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Structure-Based Drug Discovery for Tropical Diseases

Rafael V. C. Guido and Glaucius Oliva*

Laboratório de Química Medicinal e Computacional, Centro de Biotecnologia Molecular Estrutural, Instituto de Física de São Carlos, Universidade de São Paulo, Av. Trabalhador São–Carlense 400, 13560-70, São Carlos -SP, Brazil

Abstract: Parasitic diseases are amongst the foremost threats to human health and welfare around the world. In tropical and subtropical regions of the world, the consequences of parasitic infections are devastating both in terms of human morbidity and mortality. The current available drugs are limited, ineffective, and require long treatment regimens. To overcome these limitations, the identification of new macromolecular targets and small-molecule modulators is of utmost importance. The advances in genomics and proteomics have prompted drug discovery to move toward more rational strategies. The increasing understanding of the fundamental principles of protein-ligand interactions combined with the availability of compound libraries has facilitated the identification of promising hits and the generation of high quality lead compounds for tropical diseases. This review presents the current progresses and applications of structure-based drug design (SBDD) for the discovery of innovative chemotherapy agents for a variety of parasitic diseases, highlighting the challenges, limitations, and future perspectives in medicinal chemistry.

Keywords: Tropical diseases, parasites, structure-based drug design, molecular target, inhibitor.

INTRODUCTION

Infectious disease is one of the most serious global problems and represents a major socio-economic challenge facing humanity [1-4]. In fact, several infectious diseases are amongst the top 20 leading causes of deaths in the world [5]. They are caused by pathogenic organisms such as bacteria, viruses, fungi, or parasites that invade the host for reproduction. In developing countries the problem is aggravated by the lack of well-established public health policies and the failure in the implementation of sanitation programs. Particularly, this scenario contributes to the spread and prevalence of parasitic diseases.

The parasitic diseases are also known as tropical diseases (or neglected diseases) and include illnesses such as malaria, tuberculosis, African trypanosomiasis (sleeping sickness), Chagas' disease, dengue, leishmaniasis, schistosomiasis, onchocerciasis and lymphatic filariasis. Endemic in many developing countries, it is estimated that they affect about one person in six in the world [6-8]. In spite of the alarming health, economic, and social consequences of these parasitic infections, the limited existing drug therapy suffers from a combination of drawbacks including poor efficacy, resistance, and serious side effects [7-9]. Therefore, there is an urgent need for new, safe and effective drugs for human use. Nevertheless, the low economic profile of the affected developing countries is not sufficient to attract investments to the discovery and development of new antiparasitic agents [9]. Aiming at overcoming this limitation, new approaches to strength drug discovery for tropical diseases have been fomented. These include the publicly-funded sequencing of the genomes of several parasites and the establishment of new public–private partnerships (PPPs) whose focus is specifically on tropical diseases [7-11].

The PPP initiative has been forging a close relationship between the public-sector and the pharmaceutical companies. Hence, a new scenario of drug discovery for tropical diseases has emerged, including important pharmaceutical industries such as Pfizer, Sanofi-Aventis, Bayer, Pharmacopeia and Merck Serono. The role of these companies in the drug discovery campaign involves: (i) compound supply for testing; (ii) high-throughput screening (HTS) assays; (iii) medicinal chemistry/pharmacokinetic development and (iv) training. Simultaneously, several other large companies, such as GlaxoSmithKline, Novartis, AstraZeneca and Eli Lilly, have founded research institutes dedicated to research into tropical diseases [7,10].

The PPPs are coordinated initiatives structured in line with industry management practices and funded by national governments and philanthropic institutions. They involve multi-disciplinary networks of investigators in a venture including industry, public-sector and academia from developed and developing countries that proved to be cost-efficient vehicles for clinical-product development [11]. The mission of these organizations relies on delivering efficacious and affordable drugs to the patients in developing countries. In addition, the main goals include: (i) the development of new tools and methodologies for disease diagnosis and control; (ii) strength the research capabilities in disease-endemic countries and (iii) technology transfer [8]. Examples of successful PPPs are the Medicines for Malaria Venture (MMV) [12], the Institute for OneWorld Health (iOWH) [13], the Global Alliance for Tuberculosis Drug Development (GATB) [14], the Drugs for Neglected Diseases initiative (DND*i*) [15] and the Special Programme for Research and Training in Tropical Diseases (TDR) [16],

*Address correspondence to this author at Centro de Biotecnologia Molecular Estrutural, Instituto de Física de São Carlos, Universidade de São Paulo, Av. Trabalhador São-carlense 400, 13560-970, São Carlos-SP, Brazil; Tel: +55 16 3373–9868; Fax: +55 16 3373-9881;
E–mail: oliva@ifsc.usp.br

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which have favorably contributed to boost the discovery and development of novel antiparasititc drugs.

STRATEGIES FOR DRUG DISCOVERY FOR TROPICAL DISEASES

Due to the fact that antiparasitic drug discovery is not primarily commercial-driven, other approaches to the development of novel drugs have been exploited (Table **1**). Many drugs currently indicated for the treatment of parasitic infections were first developed for other diseases. This approach, also known as label extension, has clear advantages, including short term development time and cost reduction. Since the drug was first investigated for other indication and is already approved for therapy, the medicine shows acceptable pharmacokinetics and toxicity profile. In contrast, some pharmaceutical companies, aware of the risk of unexpected toxic effect, are reluctant in allowing further investigation of their drugs for neglected diseases [9,11].

A similar approach that relies on already existing medicines is drug combinations [9,11,17]. This strategy has been very successful and continues to play an important role in the drug development paradigm for parasitic diseases [18,19]. For instance, drug combination therapy is employed for the treatment of sleeping sickness (*e.g.*, eflornithine and melarsoprol) [20], schistosomiasis (*e.g.*, praziquantel and oxamniquine) [21] and malaria (*e.g.*, sulphadoxine and pyrimethamine) [22]. Improved efficacy associated with lower cost and toxicity is usually observed in several combination regimens [17].

Although their notably importance for the treatment of parasitic infections, combination strategies do not provide the opportunity to identify new molecular entities with biological activity. Particularly, the discovery of novel anti-infective compounds is important to overcome current treatment limitations (*e.g.*, high cost, drug resistance, loss of efficacy, lack of selectivity and poor pharmacokinetic profiles) [23-27]. A start point to tackle these issues is the molecular manipulation of known drugs or active classes of compound to generate new scaffolds with improved potency, safety or straightforward synthesis. Currently, analogues of existing antiparasitic drugs are under investigation for a variety of neglected diseases [28-37].

An alternative approach based on known active compounds classes is the *piggy-back* discovery [38]. Commonly, the strategy is applied when active compounds developed for other therapeutical indications are employed as good chemical starting points to design new drugs against molecular targets from parasites. In this scenario, the lead compounds are already developed in terms of medicinal chemistry, pharmacokinetics, and toxic profile. Nevertheless, further structure-activity relationships (SAR) investigation is required to generate clinical candidates with enhanced biological properties towards the pathogenic organism (*e.g.*, selectivity). Notable successes have been achieved with this strategy, for instance, eflornithine (Ornidyl®, Sanofi-Aventis) and miltefosine (Impavido®, Zentaris) were originally developed for cancer and later used for African trypanosomiasis and visceral leishmaniasis, respectively. Ivermectin (Stromectol®, Merck), first developed for dog heartworm, was subsequently applied for human onchocerciasis. More recently, inhibitors of cysteine protease and protein farnesyl transferase, which were identified as promising drugs for osteoporosis and cancer [38,39], have been investigated for the chemotherapy of malaria and trypanosomiasis [38,40].

Table 1. Antiparasitic Drug Discovery Strategies Features

Impact	Project risk	Possibility	Limitation	Example	Indication
Label extension					
Short-term	Low	Reduction in cost and development time	Companies reluctance of risk uncovering toxicities	Pafuramidine; miltefosine	Sleeping sickness; Leishmaniasis
Drug combination					
Short-term	Low	Synergy, lower dose, reduced toxicity	Development of resistance	Eflornithine- melarsoprol	Sleeping sickness
Piggy-back discovery					
Medium-term	Medium	Quick identification of lead series; NCE	Homologous target has to be crucial to parasite survival and being investigated for other commercial indication	Screening of leads series of histone deacetylase; cysteine protease; farnesyl transferase inhibitors	Malaria; Sleeping sickness; Chaga's disease
De novo discovery					
Long-term	High	Innovative drugs; new targets; novel and affordable treatments	Higher cost; attrition rate	HTS campaigns; lead identification	Malaria; Sleeping sickness; Chagas' disease; Leishmaniasis

Advances in medicinal chemistry at the interface of chemistry and biology have created an important foundation in the search for new drug candidates that possess a combination of optimized pharmacodynamic and pharmacokinetic properties [41]. In this regard, the most attractive strategy for the development of clinically relevant agents for the treatment of parasitic diseases is the *de novo* discovery approach [9,11]. It is focused on the identification of new chemical entities (NCE), both synthetic compounds and natural products, against new validated molecular targets from parasites. Owing to its intrinsic innovative nature, *de novo* discovery strategy offers the opportunity to develop active substances that match the desired profile for anti-parasitic drugs, which includes: (i) low cost pharmaceuticals; (ii) efficacy against drug-resistant strains; (iii) cure within a reasonable time to ensure good compliance; (iv) safety; and (v) appropriate formulations for oral administration [17].

