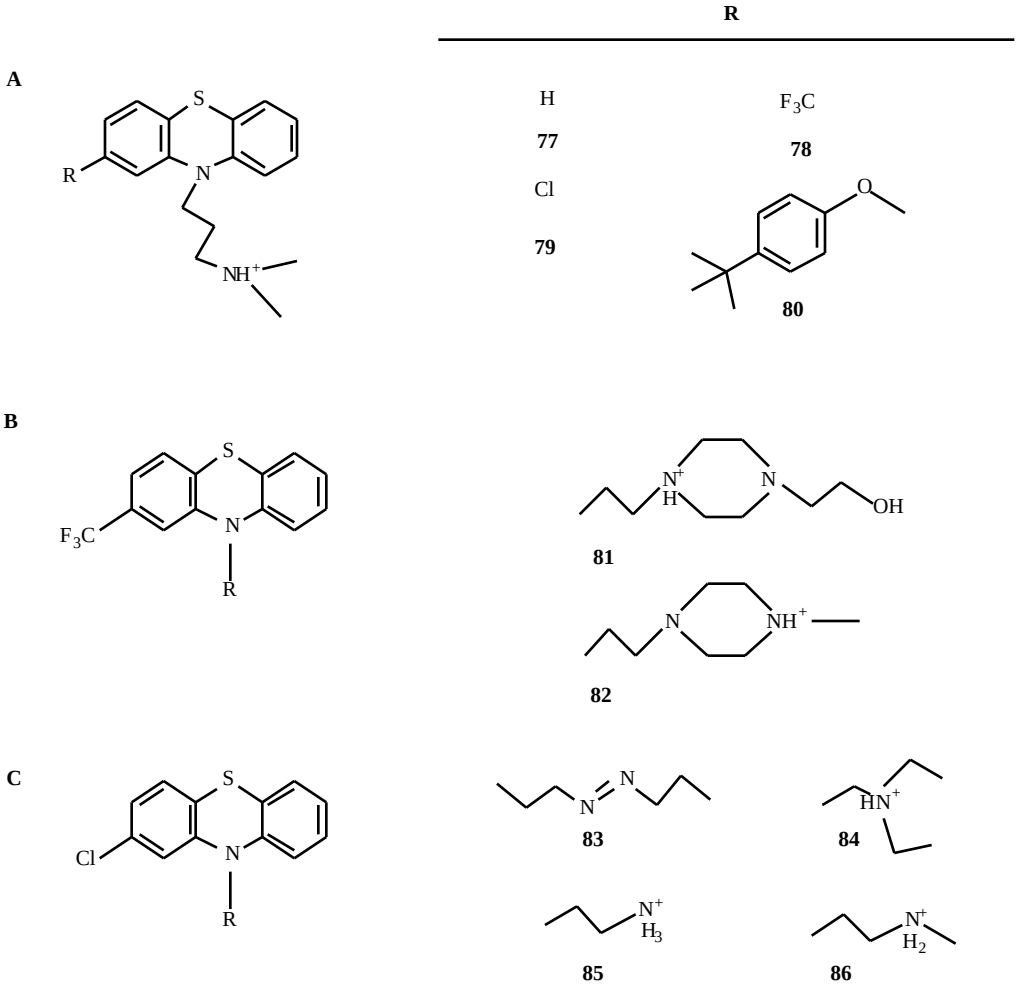

Fig. (14). Selective phenothiazine derivatives against *T. cruzi* TR reported in [178]. A: 2-promazines; B: 2-(trifluoromethyl)phenothiazines; C: 2-chlorophenothiazines; 77: promazine; 78: trifluoropromazine; 79: chlorpromazine.

result, a 140 (inhibitors sample) X 30 (variables) input matrix was built. Some molecular descriptors with high Principal components (PC) values were obtained for the assayed molecules, most notably the surface area (PC1 value = 0.743) and the (positive) charge of fragment 3 in Fig. (**15)** (PC1 value = -0.592, PC2 value = -0.427 and PC3 value = -0.425). Among the dependent variables, *T. cruzi* TR inhibition (PC2 value = 0.839 and PC3 value = -0.365) stood out. A correlation involving these variables was also suggested.

These theoretical results are also in line with previous experimental work where it was shown that chlorpromazine and analogs carrying a net positive charge selectively inhibited *C. fasciculata* TR [182]. In addition, the positively charged chlorpromazine inhibited *C. fasciculata* growth *in vitro* while a negatively charged analog failed at the same task. All this information is hinting at simple charge traits as factors accounting for a significant proportion of the ligand selectivity against TR and GR.

