Chapter 2
Selection of Molecular Targets for Drug Development Against Trypanosomatids

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Abstract Trypanosomatid parasites are a group of flagellated protozoa that includes the genera *Leishmania* and *Trypanosoma*, which are the causative agents of diseases (leishmaniases, sleeping sickness and Chagas disease) that cause considerable morbidity and mortality, affecting more than 27 million people worldwide. Today no effective vaccines for the prevention of these diseases exist, whereas current chemotherapy is ineffective, mainly due to toxic side effects of current drugs and to the emergence of drug resistance and lack of cost effectiveness. For these reasons, rational drug design and the search of good candidate drug targets is of prime importance. The search for drug targets requires a multidisciplinary approach. To this end, the completion of the genome project of many trypanosomatid species gives a vast amount of new information that can be exploited for the identification of good drug candidates with a prediction of "druggability" and divergence from mammalian host proteins. In addition, an important aspect in the search for good drug targets is the "target identification" and evaluation in a biological pathway, as well as the essentiality of the gene in the mammalian stage of the parasite, which is provided by basic research and genetic and proteomic approaches. In this chapter we will discuss how these bioinformatic tools and experimental evaluations can be integrated for the selection of candidate drug targets, and give examples of metabolic and signaling pathways in the parasitic protozoa that can be exploited for rational drug design.

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Abbreviations

AK	Adenosine kinase
ALD	Fructose 1,6 aldolase
APRT	Adenine phopsphoribosyltransferase
CatB	Cathepsin B
CDK	Cyclin dependent kinase
CNS	Central Nervous System
CPA	Cysteine proteinase A
CPB	Cysteine proteinase B
CRK	cdc2 related kinase
CYC	Cyclin
CYP51	Cytochrome P-450 51
DHFR	Dihydrofolate reductase
ECK1	ERK-like, CRK-like Kinase-1
ENO	Enolase
G3DPH	Glycerol-3-phosphate dehydrogenase
GD3DPH	Glyceraldehyde 3-phosphate dehydrogenase
GK	Glycerol kinase
GSH	Glutathione
GSK-3	Glycogen synthase kinase 3
GSpS	Glutathionylspermidine
HAT	Human African trypanosomiasis
HGPRT	Hypoxanthine guanine phopsphoribosyltransferase
HK	Hexokinase
kDNA	Kinetoplast DNA
MPK	Mitogen activated kinase
PFK	Phosphofructose kinase
PGI	Phosphoglucose isomerase
PGK	Phosphoglycerate kinase
PGM	Phosphoglycerate mutase
PK	Protein kinase
POS	Posaconazole
PTR	Pteridine reductase
PYK	Pyruvate kinase
SMT	$\Delta^{24(15)}$-sterol methyltransferase
SQS	Squalene synthatase
STE7	Signaling terminal 7 extension
TPI	Triose phosphate isomerase
TryR	Trypanothione reductase
TryS	Trypanothione synthatase
TS	Thymidylate synthase
TXN	Tryparedoxin
WHO	World Health Organization
XPRT	Xanthine phopsphoribosyltransferase

1 Introduction

Trypanosomatid protozoan parasites of the genera *Trypanosoma* and *Leshmania* are infectious agents of important diseases, such as human African trypanosomiasis (HAT or sleeping sickness), Chagas disease and leishmaniases (cutaneous, mucocutaneous and visceral). According to the World Health Organization (WHO), the leishmaniases are endemic in 88 countries, with 350 million people being at risk of acquiring the infection, sleeping sickness occurs in sub-Saharan Africa and threatens more than 60 million people and Chagas disease is prevalent in Latin America and more than 25 million people are at risk of acquiring the infection. Together, these three parasitic diseases represent a huge social and economic burden since approximately 0.5 million people are infected with *T. brucei*, ten million with *T. cruzi* and an estimated 12 million with different species of *Leishmania* (Teixeira et al. 2012), and cause approximately 150,000 deaths annually (Nussbaum et al. 2010).

Since there are no human vaccines yet, the only treatment for these diseases relies solely on chemotherapeutic drugs. For HAT, the first-line treatment for second-stage cases is melarsoprol, a toxic drug in use since 1949. Increase in drug resistance and high therapeutic failure rates have been reported recently in several foci (Robays et al. 2008). Eflornithine is an alternative treatment that is better tolerated, but difficult to administer (Burri and Brun 2003). Nifurtimox, an orally administered cheap drug, has many side effects and is not yet fully validated for use in HAT (Legros et al. 2002). For the treatment of Chagas disease there are two drugs available, benznidazole and nifurtimox, which are highly toxic and ineffective in the chronic phase of infection (Castro and Diaz de Toranzo 1988). For the treatment of leishmaniases, the mostly widely prescribed drug discovered, the pentavalent antimony, was discovered almost a century ago and has various limitations, including serious side effects, prolonged course of treatment and emergence of drug resistance (Croft et al. 2006). Although newer treatments for leishmaniases exist, they are not optimal (Singh et al. 2012). Overall, current chemotherapy for trypanosomatid parasitic diseases has the pre-mentioned serious limitations, including high toxicity, low efficacy, high cost and increasing parasitic resistance. Therefore there is an urgent need to discover new drugs for the treatment of these devastating diseases.

There are several strategies for expanding the repertoire of new antiparasitic agents. Amongst these, one of the most attractive is targeted drug discovery, because in general it requires less investment to develop a molecule against the target. In addition, targeted drug discovery allows lead optimization, which is often essential to make the drug more effective and safer. In this context, identifying a suitable potential target is the first and most important step towards targeted drug discovery.