The strategy involves target-based HTS and medium-throughput screening (MTS) with *in vitro* assays against biological relevant molecular receptor as well as whole parasites. Moreover, sophisticated chemoinformatic methodologies integrated to genomics, *in silico* screening, and structural determination of the targets and their molecular complex with small ligands are commonly applied in *de novo* drug discovery.

Specific knowledge about ligand binding mode and mechanism of action is of great importance to guide the design of novel and relevant bioactive molecules. Thus, this paper presents an overview of the structure-based drug design (SBDD) approaches and recent advances for the discovery and development of novel therapeutic agents against tropical diseases.

MOLECULAR TARGETS FOR DRUG DESIGN

Several critical and unrelated macromolecules are exploited as chemotherapeutic targets. In this regard, biochemical pathways general to parasites but absent from mammalian hosts have long been an attractive source of molecular targets for anti-infective drug development [23,27]. Additionally, biochemical peculiarities of the parasites such as (i) turnover rates [42,43]; (ii) structure of biological membranes [44-47]; (iii) cell signaling [48] and (iv) protein expression and regulation [49] have also been investigated as potential targets for drug intervention [50].

The knowledge of parasite biology as well as the availability of the whole genomes and proteomics provides a wide range of novel targets for drug design [51-54]. The wealth of information generated by these experiments has created the need for robust and reliable data processing methods to analyze and identify relevant molecular targets. This scenario prompted the development of integrated approaches on the basis of the knowledge from different disciplines such as chemistry, biology and informatics. In this context, the chemogenomics is an attractive and useful strategy to define and assess the chemical and biological space [55,56]. In order to improve the efficiency of early-stage discovery and speeding up the screening process, chemogenomics methods are widely applied to assign priority to the most promising molecular receptors for further investigation, thereby enabling potential targets to be selected before expensive and time-consuming drug-screening and optimization studies are undertaken [57].

Concomitantly, the evolution of structural biology and computational methods has led to the determination of high-resolution atomic structures of many relevant targets for antiparasitic drug discovery projects. The TDR program of the World Health Organization supports and manages a network of academic and industrial centers that established a portfolio of drug targets for tropical diseases using the wealth of available genomics and proteomic information [58].

Structural data of several important drug-metabolizing enzymes is now available, which potentially enables the structure-based rational optimization of potency, selectivity and metabolism. However, the use of these data has not delivered the predicted new generation of anti-infective drugs yet. To tackle this problem, chemogenomics methods applied to pathogenic organism are employed to assign priority to targets not only on the biological relevance, but also on the basis of their "druggability" profile (*i.e.*, the probability that the targets will bind to drug-like molecules) [59]. Tables **2** and **3** show several essential parasite proteins as molecular targets with available structural information and their druggability index.

SBDD AND TROPICAL DISEASES

Structure-based approaches have become essential components of modern medicinal chemistry [41]. Over the past decade, high-performance computers, algorithms, methods and expertise have evolved and transformed SBDD methods in tools of large impact in drug discovery [60-62]. They incorporate information from the target receptor, hence, these knowledge-driven approaches require extensive informations about the target topology under investigation (*e.g.*, X-ray or NMR structure, or robust homology modeling) [63]. In this context, public databases such as the Protein Data Bank (PDB) [64], InterPro [65-67], ExPASy [68], and Relibase [69-71] are invaluable data sources to retrieve and analyze 3D structures of target proteins.

The molecular recognition phenomenon relies on properties and features of a binding pocket, which are determined by the amino acids present in the binding cavity. The spatial arrangement of the amino acids within the binding site specifies structural and physicochemical constraints that must be met by any putative ligand. Therefore, a detailed analysis of the stereo-electronic properties of the target binding pocket provides useful insights into relevant ligand-receptor interactions that can be optimized to enhance the biological properties of lead compounds [41].

The SBDD produced several success stories and has been continuously employed as an important tool to assist the design of compounds for a variety of diseases and disorders [72]. Particularly, investigations on novel anti-infective compounds led to the identification and development of distinct sets of available drugs, for instance, the anti-HIV (*e.g.*, saquinavir - Invirase®; ritonavir - Norvir®; nelfinavir - Viracept®) and antiviral (oseltamivir - Tamiflu®; zanamivir - Relenza®) agents.

Table 2. Druggability Index and 3D Structure Availability of Essential Drug Targets in Trypanosomatids

Organism	Molecular Target	Druggability Index*	PDB Code
T. cruzi	farnesyl pyrophosphate synthase	1.0	1YHK; 1YHL; 1YHM
	trypanothione reductase	0.7	1AOG; 1BZL; 1GXF; 1NDA
	glyceraldehyde 3-phosphate dehydrogenase	0.6	1K3T; 1ML3; 1QXS; 3DMT
	cyclophilin	0.6	1XO7
	triosephosphate isomerase	0.6	1CI1; 1SUX; 1TCD
	tryparedoxin peroxidase	-	1UUL
	cruzain	-	1AIM; 1EWL; 1EWM; 1EWO 1EWP; 1F29; 1F2A; 1F2B; 1F2C; 1ME3; 1ME4; 2AIM
	tyrosine aminotransferase	-	1BW0
L. major	triosephosphate isomerase	0.8	1AMK; 1IF2; 1N55; 1QDS
	glyceraldehyde 3-phosphate dehydrogenase	0.8	1A7K; 1GYP; 1GYQ; 1I32; 1I33
	pteridine reductase 1	0.7	1E7W; 1E92; 1P33; 1W0C
	cyclophilin	0.6	2HAQ
	cysteine peptidase C	0.6	1BF7
	cyclophilin a	0.6	2HAQ
	glucose-6-phosphate isomerase	0.2	1Q50; 1T10
	fructose-1,6-bisphosphate aldolase	0.2	1EPX
	adenine phosphoribosyltransferase	0.2	1MZV; 1QB7; 1QB8; 1QCC; 1QCD
	pyruvate kinase	0.2	1PKL
	coproporphyrinogen iii oxidase	0.2	1VJU
	glycerol-3-phosphate dehydrogenase	0.2	1EVY; 1EVZ; 1JDJ; 1M66; 1M67; 1N1E; 1N1G
	transketolase	-	1R9J
T. brucei	prostaglandin f synthase	0.8	1VBJ
	phosphoglycerate kinase	0.8	13PK; 16PK
	glyceraldehyde 3-phosphate dehydrogenase,	0.8	1GGA
	farnesyl pyrophosphate synthase	0.8	2EWG; 2I19
	triosephosphate isomerase	0.6	1AG1; 1DKW; 1IIG; 1IIH; 1KV5; 1ML1; 1MSS; 1MTM; 1TPD; 1TPE; 1TPF; 1TRD; 1TRI; 1TSI; 1TTI; 1TTJ; 3TIM; 4TIM; 5TIM; 6TIM
	6-phosphogluconate dehydrogenase	0.25	1PGJ
	tryparedoxin	-	1O73
	fructose-bisphosphate aldolase	-	1F2J
	enolase	-	1OEP

*The druggability index values range from 0 to 1, where a larger index score means a more likely to be druggable target. The druggability index is a composite score consisting of a weighted normalised sum, where each of the different druggability prediction methods are given different weights depending on their relative contribution to prediction [58].

Table 3. Druggability Index and 3D Structure Availability of Essential Drug Targets in *Plasmodium Falciparum*

Organism	Molecular Target	Druggability Index*	PDB Code
P. falciparum	adenosine deaminase	1.0	2AMX; 2PGF; 2PGR; 2QVN
	ribonucleotide reductase small subunit	1.0	2O1Z; 2P1I
	cell division control protein 2 homolog	0.9	1LCH; 1OB3; 1V0B; 1V0O; 1V0P
	casein kinase 1	0.9	1LHX
	glutathione reductase	0.9	1ONF
	NADP-specific glutamate dehydrogenase	0.8	2BMA
	farnesyl pyrophosphate synthase	0.8	2IHI
	adenylosuccinate synthetase	0.8	1P9B
	multidrug resistance protein 2	0.7	2GHI
	deoxyuridine 5'-triphosphate nucleotidohydrolase	0.6	1VYQ
	triosephosphate isomerase	0.8	1LYX; 1LZO; 1M7O; 1M7P; 1O5X; 1VGA; 1WOA; 1WOB; 1YDV; 2FI6
	ubiquitin-conjugating enzyme	0.28	2PWQ
	glyceraldehyde-3-phosphate dehydrogenase	0.28	1YWG; 2B4R; 2B4T
	nucleoside diphosphate kinase b	0.2	1XIQ
	small GTPase Rab11	0.2	3BFK
	dimethyladenosine transferase	0.2	2H1R
	phosphoglycerate mutase	0.2	1XQ9
	protein-L-isoaspartate O-methyltransferase beta-aspartate methyltransferase	-	2PBF
	2-Cys peroxiredoxin	-	2H01; 2H66; 2I81
	beta-hydroxyacyl-acp dehydratase precursor	-	1Z6B; 1ZHG; 2OKH; 2OKI
	fructose-bisphosphate aldolase	-	1A5C; 2EPH; 2PC4
	ribosomal RNA methyltransferase	-	2PLW
	pyrroline carboxylate reductase	-	2RCY
	adenylosuccinate lyase	-	2HVG; 2QGA
	2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	-	3B6N

*The druggability index values range from 0 to 1, where a larger index score means a more likely to be druggable target. The druggability index is a composite score consisting of a weighted normalised sum, where each of the different druggability prediction methods are given different weights depending on their relative contribution to prediction [58].