Phenothiazine compounds also benefit from their rigid cyclic structures. In effect, since cyclization brings about order in a chemical structure, the entropy penalty associated with the binding of this type of molecules to a given receptor is comparatively small. In turn, the corresponding free energy acquires more favorable (i.e., negative) values. For a more detailed description of the advantages of rigid compounds as ligands for foreign receptors see [163].

A final advantage of phenothiazine derivatives is that their pharmacology and toxicology have been studied in detail. In particular, the side-effects are much less important than those of nitrofuranes for instance. In fact, as already stated, a few phenothiazine drugs are routinely prescribed in

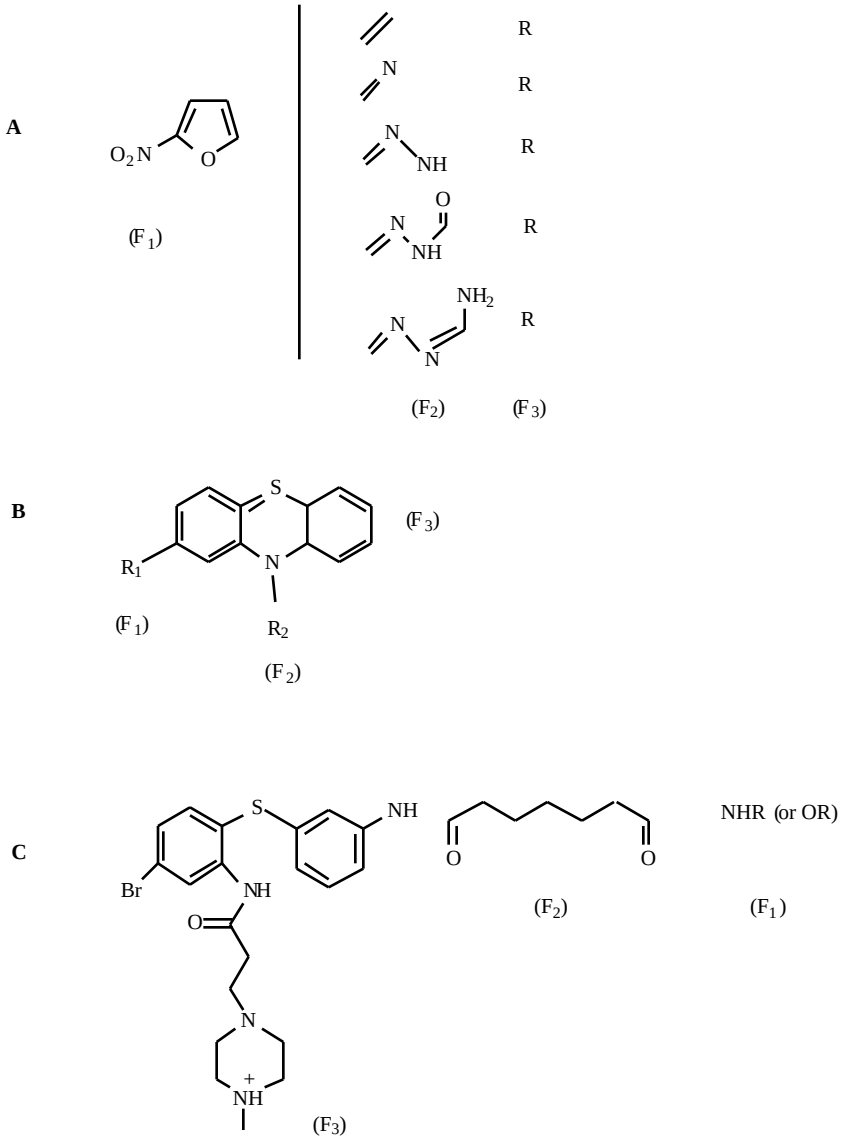

Fig. (15). Generic structures of A: nitrofuranes; B: phenothiazines; C: aminodiphenylsulfides compounds used in molecular modeling and PCA studies (see text for details). Three different conserved fragments (substructures) can be identified for each group of molecules ((F_1), (F_2) and (F_3)). For A and C, compound molecules are formed by succesively joining fragments in the same order as depicted in the picture. F_3 for A and C, and F_2 for B, correspond to the moieties carrying functional groups (i.e. nitrogen center) capable of protonating at physiological pH.

mental illness cases. In this regard, the only serious disorder associated with phenothiazine based treatment is the so called *Neuroleptic Malignant Syndrome* (NMS) [183], a very uncommon but often fatal complication. All these elements combined, phenothiazines appear as promising lead compounds for Chagas' chemotherapy.