2 Essential and Desirable Criteria for the Selection of a Trypanosomatid-Specific Drug Target

A good drug target has to meet different criteria. Amongst these criteria some are essential, whereas others are desirable (Fig. 2.1). One of the first essential criteria for drug target selection is its ability to be disease-modifying. For trypanosomatid parasites this means that the target must be essential for the viability of the parasite or its inhibition affects significantly the virulence of the parasite. In this context, it is necessary to select a target that participates in these processes in the "mammalian" forms of the parasite (intracellular amastigote for *Leishmania*, bloodstream form for *T. brucei* and trypomastigotes and amastigotes for *T. cruzi*). Another criterion that is essential for drug target selection is its "druggability", i.e., the likelihood of small molecule drugs being able to modulate the target. In addition, ideally the target must be either found solely in the parasite, or have differences in the drug binding region/active site that can be exploited for inhibiting more potently the parasitic enzyme, thus allowing the identification of compounds with selective toxicity. The identification of targets for achieving parasite/host selectivity is a less difficult task, as

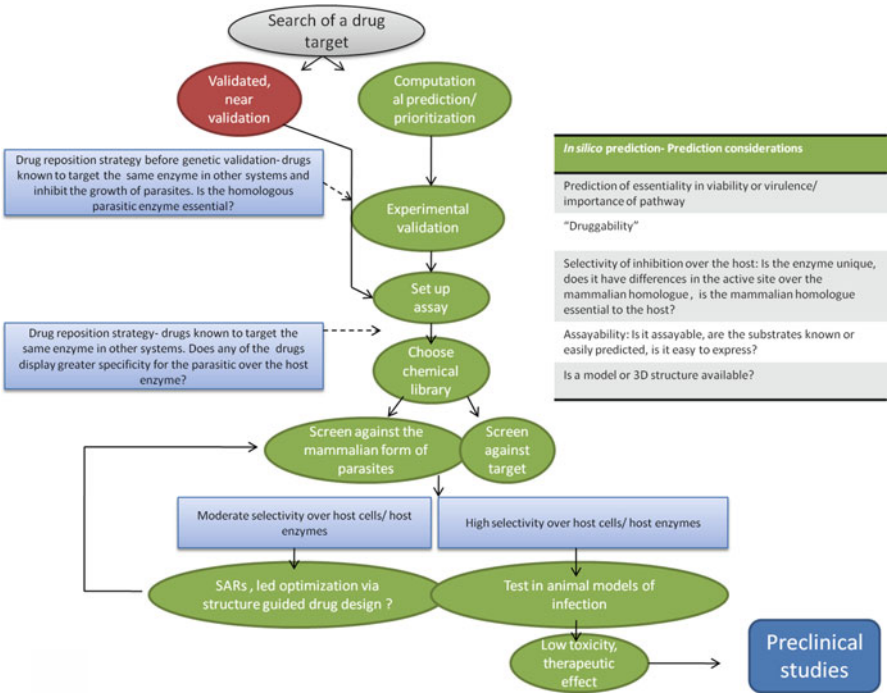

Fig. 2.1 Considerations for the selection of a trypanosomatid-specific molecular target for drug development and representation of procedures required until a drug reaches preclinical trials

potential molecular targets that are essential for the parasite could be redundant and thus not essential in mammalian cells. Moreover, an assayable target is required for performing drug screening. A simple assay that allows high throughput screening is a desirable criterion for drug target selection, facilitating the screening of a large number of compounds. The necessity of an active recombinant protein to perform screening assays has to be taken into consideration and a desirable criterion would be the feasibility to express the protein in its active form. High molecular weight proteins, or proteins that have transmembrane domains might prove difficult to express in an active form. Furthermore, it is useful if a drug target is found in more than one trypanosomatid species and also shares a function that can be disease-modifying in all trypanosomatid pathogens. Finally the presence of an available three-dimensional structure or a model is useful for drug target selection, as it opens possibilities for the development of structure-activity relationship studies (SARs) for lead optimization and structure-guided drug design.

3 Computational Approaches that Aid Drug Target Prioritization

Prerequisite for the identification of a new drug target is its experimental validation. This process is expensive and time consuming. Thus for the selection of a new drug target, an *in silico* approach is often essential. To this end, the completion of the genome project of trypanosomatid pathogens (http://www.genedb.org), including *Leishmania* (*L. infantum, L. major* and *L. braziliensis*) and *Trypanosoma* (*T. brucei* and *T. cruzi*) (Berriman et al. 2005; El-Sayed et al. 2005a, b; Ivens et al. 2005) has opened new possibilities for guided drug discovery. These parasites encode from 8,300 to 12,000 protein genes, of which 6,500 are common to *L. major*, *T. brucei* and *T. cruzi* genomes. In addition, another database, the TriTrypDB (Aslett et al. 2010) (http://tritrypdb.org/tritrypdb/), allows the integration of datasets from *L. braziliensis*, *L. infantum*, *L. major*, *L. tarentolae*, *T. brucei* and *T. cruzi* and enables a user to construct complex queries combining multiple data types. The TriTrypDB database gives information on individual genes or chromosomal spans in their genomic context, including syntenic alignments with other kinetoplastid organisms. The TriTrypDB database has allowed *in silico* metabolic pathway analysis using genome information (Alves-Ferreira et al. 2009). An inventory of predicted metabolic components and integrated metabolic networks of TriTryp is collected in publicly available databases, and a comprehensive review of many of these databases is provided in detail by Myler (Myler 2008). Amongst these, the database BRENDA (Braunschweig Enzyme Database, http://www.brenda-enzymes.org/), contains a plethora of metabolic enzymes that belong to more than 10,000 different organisms, including entries from *T. brucei*, *T. cruzi* and *L. major*. This database includes Enzyme Commission classification, function (including pathways, inhibitors, reaction types and substrates) and structure (including 3D structures), isolation and stability and links to bibliographic references. Another database,

the KEGG (Kyoto Encyclopedia of Genes and genomes, http://www.genome.jp/kegg/), is dedicated to the understanding of high-level function utilities and integrates information and maps of metabolic pathways and associated human diseases, including infectious, and prescription drugs. In addition, it contains enzymatic families and reactions from 2,185 organisms including five trypanosomatids (*L. infantum*, *L. major*, *L. braziliensis*, *T. cruzi* and *T. brucei*). LeishCyc is a pathway/genome database (http://biocyc.org/LEISH/organism-summary?object=LEISH) that captures information about *Leishmania* metabolic pathways from genome annotation and literature resources and organizes this information into a structured database supported by a publicly available ontology. It contains 1,027 enzymes, 573 compounds and 143 metabolic pathways and allows the analysis and visualization of *Leishmania* high-throughput (metabolic 'omics' data), including metabolomics and proteomics (Doyle et al. 2009). In addition, pathway tools available in this database allow the identification of reactions that consume unique substrates or participate in unique reactions, the so-called network "chokepoints" (Doyle et al. 2009), which can be particularly useful for drug target selection.