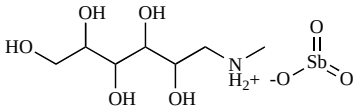
Experimental and computational structure-based methods applied to tropical diseases contribute to shed some light on both the understanding the biology of infectious organisms and the enabling competence for rational drug design. Additionally, the combination of these techniques is expected to accelerate the process of hit identification, lead optimization and NCE generation. The relevance of this strategy for the development of novel chemotherapeutic agents against tropical diseases is highlighted by several successful examples in the following sections.

TRYPANOSOMIASIS AND SBDD

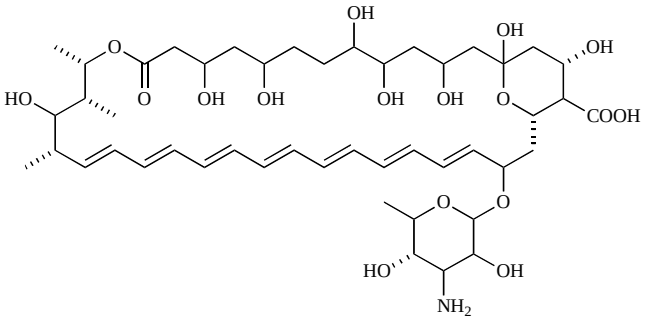
Parasitic infections caused by trypanosomatids represent several of the major neglected diseases, including leishmaniasis, sleeping sickness (human African trypanosomiasis, HAT) and Chagas' disease (American trypanosomiasis). The causative agents of these illnesses are *Leishmania ssp.*, *Trypanosoma brucei ssp*. and *Trypanosoma cruzi*, respectively. They occur in both tropical and subtropical regions worldwide and affect about 30 million people with another 400 million living in high-risk areas [73-75]. All existing therapeutic treatments have inadequate efficacy and unacceptable toxicity profiles.

Several different classes of compounds were approved for the treatment of leishmaniasis (Fig. **1**) [19]. Since the 1940s the basis of antileishmanial chemotherapy consist of pentavalent antimonial drugs (*e.g.*, meglumine antimoniate and sodium stibogluconate), however, these agents require a

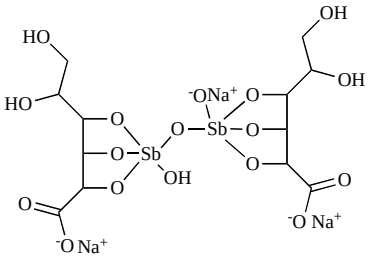
Lieshmaniasis



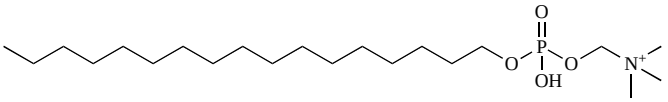
meglumine antimoniate.



amphotericin B

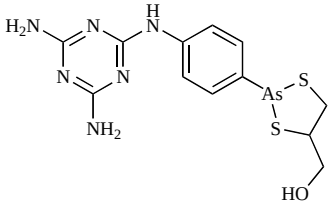


sodium stibogluconate

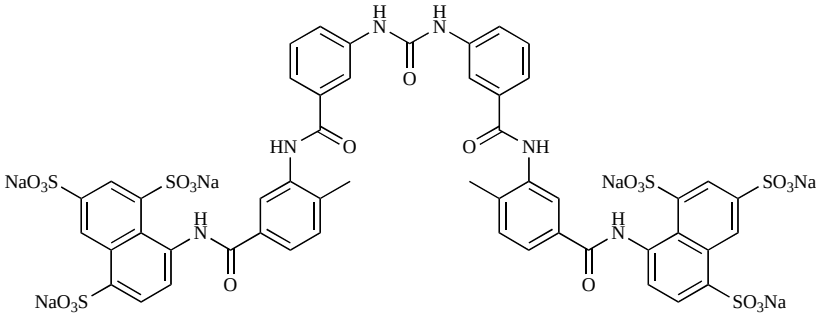


milte fosine

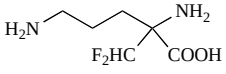
Sleeping sickness



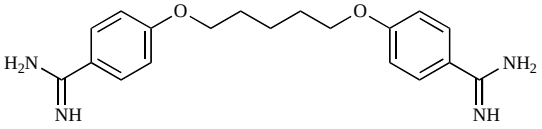
melarsoprol



suramin

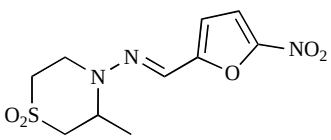


eflornithine

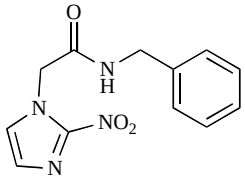


pentamidine

Chagas' disease



nifurtmox



benznidazol

Fig. (1). Molecular structure of approved drugs for trypanosomiasis.

long course of parenteral administration, and many cases of lack of efficacy were reported [19,23]. Amphotericin B has been used as an alternative therapeutic option, nonetheless, it must also be administered parenterally, and causes serious toxic effects (*e.g.*, nephrotoxicity). To circumvent this issue, novel formulation approaches such as drug liposome encapsulation were developed. This new presentation is much less toxic than the standard one, however, it is very expensive to be used for routine antileishmanial chemotherapy in the developing world. More recently, miltefosine, the first oral drug for the treatment of visceral leishmaniasis was registered. Unfortunately, the drug shows severe gastrointestinal side effects and must be given over a long course of treatment [76].

The current therapeutic arsenal for sleeping sickness mainly consists of three drugs, which were introduced more than half a century ago (suramin, pentamidine and melarsoprol) and one more recently registered (eflornithine) (Fig. **1**). Suramin and pentamidine are only active in the first stage of the disease when the parasites reside in the bloodstream and lymphatic system. Alternatively, melarsoprol and eflornithine are used for the treatment of sleeping sickness when the parasites have established themselves in the central nervous system (late-stage). Unfortunately, a significant number of patients with secondary-stage sleeping sickness show resistance to melarsoprol, whilst eflornithine is ineffective against *T. brucei rhodesiense* (prevalent in eastern and southern African countries) and only used to treat the disease caused by *T. brucei gambiense* (prevalent in western African countries) [19,23].

One hundred years have passed since the discovery of Chagas' disease [77], however, the only clinically available alternatives for the chemotherapy are nifurtimox and benznidazol (Fig. **1**). Both compounds have serious side effects and are active only in the acute phase of the disease. In addition, their usefulness in the intermediate and late chronic stage of the infection is highly questionable [78].

As stated above, the identification of new macromolecular targets and small-molecule modulators is of utmost importance for the development of novel and effective anti-trypanosomatids agents. Thus, a brief review of the recent discoveries based on SBDD approaches for trypanosomiasis is presented in the following sections.

Pteridine Reductase Inhibitors

Enzymes involved in the provision and use of reduced folate cofactors such as dihydrofolate reductase (DHFR, EC 1.5.1.3) and thymidylate synthase (TS, EC 2.1.1.45) are suitable drug targets for the treatment of several diseases (*e.g*, bacterial infections, cancer, and malaria). Inhibition of DHFR or TS reduces the cellular pool of 2'-deoxythymidine-5'-monophosphate, impairing DNA replication and resulting in cell death [79,80]. Since trypanosomatids are auxotrophic for folates, the inhibition of the enzymes is expected to kill the parasites. However, these organisms also have the pteridine reductase enzyme (PTR1, EC 1.5.1.33), essential for salvage of pterins by parasitic trypanosomatids that shows a broad range of activity catalyzing successive reductions of conjugated (folate) and unconjugated (biopterin) pterins. In the presence of DHFR-TS inhibitors, PTR1 is overexpressed in trypanosomatids leading to sub-optimal concentration of the inhibitors to ensure parasite death [81,82]. This biological mechanism of resistance suggests that inhibition of both DHFR and PTR1 could be useful for the treatment of trypanosomatids infections.

To confirm this assumption, a database of 440 synthetic folate-like compounds was analyzed aiming at identifying new inhibitors of both enzymes [83]. A series of physico-chemical- and pharmacokinetic-based filters was applied to the database retrieving 131 molecules that fit the selection criteria. The inhibitory activity of the compounds were assessed against a panel of folate-dependent enzymes, including DHFR/TS from *L. major*, and PTR1 from *L. major* and *T. cruzi*. Additionally, inhibition data of DHFR/TS human homologues were used to estimate the toxicity and selectivity of the compounds. The biological assays identified 9 selective and potent compounds against the parasite enzymes. Compound (**1**) was the best inhibitor of *Lm*PTR1 and *Lm*DHFR (K_i value of 0.1 μM and 4 μM, respectively) and one of the most selective inhibitors in the series (Fig. **2A**). Crystallographic assays were undertaken to shed light on the molecular mechanism and binding mode of the lead compound. The ternary complex *Lm*PTR1–NADPH-**1** analysis revealed that the inhibitor adopts a substrate-like orientation (Fig. **2B**). Based on this finding, a similarity search was conducted to retrieve new potential inhibitors from the library. This procedure identified 4 compounds which were tested against *Lm*PTR1 and human DHFR. The best analogue, compound (**2**), a methylated derivative of (**1**) (Fig. **2A**), exhibited K_i values of 0.037 and 0.820 μM against *Lm*PTR1 and human DHFR, respectively. Further crystallographic studies revealed that compound (**2**) orientates the methyl group toward a small hydrophobic pocket within the catalytic site (Leu226 and Leu229, Fig. **2B**) which favorably contribute to the inhibitory activity.