To avoid the disadvantages of the neuroleptic activity of phenothiazines, some 2-amino diphenylsulfides were synthesized, including a few compounds with spermidine-like chains [184-186]. Their inhibitory capacity towards *T. cruzi* TR was studied and several compounds displayed unusually low IC_{50} values (<20μM).

Another tricyclic compound with trypanocidal activity is mepacrine, an acridine derivative. Like phenothiazines, mepacrine is a reversible competitive inhibitor of TR but not of GR [187]. Unlike mepacrine, acridine has no measurable activity on TR. A few years ago, mepacrine was crystallized at the active site of *T. cruzi* TR and the corresponding crystallographic coordinates were reported [111]. This helped elucidate the structure of the enzyme-ligand complex, which to date is the only crystallographic TR-inhibitor complex available.

CONCLUSIONS

Despite progress towards new drugs for the treatment of Chagas' disease has become rather stagnant over the last few years several new targets for chemotherapy have been identified. In some cases, promising drug candidates were found either by traditional chemistry-based approaches or through the use of genomics, proteomics and bioinformatics.

However, to date, none of the few compounds that have reached the stage of clinical trial in Chagas' disease, is considered to be a safe, effective, convenient and inexpensive agent for the extensive use in man. Thus, the development of new molecule alternatives to the currently used nifurtimox and benznidazole remains a research subject of prime interest.

It is worth noting here the recently reported Inactine™ technology, which has proven successful in inactivating *T. cruzi* and other parasites in red blood cell concentrates (RBCC) [188,189]. This technology, currently in the clinical trial phase, holds promise in preventing infection *via* blood transfusion.

From the target-based drug discovery standpoint, much of the work has been devoted to the trypanothione system of trypanosomatids, an epitome of differential metabolism between host and parasite. This line of work should certainly continue but additional effort has to be directed to explore other targets (c.f. previous sections) and, if possible, identify new valuable chemotherapeutic targets. In this sense, with the possibilities that the almost complete (14X) *T. cruzi* genome project provides, the perspectives never looked brighter.

Chagas' disease is not an exclusive Thirld World concern. Developed countries, in the last few years, have seen an increase in the predominance of this and other trypanosomatid disease burdens; in particular, leishmaniasis and malaria. Given the apparent similarities among the various trypanosomatid species, one can hope that an eventual cure for Chagas' disease could be applied to treat other related parasitosis.

In the authors' opinion, the search for new drugs should continue by means of nucleating existing chemotherapy efforts in joint endeavors aiming at providing a suitable framework to improve the chances of obtaining the hitherto elusive chemotherapeutic solution. In fact, there are multi-disciplinary research projects currently in progress, involving several laboratories across the Americas, which tackle the problem taking advantage of the synergy between gene and protein research programs with medicinal chemistry and structure optimization programs, in order to identify anti-chagasic agents based on rational drug design and natural products screening.

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LIST OF ABBREVIATIONS

HTS	=	High Throughput Screening
CP	=	Cystein Proteinase
HGPRT	=	Hypoxanthine-Guanine Phosphoribosyltransferase
GAPDH	=	Glyceraldehyde-3-phosphate Dehydrogenase
DHFR	=	Dihydrofolate Reductase
PTR	=	Pteridine reductase
FPPS	=	Farnesylpyrophosphate synthase
ADC	=	Arginine Decarboxylase
ODC	=	Ornithine Decarboxylase
DFMO	=	Difluoromethylornithine
DFMA	=	Difluoromethylarginine
$T(SH)_2$	=	Trypanothione
$T(S)_2$	=	Trypanothione disulfide
GCS	=	γ-Glutamylcysteine Synthetase
GSH	=	Glutathione
GSSG	=	Glutathione disulfide
SPDSYN	=	Spermidine Synthase
TS	=	Trypanothione Synthetase
ABC	=	ATP-Binding Cassette
MRP-1	=	Multidrug Resistance-Associated Protein 1
MRP-A	=	Multidrug Resistance-Associated Protein A

PGPA = Phosphatidylglycerophosphatase A

TR = Trypanothione Reductase

GR = Glutathione Reductase

LipDH = Lipoamide Dehydrogenase

SOD = Superoxide Dismutase

CoMFA = Comparative Molecular Fields Analysis

SIMCA = Structured Implementation Methodology for Complex Applications

ROS = Reactive Oxygen Species

PCA = Principal Components Analysis

RBCC = Red Blood Cell Concentrates

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