In silico drug target prioritization for major tropical disease pathogens, including the trypanosomatids *L. major*, *T. brucei* and *T. cruzi*, is now possible and can be implemented by TDRtargets.org (http://tdrtargets.org, Crowther et al. 2010). In TDRtargets.org each criterion is assigned a subjective value and targets earn points of each criterion they meet, in a flexible ranking system that allows BOOLEAN intersection of criteria. The criteria used for drug target selection include the availability of structure (PDB structures or ModBased models), phylogeny (for the selection of unique proteins not present in the host), essentiality, druggability, assayabilty (information regarding the availability of recombinant protein) and specific criteria that are applicable in some species, like location, pathway, expression in distinct morphological stages, phenotype and others (Crowther et al. 2010). In addition, the database is not only restricted to drug targets, but also allows the search for compounds (Crowther et al. 2010). Although computational searches may save money and time for drug target identification, experimental validation is always required. The knowledge of important parasitic pathways for virulence or survival will aid this step. Several of these pathways and drug targets are already validated and herein some examples of these are listed.

4 Examples of Putative or Validated Drug Targets in Parasitic Metabolic and Signaling Pathways

Metabolic pathways in trypanosomatids have peculiarities that may be exploited for targeted drug discovery. Since excellent descriptions of these pathways are present (Lakhdar-Ghazal et al. 2002; de Souza and Rodriguez, 2009; Flohe, 2012), herein we will focus only specific points and data that describe validated or potential trypanosomatid drug targets.

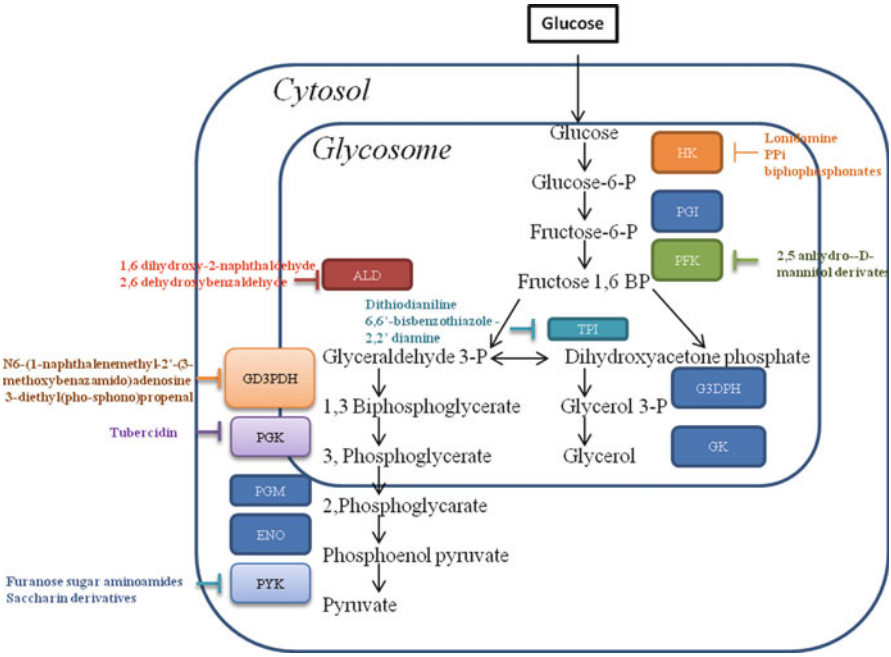

Fig. 2.2 Drug inhibition of the glycolytic pathway in trypanosomatids. The figure shows the glycolytic pathway in trypanosomatids, and drugs known to inhibit the parasitic glycolytic enzymes

4.1 Glycolysis

Glycolysis is an important pathway for drug development in trypanosomatid parasites, not only because it has a critical role for ATP production (the only way for bloodstream *T. brucei*), but also because it has many unique features reflecting both the peculiar glycosomal compartmentalization and differences in structure-activity relationships (Verlinde et al. 2001) (Fig. 2.2). To this end, the available crystal structures of many trypanosomatid glycolytic enzymes have been resolved, revealing significant differences compared to the corresponding mammalian enzymes. These differences point out the importance of glycolytic enzymes as good drug target candidates (Fig. 2.2).

In this respect, the first enzyme of glycolysis, hexokinase (HK), has been evaluated as a potential drug target. HK is encoded by two very similar enzymes in *Leishmania* and *T. brucei* (HK1 and HK2) and one in *T. cruzi*. The hexokinases from *T. brucei* (*Tb*HK1 and *Tb*HK2) have distinct C-terminal domains that result in distinct oligomer formation (Morris et al. 2006). The knocking down of *Tb*HK1 by RNA interference (RNAi) revealed that this protein was essential for viability (Chambers et al. 2008a), and this enzyme was inhibited by lonidamine, a drug that killed effectively *T. brucei* parasites (Chambers et al. 2008b). The hexokinase

from *T. cruzi* (*Tc*HK) displayed significant kinetic differences from its mammalian orthologue, as this enzyme was not inhibited by mammalian HK regulators such as D-glucose-6-phosphate, fructose 1,6 diphosphate, phosphoenolpyruvate, lactate or citrate (Racagni and Machado de Domenech 1983; Urbina and Crespo 1984). In addition, inorganic pyrophosphate (PPi) inhibited this enzyme (Caceres et al. 2003). The synthesis of a series of 42 bisphosphonates confirmed that these compounds could inhibit *Tc*HK, whereby the most potent of which displayed an IC_{50} of 2.2 μM against intracellular amastigotes (Hudock et al. 2006). Another series of non-competitive or mixed *Tc*HK inhibitors, the aromatic arinomethylene biphosphonates, also displayed antiparasitic activity against intracellular amastigotes (Sanz-Rodriguez et al. 2007).

Two other enzymes of the glycolytic pathway, phosphoglucose isomerase (PGI) and phosphofructose kinase (PFK), are promising molecular targets for drug discovery. The crystal structures of PGI from *T. brucei* (Arsenieva et al. 2009) and *L. mexicana* (Cordeiro et al. 2004) and the *Tb*PFK (Martinez-Oyanedel et al. 2007; McNae et al. 2009) have been determined and reveal unique features compared to mammalian orthologues. Moreover, the evaluation of a series of 2,5anhydro-D-mannitol derivatives have been described and determined as inhibitors of the *T. brucei* and *L. mexicana* PFK (Nowicki et al. 2008).

The crystal structure of *L. mexicana* and *T. brucei* fructose 1,6 aldolase (ALD) is also available, and allows the design of specific inhibitors (Chudzik et al. 2000). In this context, selective inhibitors of the trypanosomal over the corresponding mammalian enzyme that belong to the series of 1,6-dihydroxy-2-naphthaldehyde and 2,5 dihydroxybenzaldehyde were designed. However, they displayed low antiparasitic activity, possibly due to their poor potential to cross membranes (Dax et al. 2006).