Subsequently, *in vitro* assays against the whole *L. major* and *T. cruzi* parasites were carried out. The inhibitors showed poor efficacy when tested independently, however, when used in combination with known DHFR inhibitors, a synergic and deleterious effect to parasites was observed. These results indicate that essential biochemical processes for parasite viability were targeted and corroborated the previous assumptions. Furthermore, these findings provide an alternative therapeutic approach against trypanosomatids.

Cruzain inhibitors

Cysteine proteases are widespread in nature [84]. Their implication in numerous vital processes of several parasitic protozoa makes them highly attractive targets for drug design [85,86]. Cruzain (EC 3.4.22), the major cysteine protease of *T. cruzi*, plays a pivotal role during the infection of host cells, replication, and metabolism, and has been extensively investigated as an attractive target for the development of a new generation of antitrypanosomal agents [87-96]. Indeed, cruzain inhibitors are amongst the most advanced compounds in the drug development pipeline for Chagas' disease. For instance, **K11777**, a dipeptidyl vinyl sulfone derivative (Fig. **3**), is currently in preclinical trials [96,97].

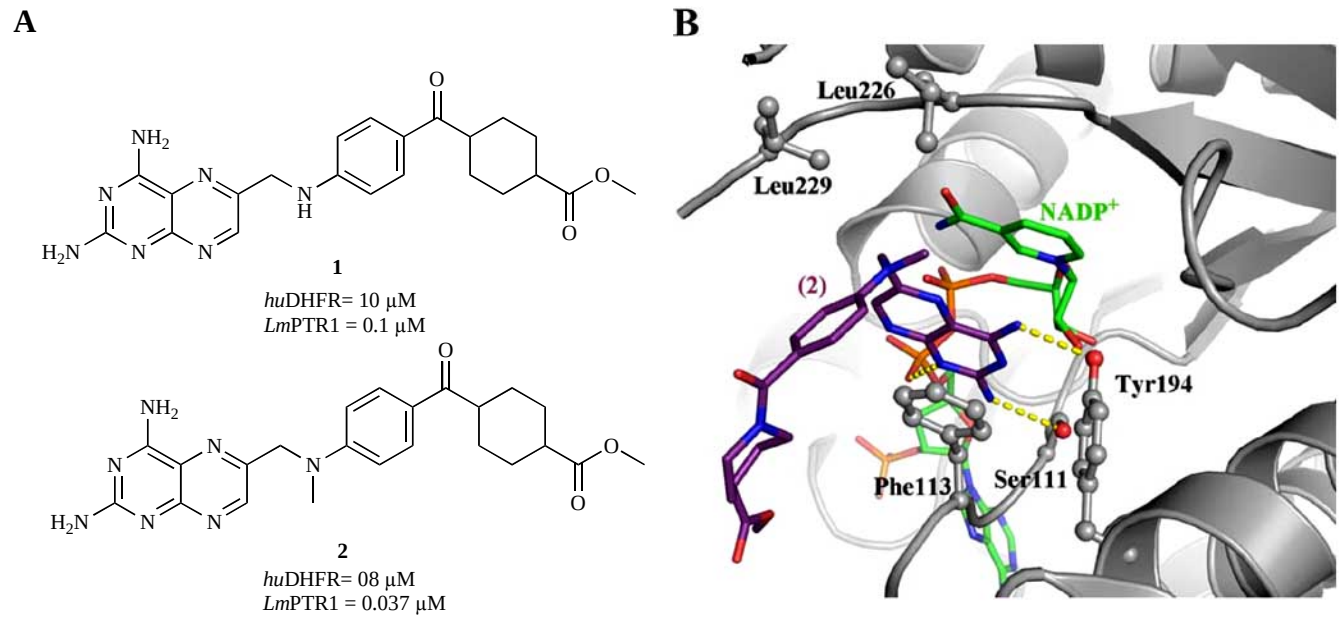


Fig. (2). (**A**) Molecular structures and biological activity of the best PRT1 inhibitors. (**B**) Binding mode of (**2**) to PTR1 binding site (PDB ID, 2QHX). The protein is indicated as cartoon and the binding site residues as ball-and-stick model. The ligand and cofactor molecules are represented as stick model and hydrogen-bond as broken lines.

Aiming at developing nonpeptidic inhibitors of cruzain with improved biological activity and pharmacokinetic properties, an alternative approach based on substrate activity screening (SAS) was employed to screen a library of protease triazole-based substrates [98]. The SAS method consists of three main steps: (i) the identification of nonpeptidic substrate; (ii) substrate optimization and (iii) conversion of optimal substrates to inhibitors [99]. The first step of the procedure identified compounds (**3**) and (**4**) as potential hits in the focused library (Fig. **3**). Subsequently, a series of iterative cycles of organic synthesis and biological evaluation was carried out to enhance the cleavage efficiency. The optimization step was guided by molecular modeling based on the structure of a closely related ketone triazole analogue in complex with cathepsin S. Significant structural differences in the S3 pockets of the enzymes allow the design of cruzain-specific substrate incorporating planar heterocycles in place of the phenyl ring of the benzamide moiety (*i.e.*, cruzain S3 pocket is larger and hydrophobic) (Fig. **3**). The most promising substrates synthesized were the quinoline and benzothiazole derivatives (compounds (**5**) and (**6**), respectively) with 7–9 fold improvement in cleavage efficiency related to compound (**3**) (Fig. **3**). The structure-based studies also indicated that the benzamide carbonyl of the substrate is solvent exposed and therefore, could be replaced. Based on this hypothesis, amine and amide derivatives were obtained and assessed. The enzymatic assay confirmed the modeling hypothesis and identified the quinoline amine (**7**) and benzothiazole amine derivatives (**8**) as the most potent substrate with cleavage efficiency 19-fold greater than the unsubstituted benzamide (**3**) (Fig. **3**).

Since potent and promising substrates were obtained, the final step of the SAS method was initiated. The strategy to convert substrates into cruzain inhibitors was based on the replacement of the aminocoumarin group with several mechanism-based pharmacophores (*e.g.*, vinyl sulfone (**9**), β-chloro vinyl sulfone (**10**), acyloxymethyl ketone (**11**) and aryloxymethyl ketone (**12**)) (Fig. **3**). Time-dependency assays revealed that compounds (**11**) and (**12**) were authentic irreversible inactivators of cruzain with excellent inhibitory acitvity [98]. Then, cell culture assays were employed to evaluate the inhibitors effectiveness in eliminating *T. cruzi* infection. On one hand, the experiment revealed that compound (**11**) significantly delayed *T. cruzi* intracellular replication at 5 μM (trypanostatic agent), however, it showed toxic effects at this concentration. On the other hand, compound (**12**) was trypanocidal at 10 μM and had completely eradicated the *T. cruzi* parasite from the infected cells [98]. The biological properties of (**12**) are similar to the most advanced cruzain inhibitor (**K11777**, 100% inhibition of intracellular growth of *T. cruzi* amastigotes at 20 μM; oral bioavailability ~ 20%) [95,97], suggesting that this molecule is a potential lead compound for the development of a new generation of nonpeptidic inhibitors as antitrypanosomal agents [98].

Phosphofructokinase and Pyruvate Kinase Inhibitors

The glycolytic pathway of trypanosomatids has been investigated as a promising biochemical target for drug intervention [27]. The bloodstream form of trypanosomatids has no functional tricarboxylic acid cycle and is highly dependent on glycolysis for ATP production [100,101]. Additionally, the parasite enzymes within the pathway possess significant structural differences with respect to the human homologues. Therefore, the vital dependence on glycolysis as the main source of energy for the parasites, combined to distinct molecular properties when compared with their human counterparts makes the glycolytic enzymes attractive targets for drug design. Structure- and ligand-based approa-