The trypanosomatid triose-phosphate isomerase (TPI) from *T. cruzi* (Maldonado et al. 1998), *T. brucei* (Wierenga et al. 1991a, b) and *L. mexicana* (Williams et al. 1999) is another glycolytic enzyme with available structure that predicts important differences in the parasitic enzymes compared to the mammalian enzyme. Thus, specific inhibitors of the trypansomatid TPI that are also potent antiparasitics have been developed. More specifically, dithiodianiline and 6,6′-bisbenzothiazole-2,2′ diamine were potent inhibitors of *Tc*TPI and trypanosomatid TPIs respectively, without significantly affecting human TPI (Olivares-Illana et al. 2006, 2007).

The next enzyme in the glycolytic pathway is glyceraldehyde-3-phosphate dehydrogenase (GD3PDH). Trypanosomatid GD3PDH has been also evaluated as a potential drug target. The structures of GD3PDH from *L. mexicana* (Kim et al. 1995), *T. cruzi* (Souza et al. 1998) and *T. brucei* (Vellieux et al. 1993) are resolved and specific inhibitors based on its structure have been designed. A series of adenosine competitive inhibitors that compete for NAD^+ binding with substitutions at the 2′positions of the ribose and N^6 position of adenine inhibited the leishmanial enzyme. In particular, one of the analogs [N6-(1-naphthalenemethyl-2′-(3-methoxybenazamido) adenosine] displayed potent antiparasitic activity at the nanomolar range (Suresh et al. 2001). One of the best inhibitors of *Tb*GD3PDH is 3-diethyl (phosphono) propenal, which kills cultured trypanosomes with an LD_{100} of 300 nM (Willson et al. 1994).

The crystal structure of *T. brucei* phosphoglycerate kinase (PGK) is also available (Bernstein et al. 1997, 1998), and the comparison with the porcine PGK suggested that the ATP/ADP binding pockets display significant differences. The resolved crystal structures suggest that the ATP/ADP binding pockets between porcine and trypanosomatid PGKs display significant differences. Thus, the adenosine analogue tubercidin, which displays antitrypanosomal activity, has been shown to block the *Tb*PGK (Drew et al. 2003). The trypanosomatid enolase (ENO) crystal structure is available, and the enzyme has a more flexible active site from its mammalian counterpart that might allow the design of specific and potent inhibitors (de AS Navarro et al. 2007).

Pyruvate kinase (PYK) catalyzes the last reaction of glycolysis and the enzyme from *L. mexicana* has an available crystal structure (Morgan et al. 2010; Tulloch et al. 2008). From this information, unique features of the effector binding site become apparent that could be used for drug design. Stepwise library synthesis and inhibitor design from a rational starting point identified furanose sugar amino amides (Nowicki et al. 2008) and saccharin derivatives as novel inhibitors of trypanosomatid PYK (Morgan et al. 2012). Overall, these data suggest that the glycolytic pathway enzymes are promising drug target candidates for future studies.

4.2 Purine Salvage Pathway

Trypanosomatid parasites are totally deficient in the *de novo* biosynthesis of purines, and rely on the scavenging from the host (Boitz et al. 2012). Research on purine transport (nucleobase/nucleoside) has focused on the use of purine antimetabolites or specific inhibition of the host nucleoside transporters (de Koning et al. 2005). Moreover the purine transport system can be exploited for the selective transfer of antiparasitic drugs, as in the case of melaminophenyl arsenicals that are efficiently accumulated through the *T. b. brucei* (De Koning 2008). Other genes involved in the purine salvage pathway include: (i) three phosphoribosyltransferases, hypoxanthine guanine phosphoribosyltransferase (HGPRT), xanthine phosphoribosyltransferase (XPRT), and adenine phosphoribosyltransferase (APRT), which catalyzes purine phosphoribosylation, (ii) adenosine kinase (AK) that phosphorylates adenosine (Datta et al. 1987; Iovannisci and Ullman 1984), and a multitude of interconversion enzymes (LaFon et al. 1982; Looker et al. 1983; Marr et al. 1978) (Fig. 2.3). Although none of the enzymes that convert host purine nucleobases or nucleosides to nucleotides are essential, genes in this pathway display properties that allow their exploitation for targeted drug design.

One such example is HPGPRT, which displays differences from the mammalian homologue with respect to substrate specificity (Monzani et al. 2007). Allopurinol, an effective drug against visceral and cutaneous leishmaniasis (Kager et al. 1981; Martinez and Marr 1992) and Chagas disease, is metabolized more efficiently by parasitic HGPRT than the mammalian homologue (Eakin et al. 1997) and therefore incorporated into RNA during transcription, resulting in its degradation and

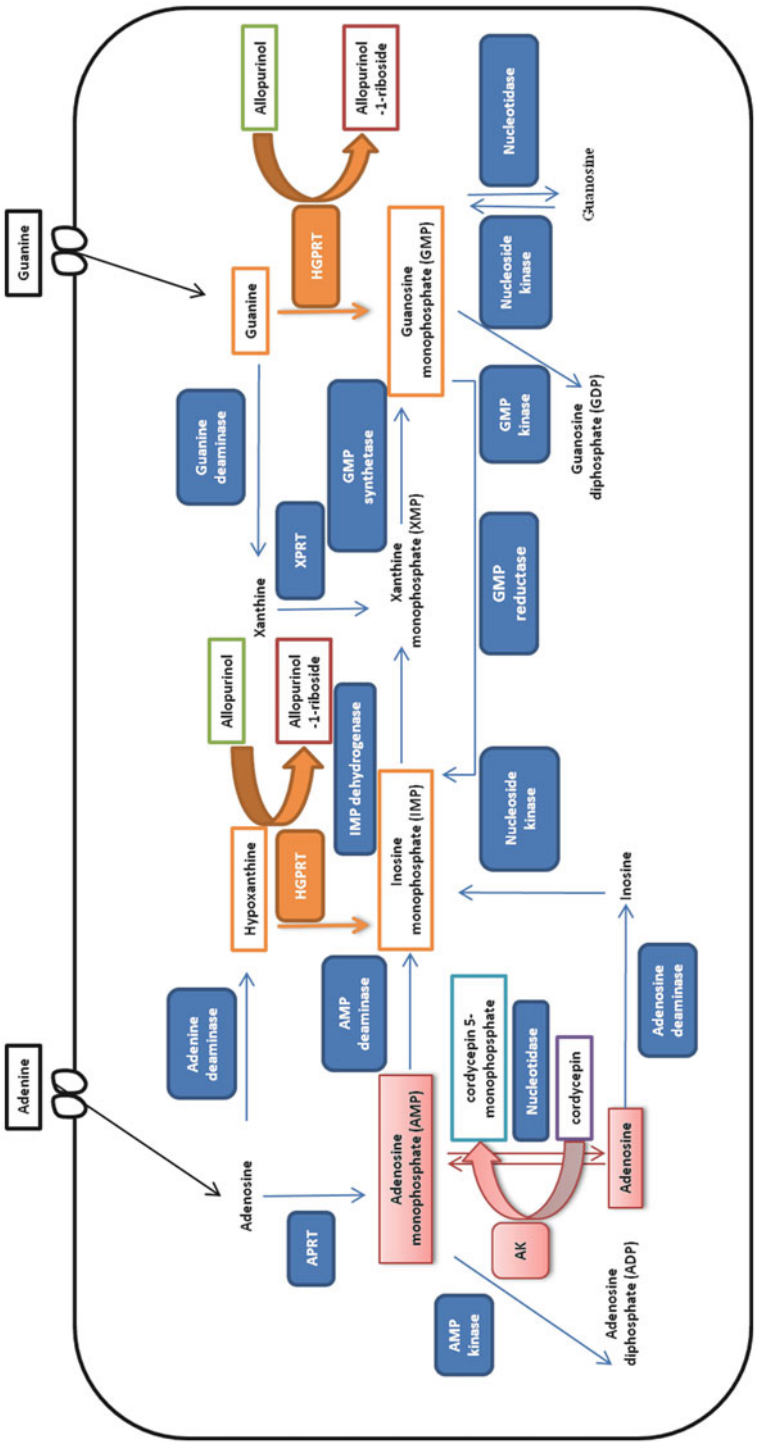

Fig. 2.3 Simplified scheme of the purine salvage pathway in trypanosomatids. The scheme includes drugs which are converted to toxic metabolites by enzymes of the purine salvage pathway

inhibition of protein synthesis (Marr and Berens 1983). Freymann et al., using a structure-based docking method based on the crystal structure of HGPRT from *T. cruzi* (*Tc*HGPRT) provided a remarkably efficient path for the identification of HGPRT inhibitors with potent antiparasitic activity against *T. cruzi* trypomastigotes (Freymann et al. 2000).

Another example involves *T. brucei* AK. This enzyme activates adenosine antimetabolites, like cordycepin (3′-deoxyadenosine), and consequently contributes to their incorporation into RNA, ceasing protein synthesis (Luscher et al. 2007). *In vitro* incubation with cordycepin reduced not only the growth of *T. brucei* but also of *T. cruzi*, as well as *L. major* and *L. amazonensis*, suggesting that cordyceptin is activated by all the corresponding parasitic AKs (Rottenberg et al. 2005). In addition, this drug was active in mouse models of Chagas disease and African trypanosomiasis, when co-administered with deaminase inhibitors (Rottenberg et al. 2005). Thus the purine salvage pathway and the enzymes therein can be "druggable" targets with good potential.

4.3 The Trypanothione System

Trypanothione is a low-molecular mass thiol, unique in trypanosomatids, that adopts the metabolic roles of glutathione (GSH) from other systems. It is implicated in the defense against oxidants, xenobiotics and regulatory proteins and is essential for parasitic survival (Flohe 2012). The biosynthesis and use of trypanothione relies on enzymes that are unique in trypanosomatids and absent from the mammalian host. Trypanothione is known to be synthesized by trypanothione synthetase (TryS) from GSH and spermidine or glutathionylspermidine synthesized by glutathionylspermidine synthase (GSpS). Reduction of oxidized trypanothione is NADPH-dependent and occurs by trypanothione reductase (TryR). Reduced trypanothione then participates in the reduction of glutaredoxin (Ceylan et al. 2010), and dehydroascorbate (Krauth-Siegel and Comini 2008) and tryparedoxin (TXN) (Flohe 2012). The latter mediates the reduction of other regulatory proteins (Flohe 2012). Most of the components of the trypanothione system are essential for parasite viability and allow selective inhibition over the mammalian corresponding enzymes. In addition, the structure of most of the enzymes in this pathway has been determined, making this pathway attractive for therapeutic interventions (Urbina 2010). One of the most attractive targets for drug design is TryR. Numerous reports in the literature exist on the inhibition of TryR, including tricyclic antidepressants and congeners, 2-aminodiphenylsulfides, quaternary alkylammonium, polyamine derivatives and others [reviewed in (Khan 2007)]. However, despite the multitude of TryR inhibitors, only a few were efficacious in animal models of leishmaniasis and trypanosomiasis (Flohe 2012). The differences observed between *in vitro* and *in vivo* antiparasitic effects of TryR inhibitors can be attributed to the pleiotropic effects of these inhibitors (Urbina 2010), to their metabolic instability (Khan 2007) and finally to the fact that redox metabolism of trypanosomatids is normal unless the residual activity of TryR

is below 5 % of wild-type activity (Krieger et al. 2000). Apart from TryR, TryS, the enzyme that catalyzes the synthesis of trypanothione, has been validated as a drug target. Interestingly, compounds that inhibit this enzyme proved to effectively kill *T. brucei* parasites (Torrie et al. 2009). Thus, it is clear that the unique trypanothione system provides opportunities for the development of novel inhibitors with limited "off-target" activity to the host.

4.4 Sterol Biosynthesis

The abundant supply of cholesterol present in their mammalian hosts cannot be used by trypanosomatid protozoa. Instead, *Trypanosoma* and *Leishmania* parasites have a strict requirement for specific endogenous sterols (ergosterol and other sterols) for survival and growth. The main sterols of trypanosomatids belong to the C28-ergostane or C29-stigmastane [reviewed in (de Souza and Rodrigues 2009)]. An exception to this rule is the membrane composition of bloodstream *T. brucei* parasites, which contains predominantly cholesterol, incorporated through a receptor-mediated endocytic process by suppressing the *de novo* ergosterol biosynthesis (Coppens et al. 1988). The sterol biosynthesis pathway is a promising target for the development of new anti-trypanosomatid drugs. Several drugs available today are known to interfere with the sterol biosynthesis pathway (Fig. 2.4).