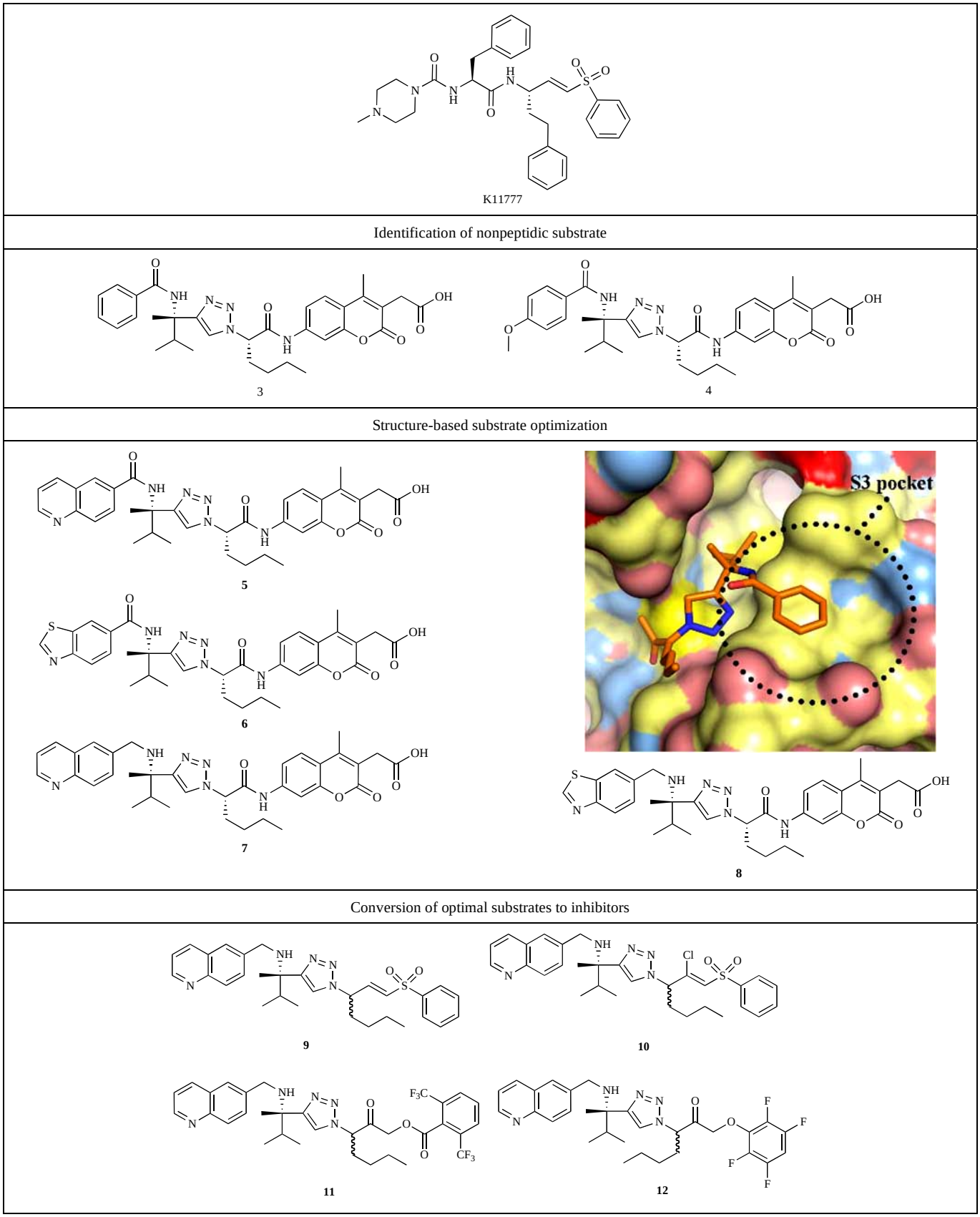


Fig. (3). Substrate activity screening (SAS) approach and molecular structure of the most promising compounds identified in each step. Insert, predicted binding mode of a ketone triazole analogue (orange) within cruzain binding site (PDB ID, 1F2C). The protein Connolly surface is indicated with protein atoms colored according to the physicochemical property (hydrophobic atom in pale yellow; positively charged atom in blue; negatively charge atom in red, polar nitrogen atom in light blue, polar oxygen atom in light red, and sulfur atom in yellow).

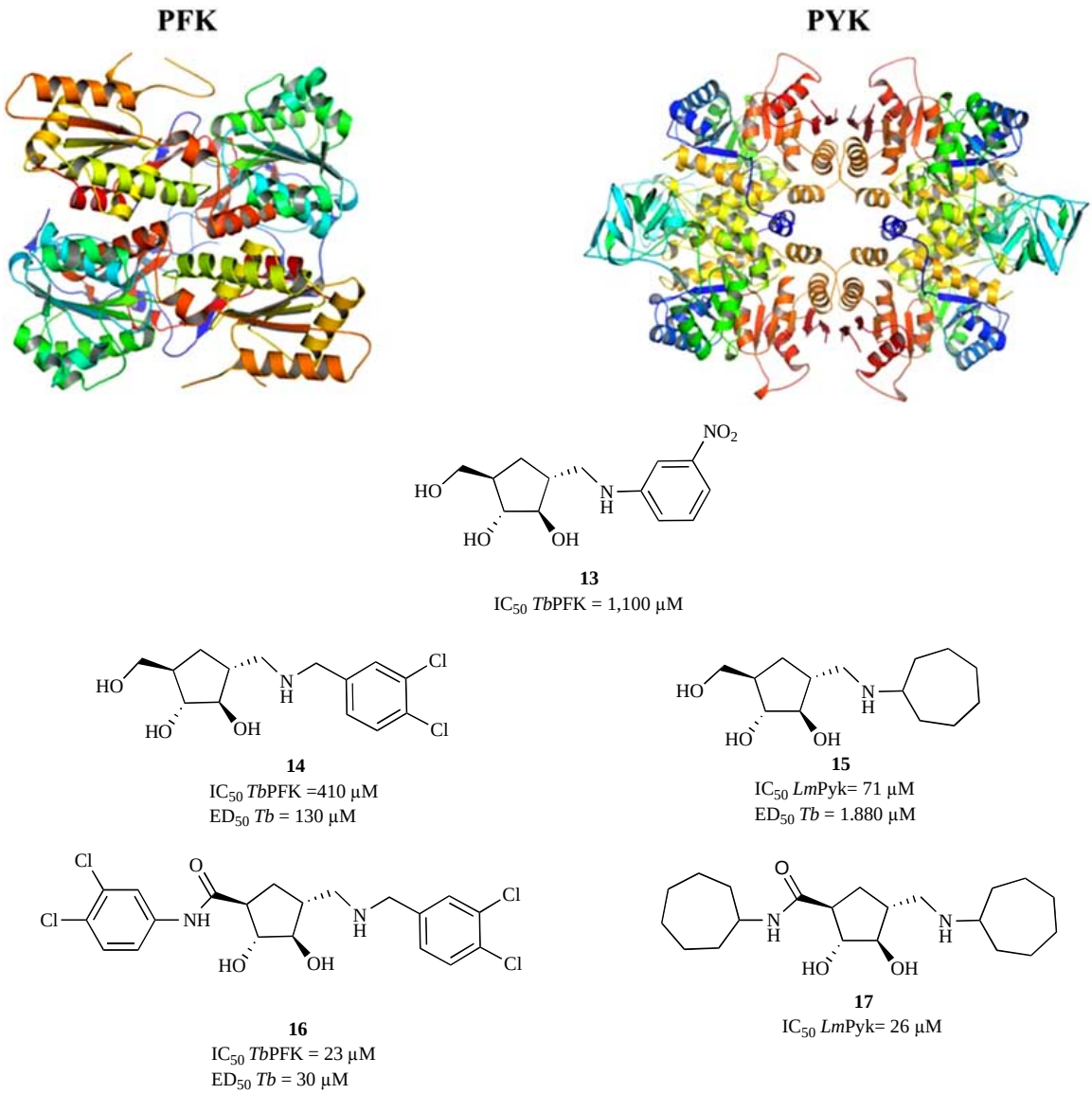


Fig. (4). Upper panel. Crystallographic structures of PFK (PDB ID, 2HIG) and PyK (PDB ID, 1PKL). Lower panel. Molecular structure and related biological properties.

ches on enzymes of the carbohydrate metabolism pathway have identified several promising inhibitors, as well as shed light on the structural basis for selective inhibition [102-117].

Phosphofructokinase (PFK, EC 2.7.1.11) and pyruvate kinase (PyK, EC 2.7.1.40) (Fig. **4**) play important role in trypanosomatids glycolytic flux and were explored as molecular target for the development of trypanocidal agents [118]. Previously, pioneering kinetic and inhibition studies conducted on *T. brucei* PFK (*Tb*PFK) substrate analogues have identified a nonphosphorylated mannitol derivative ((**13**), IC_{50} = 1,100 μM) as an interesting starting point for inhibitor design [119] (Fig. **4**). Due to the structural similarities between fructose 6-phosphate (F6P) and fructose 2,6-bisphosphate (F2,6BP), respectively substrate and allosteric regulators of PFK and PyK, *L. mexicana* PyK (*Lm*PyK) was also included in the inhibition assays.

Aiming at exploring the SAR within this series, a focused library of amine derivatives of (**13**) was synthesized and evaluated against *Tb*PFK and *Lm*PyK. On one hand, compound (**14**), bearing a 3,4-dichlorobenzyl substituent, was the most potent inhibitor against *Tb*PFK (IC_{50} = 410 μM). On the other hand, compound (**15**), a cycloheptyl mannitol derivative, exhibited good inhibitory activity against *Lm*PyK (IC_{50} = 71 μM) (Fig. **4**). These findings prompted further investigation on the inhibitor scaffold that explored the chemical diversity of mannonamide derivatives. Significant improvements in potency against *Tb*PFK was observed when the 3,4-dichlorobenzamide substituent was obtained ((**16**), IC_{50} = 23 μM). Similarly, the addition of a cycloheptyl-amide moiety to (**15**) led to some enhancement in the inhibitory activity against *Lm*PyK ((**17**), *Lm*PyK IC_{50} = 26 μM) (Fig. **4**).

Molecular docking studies were undertaken with both enzymes to elucidate the structural determinants underlying

the biological properties within this series. Regarding *Tb*PFK, the model indicated that the increase in potency observed for (**16**) could be explained by hydrogen-bonds and hydrophobic interactions between the 3,4-dichlorobenzamide substituent and the aminoacid residues within active site. Comparable results were obtained when (**17**) had been modeled within *Lm*PyK binding pocket. The enhanced potency might be due to the increase in hydrophobic bulk. Therefore, the molecular models correlate well with SAR data and provide a reasonable explanation for the observed biological activities.