Amongst these are quinuclidines that target squalene synthase (SQS, E.C. 2.5.1.21), the enzyme that catalyzes the first committed step in sterol biosynthesis (Fig. 2.4). This enzyme is a very attractive target and has been validated not only for treating hypercholesterolhaemia in humans but also for treating trypanosomatid diseases (Suckling 2006; Urbina et al. 2002). Two quinuclidines, ER-119884 and E5700, had potent *in vitro* anti-*Leishmania* (Fernandes Rodrigues et al. 2008) and anti-*T. cruzi* activity and the latter compound provided full protection against death in an *in vivo* murine model of Chagas disease (Urbina et al. 2004). Despite the fact that most SQS inhibitors tested in the parasite also block the mammalian host enzyme, this inhibition is tolerable in mammals. In addition, it has been possible to develop specific antiparasitic SQS inhibitors (Orenes Lorente et al. 2005; Sealey-Cardona et al. 2007), such as aryloxylethyl thiocyanates (WC-9) (Elhalem et al. 2002; Urbina et al. 2003) and 2-alkylaminoethyl-1,1-bisphosphonic acids (Rodrigues-Poveda et al. 2012). Another enzyme in the parasitic sterol biosynthesis pathway is squalene epoxidase (EC1.4.99.7) that converts squalene to 2,3 oxidosqualene. The antifungal drug terbinafine displayed potent antileishmanial activity against promastigotes and intracellular amastigotes by inhibiting this enzyme (Goad et al. 1985; Vannier-Santos et al. 1995). This drug displayed a synergistic effect with ketoconazole, another antifungal drug that also interferes with ergosterol biosynthesis (Vannier-Santos et al. 1995). The same drug combination also displayed a synergistic antiproliferative effect against epimastigotes and amastigotes of *T. cruzi* (Urbina et al. 1988). Lanosterol synthase

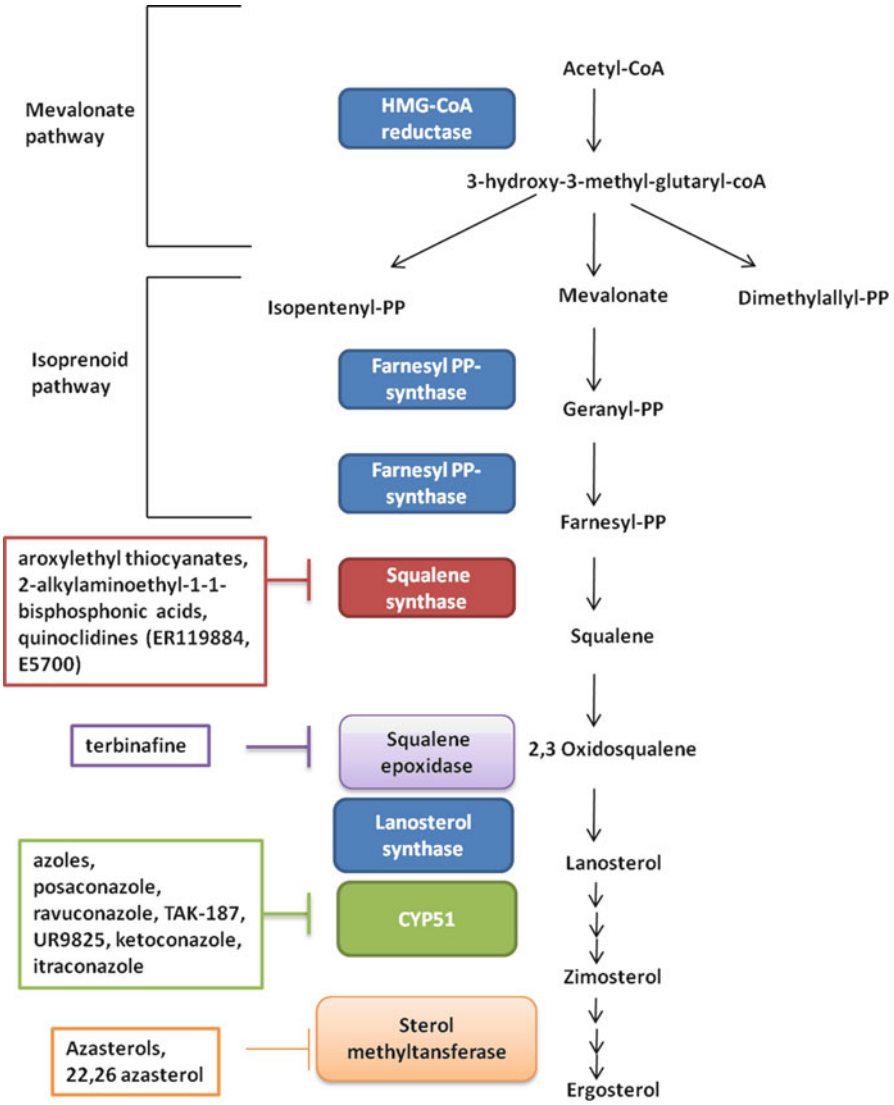

Fig. 2.4 Simplified scheme of sterol biosynthesis in trypanosomatids and known inhibitors of the pathway

(or oxidosqualene cyclase) is a key enzyme in sterol biosynthesis as it catalyzes the cyclization of 2,3 oxidosqualene (Fig. 2.4). This enzyme has already been validated as a chemotherapeutic target against *T. brucei* and *T. cruzi* (Buckner et al. 2001, 2000; Urbina and Docampo 2003).

Another reaction in sterol biosynthesis is the formation of zimosterol from lanosterol. This reaction is catalyzed by C14-demethylase (CYP51), a very diverse enzyme amongst kingdoms that is known to be inhibited by azoles (Urbina 2010). Several studies have shown that commercial inhibitors of yeast CYP51 available for the treatment of fungal infections (like ketoconazole, itraconazole) have a suppressive effect against Chagas disease in humans or in animals (Urbina 2002; Urbina and Docampo 2003). More potent and specific fungal and parasitic CYP51 inhibitors such as D0870 and posaconazole (POS) (Molina et al. 2000), a structural analogue of itraconazole, could completely cure experimental acute and chronic Chagas disease (Urbina et al. 1996a). Posoconazole is now poised to enter clinical trials to evaluate the effectiveness in treatment of Chagas disease (Robertson and Renslo 2011). POS has been tested in leishmaniasis in experimental murine models of leishmaniasis, showing a good efficacy against cutaneous leishmaniasis and to a lesser extent against visceral leishmaniasis due to *L. donovani* infection (Al-Abdely et al. 1999). Other triazoles like TAK-187, UR-9825 and ravuconazole displayed potent antiparasitic activity (Urbina 2010).

Finally, another important enzyme that catalyzes the C24 transmethylation reaction in the sterol biosynthesis is $\Delta^{24(25)}$-sterol methyltransferase (SMT) (EC 2.1.41), which is unique in trypanosomatids, but absent in the mammalian host. This enzyme has been shown to be inhibited by various azasterols, leading to *in vitro* antiproliferative effects against *L. amazonensis* and *T. cruzi* parasites (Lorente et al. 2004; Magaraci et al. 2003; Rodrigues et al. 2002; Urbina et al. 1995). One of the most potent inhibitors of SMT, 22,26 azasterol, displays selective antiparasitic activity in a murine model of acute Chagas disease (Urbina et al. 1996b). Overall, the data presented here suggests that the enzymes within the sterol biosynthesis pathway may be utilized for the design of potent and specific antitrypanosomatid inhibitors.

4.5 Pteridine Metabolism

Trypanosomatid parasites lack a *de novo* pathway for the synthesis of pteridines (folate and pteridines) and rely on salvage from the host (Beck and Ullman 1990). Among the key proteins of the pteridine salvage network that mediates the accumulation and reduction of pteridines, there is a bifunctional enzyme (DHFR-TS) that has activities of dihydrofolate reductase (DHFR) and thymidylate synthase (TS) -unlike the monofunctional enzyme of mammalian hosts-, and pteridine reductase (PTR1) that reduces both folate and biopterin (Fig. 2.5). These two enzymes have been proposed as drug targets in trypanosomatids, since they participate in essential pathways for the parasite metabolism, like thymidylate production by folates and oxidant resistance by the reduction of pterins (Moreira et al. 2009; Nare et al. 2009). Drugs that have been used to inhibit DHFR have proven ineffective in *Leishmania*

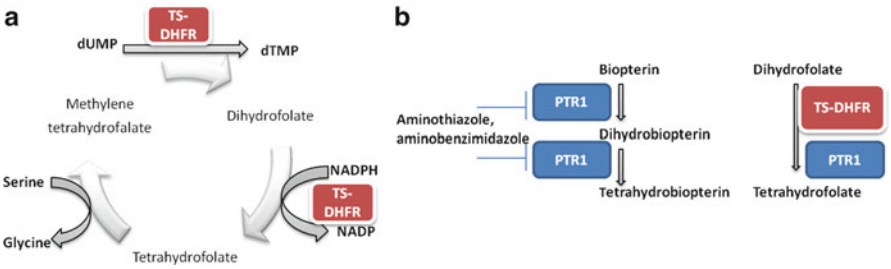

Fig. 2.5 Pteridine metabolism in trypanosomatids. (**a**) Thymidylate synthesis. The figure shows the thymidilate cycle in which thymidilate is synthesized by DHFR-TS, with the use of methylene tetrahydrofolate and its convertion to dihydrofolate. The same enzyme converts dihydrofolate to tetrahydrofolate. (**b**) Contribution of PTR1 to the reduction of pterins and folates and known inhibitors of this enzyme

(Neal and Croft 1984). This observation can be explained by the emergence of drug resistance. As PTR1 may reduce both, pterins and folates, this enzyme can act to override the inhibition of DHFR-TS (Nare et al. 1997). Thus, in *L. major* resistant strains, the amplification of PTR1 was observed (Nare et al. 1997). On the other hand, DHFR-TS is a validated drug target in *T. brucei* (Sienkiewicz et al. 2008), as null mutants had an absolute requirement for thymidine. Removal of thymidine from the medium resulted in growth arrest, followed by cell death and PTR1 was not able to compensate for loss of DHFR activity (Sienkiewicz et al. 2008). Mice infected with DHFR-TS null mutants were incapable of establishing infections (Sienkiewicz et al. 2008). The above suggests that both DHFR and TS activities in *T. brucei* are required for thymidylate synthesis and that thymidine concentrations in the host are limiting. Thus, the targeting of this enzyme could prove fruitful for improving/curing HAT.

On the other hand, research on the inhibition of trypanosomatid PTR1 has been more intense. PTR1 is an essential enzyme for viability, as PTR1 null mutants in *T. brucei* are not viable (Sienkiewicz et al. 2008). Virtual screening identified aminothiazole and aminobenzimidazole inhibitors as potent and selective *Tb*PTR1 inhibitors (Fig. 2.5). Despite the good potency against PTR1 and favorable physicochemical properties, representative members of the scaffolds were not trypanocidal (Mpamhanga et al. 2009). The reason for this lack of correlation between enzymatic inhibition and antitrypanosomal activity is currently under investigation (Mpamhanga et al. 2009). As both PTR1 and DHFR-TS catalyze the same reactions, an attempt to inhibit both of these enzymes would be desirable. A screen against *L. major* PTR1 identified a number of compounds inhibiting both PTR1 and DHFR-TS. Four of these compounds displayed antiparasitic activity, suggesting that the inhibition of both of these enzymes is required for the efficacy of new drug candidates (Hardy et al. 1997).

4.6 Drug Targets in Signaling and Other Pathways Essential for Parasitic Survival

4.6.1 Topoisomerases

Topoisomerases use DNA strand scission, manipulation and rejoining activities to deal with DNA torsional stress, which makes them potential targets for treating parasitic diseases. As topoisomerases are involved in replication, transcription, chromosomal condensation and segregation, inhibitors of these enzymes have a drastic inhibitory effect on the growth of trypanosomatid parasites (Balana-Fouce et al. 2006; Das et al. 2006a; De Sousa et al. 2003; Deterding et al. 2005; Douc-Rasy et al. 1988). These enzymes are grouped into type I and II, depending on their ability to cleave single- and double-stranded DNA, respectively. Trypanosomatid parasites possess both types of topoisomerases (Bakshi and Shapiro 2004; Balana-Fouce et al. 2006; Das et al. 2006b; De Sousa et al. 2003; Douc-Rasy et al. 1988; Strauss and Wang 1990). Type I topoisomerases include type IA and IB subclasses, which are grouped on the basis of differences in their aminoacid sequences and mechanism of action (Champoux and Dulbecco 1972). Topoisomerase IB in trypanosomatids acts as an unusual bi-subunit enzyme, and it is localized in both the nucleus and the kinetoplast (Bakshi and Shapiro 2004; Das et al. 2004).