Since *in vitro* activity evidences are desirable to help with subsequent lead identification and modification, inhibitory evaluation against the whole parasite was carried out to confirm the biological activity of the designed inhibitors. The biological assay against *T. brucei* revealed that the inhibitors exhibit some modest activity against the cultured parasite (compound (**16**) was the most potent inhibitor with ED_{50} value of 30 μM) [118].

In summary, this work highlights the first steps in drug design for parasitic diseases which involved (i) the optimization of a series of previously identified inhibitor; (ii) structural insights into the inhibitor binding mode through molecular modeling studies and (iii) preliminary *in vivo* activity evaluation.

MALARIA AND SBBD

Malaria is spread over 90 countries worldwide and is responsible for approximately 300 million acute cases each year, with 1.5 to 2.0 million fatalities. Nearly 90% of the deaths occur in sub-Saharan African regions. The protozoan *Plasmodium falciparum* is the etiological agent of the most severe and life-threatening form of disease [120].

The emergence of resistance to the current most affordable drugs, such as chloroquine, mefloquine and sulfadoxine-pyrimethamine (Fig. **5**), represents a major problem for malaria control. Accordingly, many countries must consider alternative approaches to slow the development of resistance, for instance, artemisinins analogues or drug combinations (*e.g.*, atovaquone-proguanil, amodiaquine-artesunate). Fig. (**5**) shows the molecular structures of the current available drugs for malaria. However, these therapeutical choices show serious cost limitations for patients from developing countries, particularly in Africa. Moreover, various antimalarial drugs in current usage are chemically related or show similar mode of action, possibly sharing resistance mechanisms, which increases the risk of cross-resistance and clinical failure of newly introduced therapies [121].

The continuously increasing resistance of the vector toward insecticides as well as the emergence of multidrug-resistant parasites greatly necessitates the development of new anti-infective agents that have either a novel binding

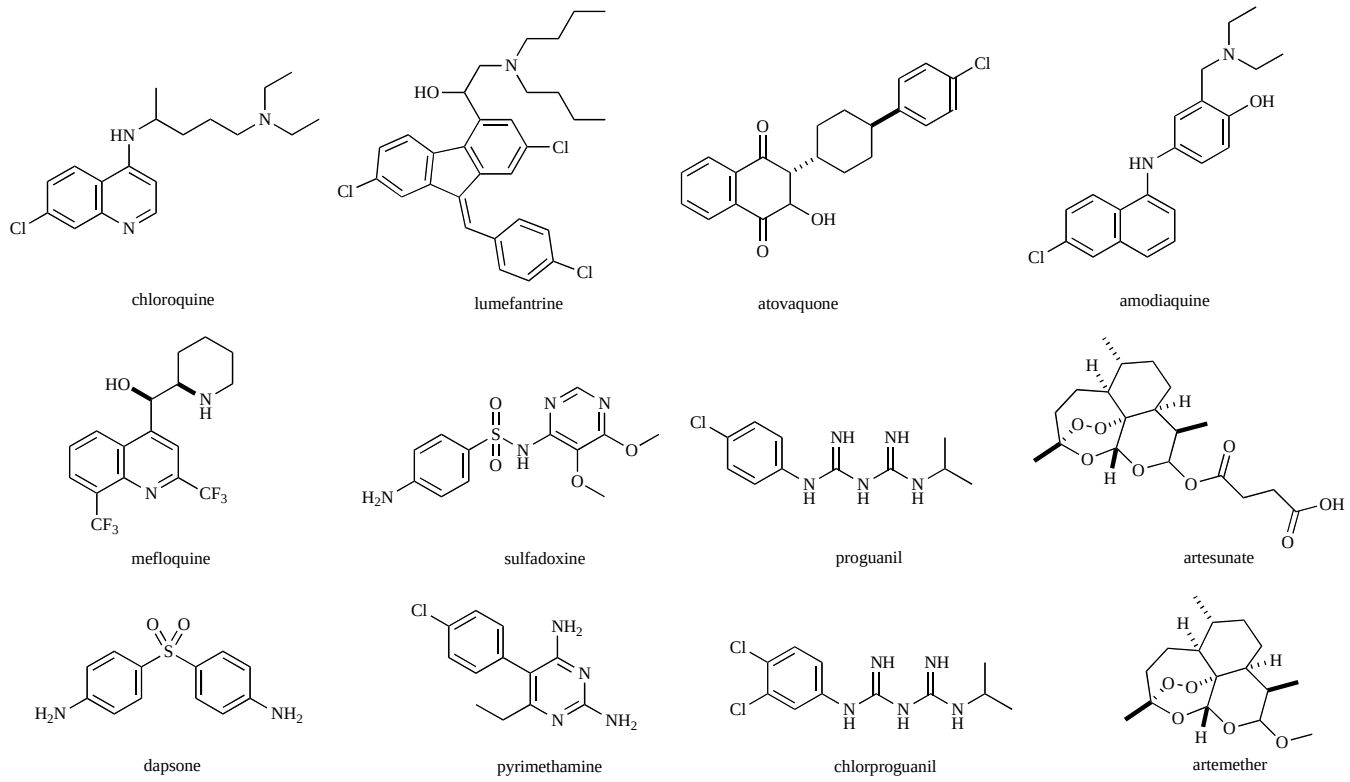


Fig. (5). Molecular structure of approved drugs for malaria. Combinations of theses drugs potentially offer a number of important advantages over monotherapies (*e.g.*, sulfadoxine-pyrimethamine; atovaquone-proguanil; artesunate-mefloquine; amodiaquine-artesunate; mefloquine-artesunate; chlorproguanil-dapsone-artesunate and lumefantrine-artemether).

mode or a new mechanism of action, which is pivotal to combat the most pernicious form of human malaria. Some recent advances toward this goal are presented in the following sections.

Farnesyltransferase inhibitors

Farnesyltransferase (PFT, EC 2.5.1.21) catalyzes the transfer of the 15-carbon farnesyl group from farnesyl pyrophosphate (FPP) to the cysteine residue in the C-terminal motif CAAX (where C: cysteine, A: aliphatic amino acid, X: serine or methionine). The enzyme is involved in several intracellular signal transduction events and has been extensively investigated for the development of novel anticancer agents [122-124]. PFTs were also identified in other eukaryotic organisms including the pathogenic protozoa parasites [125-128]. Hence, inhibition of PFT was suggested as an alternative strategy for the treatment of parasitic infections. Previously, studies on PFT inhibitors have identified and developed compounds with enhanced medicinal chemistry and pharmacokinetics properties [38]. In this context, there has been considerable research into the possible antimalarial activity of drugs designed for other diseases (*i.e.*, *piggy-back* approach) [129-133].

Aiming at discovering innovative non-thiol farnesyl-transferase inhibitors, a ligand-based pharmacophore model was employed to retrieve compounds with the desirable features [134]. Peptidomimetic compounds with a benzo-phenone core were identified as promising lead compounds. Subsequently, several cycles of lead optimization led to development of compounds with enhanced inhibitory potency capable of suppressing the growth of the multiresis-tant *P. falciparum* in the nanomolar range [135-139]. However, when tested against a murine malaria model the inhibitors were inactive. The lack of *in vivo* activity was speculated to be related to the solubility of the compounds. Therefore, new cycles of organic synthesis were performed to generate benzophenone derivatives with charged groups [140].

Nitrogen-containing heterocycles substituents were selected to be replaced into the α-position of the phenyl-acetamide moiety of the lead compound (**18**). This strategy remarkably increased the inhibitor' water solubility (from <0.06 mM for phenylacetamide derivative (**18**) to >3.33 mM for the piperazine analogue (**19**)) (Fig. **6**). Based on its high activity and relative structural simplicity, the inhibitor (**19**) was selected as the new lead compound for further optimization. Previously, investigation on benzophenone derivatives has identified chlorine as an attractive substituent to improve potency [137], thereby, the same approach was carried out yielding compound (**20**) with slightly enhanced potency with respect to the parent compound (**19**) (Fig. **6**). In order to gain further insights into the structural determinants of the binding potency underlying this series, molecular docking and homology model studies were undertaken. The model indicated that the chlorine substituent in (**20**) was orientated into a binding pocket close to the side chain of the His149. Steric hindrance between the piperazinyl moiety and some amino acid side chains of the active site might prevent the chlorophenyl substituent to adopt the optimal conformation to bind to the pocket, thereby reducing the interaction between the chlorine and the side chain of the His residue [140]. In light of this, two approaches were carried out: (i) introduction of a methylene group between the α-position and the phenyl moiety (com-pound (**21**), aiming at improving the interaction between the chlorine substituent and the side chain imidazole group; and (ii) replacement of the piperazinyl moiety by an open chain ethylenediamine substituent (compounds (**22**) and (**23**)), the enhanced flexibility of this substituent would allow for both an optimal chlorine–imidazole interaction as well as for an interaction of the terminal amine with the amino acids in the upper part of the binding site.

The inhibitory activity assays revealed that although compound (**21**) was virtually equipotent against blood stages of *P. falciparum*, it was approximately 30-fold less potent than (**20**) in farnesyltransferase inhibition assays (Fig. **6**). On the other hand, the ethylenediamine derivatives (**22)** and (**23)** displayed considerably improved *in vitro* activity with IC_{50} values of 32 and 30 nM, respectively, approximately 7-fold more active than inhibitor (**20**) (Fig. **6**). In addition, the toxicity profiles of these compounds were assessed against cultured parasites in comparison with a human cell line. The inhibitors displayed significant selectivity towards malaria parasites as well as lack of toxicity of therapeutic doses in mice [140]. Accordingly, this is an important contribution for the development of specific antimalarial farnesyltrans-ferase inhibitors.

Enoyl–acyl Carrier Protein Reductase Inhibitors

Fatty acids play a pivotal role in providing metabolic pre-cursors of biological membranes and represent an important alternative source of energy supply [141]. Inhibition of the fatty acid biosynthesis pathway has been considered an attractive target for drug development [142,143]. Fatty acid biosynthesis is an iterative process beginning with the condensation of acetyl CoA with the growing fatty acid chain. In *P. falciparum* the enoyl-acyl carrier protein reduc-tase (*Pf*ENR, EC 1.3.1.9) is responsible for the final enzymatic step in the elongation cycle: converting trans-2-enoyl-ACP to acyl-ACP in a NADH-dependent reaction [141,142]. The widely used antibiotic triclosan is a potent inhibitor of *Pf*ENR and *Plasmodium* growth both *in vitro* and *in vivo* [144]. Several efforts have been made to optimized the *Pf*ENR inhibitory potency of triclosan, how-ever, only modest inhibitors were developed (micromolar range) [143,145].

With the goal of discovering novel scaffolds with promising antiplasmodial activity, a structure-based virtual screening approach was carried out with the X-ray of *Pf*ENR in complex with triclosan [146]. The ICM molecular docking program [147] was used to screen a drug-like enriched database of 336,600 compounds from the ChemBridge Express Library (San Diego, CA) against the crystal-lographic structure of *Pf*ENR (PDB ID, 1NGH). The virtual screening protocol consisted of several hierarchical steps including (i) theoretical measurements of energy interaction; (ii) similarity index; and (iii) ADME-Tox (absorption, distri-bution, metabolism, excretion, and toxicity) properties prediction (Fig. **7A**). In the first selection step, putative ligands that exhibited ICM scoring better than the cutoff

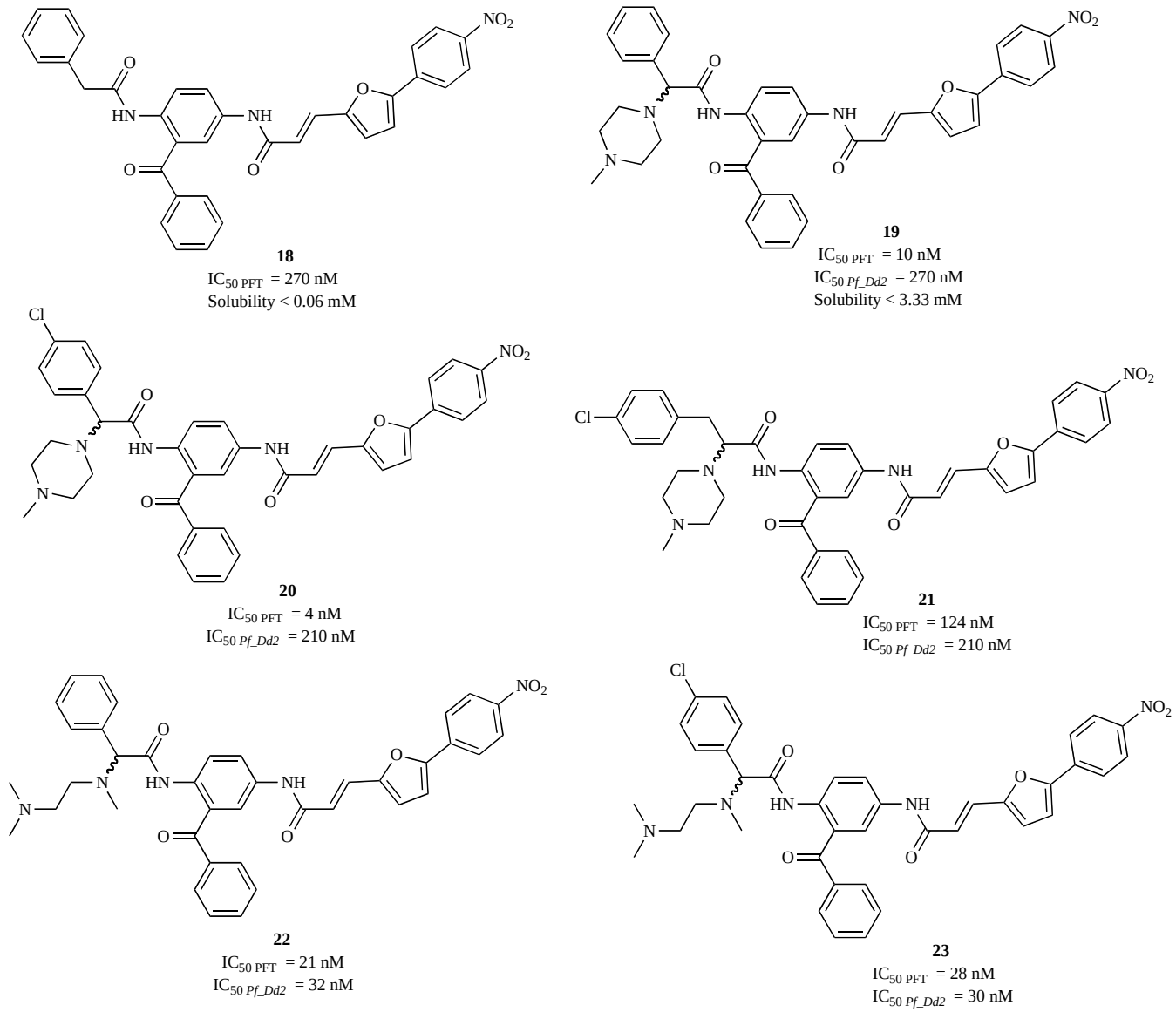


Fig. (6). Benzophenone derivatives developed as potent inhibitor of *P. falciparum* farnesyltransferase. The *P. falciparum* Dd2 strain (*Pf_Dd2*) is resistant to several commonly used antimalarial drugs (chloroquine, cycloguanil, and pyrimethamine).

value of -50 were selected (triclosan was employed as a benchmark for hit scoring, yielding a score of -40). Subsequently, a similarity cluster analysis was applied in order to reduce the redundancy as well as increasing the molecular diversity. Thus, similar compounds were eliminated reducing the initial database to 750 entries. In a final filtering step, the selected compounds were subjected to a theoretical measure of ADME-Tox features to remove compounds with predicted poor pharmacokinetic properties. The remaining 169 candidate molecules were acquired and biologically evaluated. The experimental screening assay against the *Pf*ENR identified 16 hits that showed >45% inhibitory activity at 50 μM. Those compounds were tested in cell-based experiments with two *P. falciparum* strains (*e.g.*, Dd2 and 3D7). Three of them exhibited cell growth inhibition in the micromolar range, the best inhibitor (**24**) exhibited IC_{50} values of 6 μM and 5 μM (Dd2 and 3D7 strain, respectively) (Fig. **7B**) [146].

This study was the first virtual screening strategy that has been applied to a malarial target. The identified compounds represent new leads, potentially suited for oral bioavailability, with inhibitory potency similar to triclosan. Subsequent optimization processes aimed at optimal substitutions with suitable chemical groups should be useful for the design of new structurally related *Pf*ENR inhibitors with improved *in vivo* potency as well as ADME properties.

OTHER RELEVANT TROPICAL DISEASES AND SBDD

Drug discovery programs for other relevant tropical diseases such as schistosomiasis, dengue, and onchocerciasis are not worldwide extended or advanced as the research on the diseases reviewed so far. Those diseases also represent an enormous burden on developing countries, further aggravated by the limited number of available drugs, mechanisms

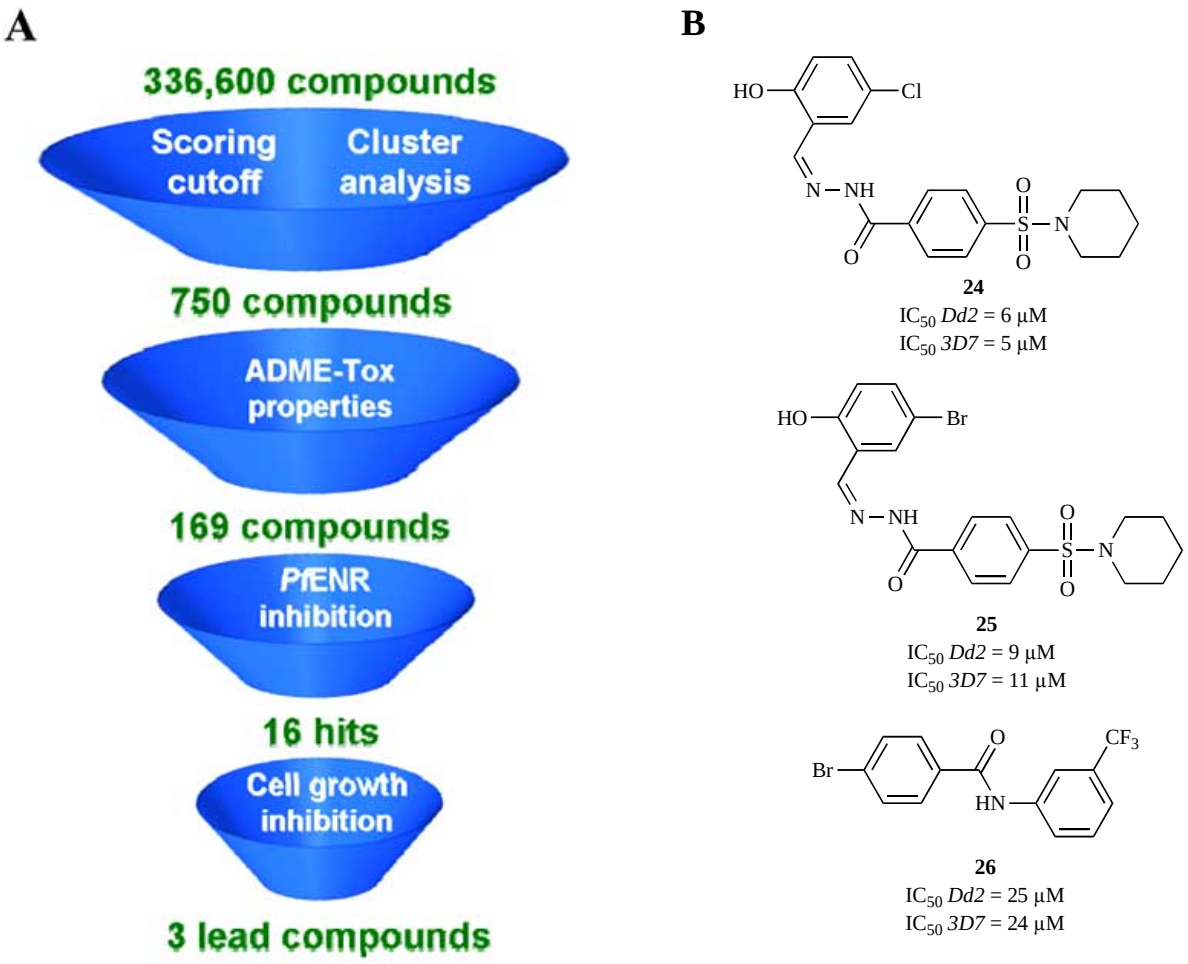


Fig. (7). (**A**) The protocol of consecutive steps applied in the virtual screening approach for novel inhibitors of *Pf*ENR. (**B**) Lead compounds identified by virtual screening.

of drug action poorly understood, and lack of efficacy of the therapeutical regimens. Hence, aiming at delivering new drug candidates for the treatment of these illnesses, some progress has been made in the identification of novel lead compounds.

For instance, the enzyme purine nucleoside phosphorylase (*Sm*PNP, EC 2.4.2.1) from *Schistosoma mansoni*, the causative agent of schistosomiasis, was selected as a molecular target for the identification and kinetic characterization, including potency, affinity and mechanism of action, of new inhibitors [148]. The 9-deazaguanines derivatives were identified as competitive *Sm*PNP inhibitors that exhibited significant inhibitory and selective properties (the best inhibitor (**27**) showed K_i value in the low nanomolar range) (Fig. **8A**). Two of those inhibitors were co-crystallized with *Sm*PNP, providing important insights on the intermolecular interactions responsible for affinity as well as gathering structural information useful for medicinal chemistry efforts in the design of new inhibitors with increased biological properties. Regarding dengue, the enzyme methyltransferase (EC 2.7.7.48) from the *Flavivirus* was explored as a molecular target for a computational and experimental approach involving (i) pharmacophore search; (ii) structure-based virtual screening; and (iii) experimental testing. The strategy identified a novel scaffold, the adamantane derivative (**28**), with IC_{50} value of 60 μM (Fig. **8B**) [149]. Concerning onchocerciasis, which is the world's second leading infectious cause of blindness and caused by the filarial worm *Onchocerca volvulus*, the structure-based development is very limited. There is only one crystal structure with a bound inhibitor for application of structure-based methods (Fig. **8C**). The crystallographic data of the major cytosolic glutathione S-transferase (GST, EC 2.5.1.18) in complex with the competitive inhibitor S-hexylglutathione provided structural insights on the substrate specificities and highlighted chemical features of the binding site that can be explored for the discovery and design of novel anti-filarial compounds [150].

CONCLUSION

In medicinal chemistry and drug design programs, the combination of innovative strategies based structural data plays a central role on our increasing understanding of the fundamental principles of protein-ligand interactions. Thus, besides providing invaluable information about the spatial arrangements of the target receptor, SBDD methods allow the investigation of intermolecular interactions underlying molecular recognition mechanisms and biological activity. In this scenario, the integration of experimental and compu-

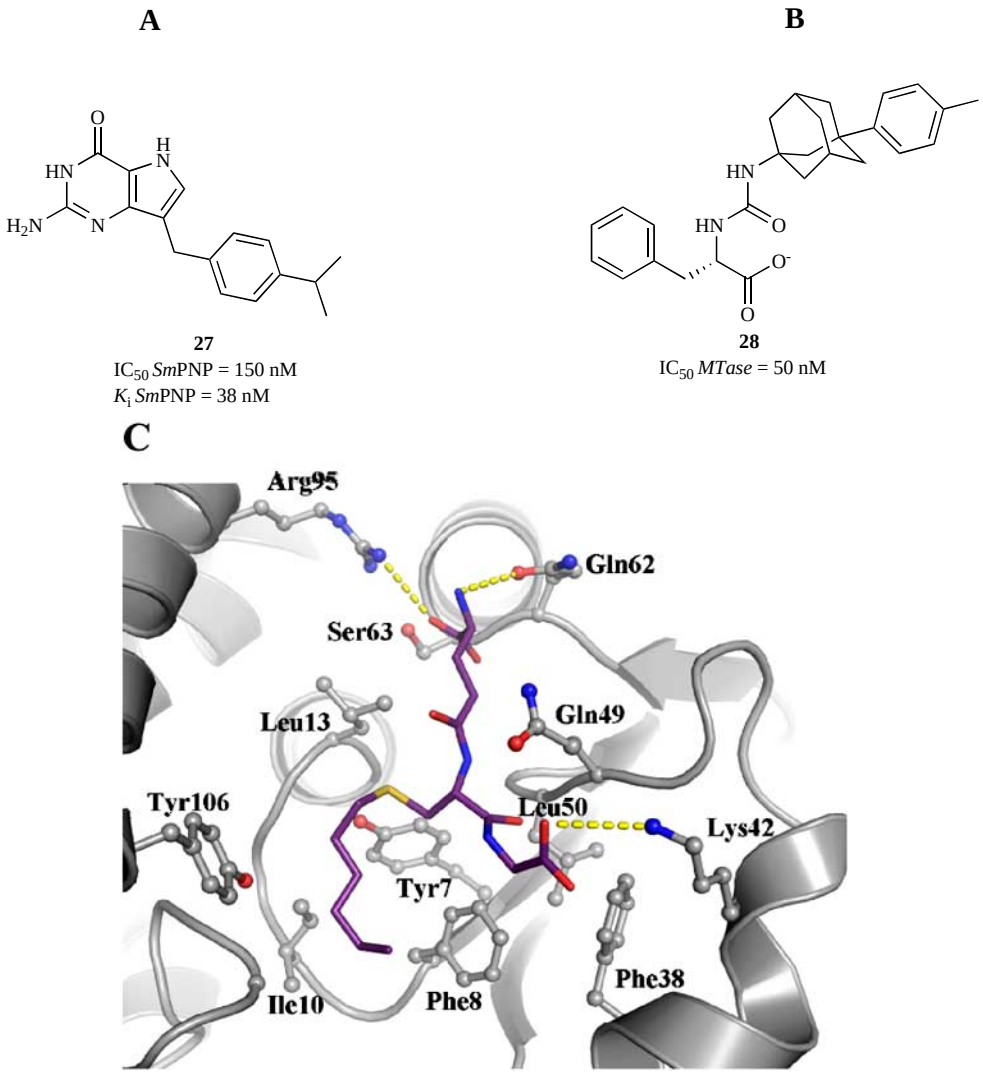


Fig. (8). Identified inhibitors of (**A**) *Sm*PNP and (**B**) *Flavivirus* MTase. (**C**) Crystallographic structures of GST from *O. volvulus* in complex with the competitive inhibitor S-hexylglutathione (PDB ID, 1TU8). The protein is indicated as cartoon and the binding site residues as ball-and-stick model. The ligand is represented as stick model and hydrogen-bond as broken lines.

tational approaches is an important component of modern drug discovery campaigns that possess the potential to boost the early phases of drug research, particularly in terms of time and cost savings. This is the major motivation for the partnerships between the public and private sectors which have remarkably promoted the drug research programs for several tropical diseases. They are stimulating the identification of promising hits, the generation of high quality leads as well as encouraging the successes of moving drug candidates into clinical trials.

In summary, SBBD methods have a great impact on the discovery of new lead compounds for several parasite infections. Very promising enzyme inhibitors were identified and optimized based on iterative cycles of structure-based approaches including biological testing, structure elucidation, computer-aided drug design and synthesis. These molecules represent new drug candidates with potent *in vitro* and *in vivo* activity as well as some improved pharmacokinetic properties. Therefore, the next step in this research activity aims to progress the drug candidates into the development phase.

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