Topoisomerase inhibitors are often used as anticancer drugs in mammalian cells. These drugs may inhibit topoisomerases via two distinct mechanisms. The first type of inhibition includes the covalent stabilization of DNA-topoisomerase complexes, and the drugs that have this action are referred to as topoisomerase poisons. The second type of inhibition is mediated by drugs that interfere with the active site of the enzyme, and are termed topoisomerase inhibitors (Steverding and Wang 2009). Camptothecins, anthracyclins, epipodophyllotoxins and quinolones are classified as topoisomerase poisons, whereas coumarin antibiotics and forstriecin analogues are topoisomerase inhibitors (Steverding and Wang 2009). Topoisomerases are essential proteins for parasite viability. Thus, topoisomerase inhibitors or poisons have been shown to have activity against trypanosomatid protozoa, mediating apoptosis-like death (Smirlis et al. 2010). Moreover, the antileishmanial compounds sodium stibogluconate and urea stibamine have been shown to act via the inhibition of type I topoisomerase (Chawla and Madhubala 2010). Camptothecin, a drug that acts as a poison against leishmanial topoisomerase I (Das et al. 2006a), has been found to block *L. donovani*, *T. cruzi* and *T. brucei* proliferation, whereas analogues have been screened against *T. brucei* bloodstream parasites and different substitutions have been identified (9,10,11-methylenedioxy analogs) that display a selectivity over the *T. brucei* parasites (Bodley et al. 1995). In addition, the flavones baicalein, quercetin and luteolinhave been shown to stabilize the DNA- *L. donovani* bi-subunit topoisomerase I cleavage complex by a differential mechanism, providing in this way additional insights into the ligand-binding properties of *L. donovani* topoisomerase I (Das et al. 2006b). Besides the abovementioned flavones, three isoflavanoids, 8-prenylmucronulatol, lyasperin H and smiranicin display antileishmanial activity

correlated with topoisomerase II inhibition and kinetoplast DNA (kDNA) linearization (Salem and Werbovetz 2005). Acridine compounds, such as 9-anilinoacridine derivatives, display antiparasitic activity against *Leishmania* and *Trypanosoma* and their mode of action is via the inhibition of topoisomerase II (Figgitt et al. 1992). The topoisomerase II poisons, belonging to the family of anthracycline antibiotics daunomycin and its hydroxyl derivative doxorubicin, had activity against *T. b. rhodesiense* affecting parasite motility and infectivity to mice (Williamson and Scott-Finnigan 1978). Moreover, doxorubicin has been shown to be highly active in an *in vivo* model of visceral leishmaniasis (Sett et al. 1992), its mechanism of action being the inhibition of leishmanial topoisomerase II (Singh and Dey 2007). Another set of topoisomerase II poisons, the fluoroquinolones like KB5426, ofloxacin and ciprofloxacin have been shown to have activity against *T. brucei* (Nenortas et al. 1999, 2003). Finally, aclarubicin, an anthracenedionemotoxantrone derivative that acts as a catalytic inhibitor of topoisomerase II, exhibited potent antiparasitic activity against *T. brucei* bloodstream parasites and a 1,000 fold selectivity index, making this drug a good drug candidate for the treatment of trypanosomiasis (Deterding et al. 2005). Despite the fact that parasitic topoisomerases are essential for survival, and that a multitude of compounds that target these enzymes are available, up to date most of the known topoisomerase inhibitors lack selectivity over the host cells. To this end it is essential to exploit differences between parasite and mammalian topoisomerases.

4.6.2 Proteases

Proteases in trypanosomatid protozoa have been shown to be involved in many important signaling processes, including cell-cycle progression, stage differentiation, autophagy, apoptosis and others (Abdulla et al. 2008; Ambit et al. 2008; Boukai et al. 2000; Galvao-Quintao et al. 1990; Leon et al. 1994; Smirlis and Soteriadou 2011). Amongst the multitude of proteases in parasitic protozoan, cathepsin-like cysteine proteases have been the focus of attention. The cathepsin L-like proteases include CPA and CPB in *Leishmania*, and cruzipain and brucipain (or rhodesain) in *T. cruzi* and *T. brucei*, respectively. On the other hand, the cathepsin B-like proteases are termed CPC in *Leishmania* and *Tb*CatB in *T. brucei*. CPB deletion mutants in *L. mexicana* displayed reduced virulence in BALB/c mice, suggesting that the pharmacological inhibition of this protease may compromise the progression of leishmaniasis (Alexander et al. 1998). Indeed, a peptide that has been characterized as a natural inhibitor of cysteine proteases and a potent inhibitor of CPB led to a reduced virulence and a Th1 response (Bryson et al. 2009). In *L. major*, a class of cathepsin inhibitors, known as aziridine-2,3-dicarboxylate derivatives, induced cell death correlated with the presence of autophagy-related lysosome-like vacuoles (Schurigt et al. 2010).

In *T. brucei*, downregulation of brucipain by RNAi showed that this protease is not essential for growth. However, a vinyl-sulfone inhibitor (K11777, see below) of this protease limited the ability of *T. b. rhodesiense* to cross the blood brain barrier

in brain endothelial cells *in vitro*, suggesting its potential role ameliorating central nervous system (CNS) damage during *T. brucei* infection. Despite the fact that brucipain was not proven to be essential for *T. brucei* viability, *Tb*CatB was required for parasite growth, participating in iron acquisition by degrading host transferrin (Abdulla et al. 2008; O'Brien et al. 2008). Thus, a series of selective purine nitrile inhibitors with enhanced potency for *Tb*CatB were developed via structure-guided optimization. These compounds were trypanocidal, reaffirming the potential of *Tb*CatB as a therapeutic target, whereas a lead compound of the series significantly prolonged the life of infected mice with *T. brucei* (Mallari et al. 2009, 2010).

In *T. cruzi*, cruzipain is required for all the major proteolytic activities of the parasite life cycle (Cazzulo 2002; Urbina and Docampo 2003). Inhibition of this protease by K-11777 resulted in cessation of proliferation in epimastigotes and intracellular amastigotes and arrested metacyclogenesis. In murine models of acute and chronic Chagas disease, the inhibitor significantly reduced parasitemia and prolonged survival, conferring minimal toxicity to the host (Engel et al. 1998). In addition, the same compound displayed therapeutic activities in an immunodeficient mouse model of Chagas disease (Doyle et al. 2007). In a canine model of acute Chagas disease, K-11777 significantly reduced cardiac damage (Barr et al. 2005). Following these promising results in animal models of Chagas disease, the development of K-11777 as a new treatment for Chagas diseases was initiated. Despite the hepatotoxicity and other serious problems with this compound that resulted in terminating the project, this effort demonstrated that cruzipain inhibitors could prove promising drugs for the treatment of Chagas disease. Thus, attempts to identify potent and specific inhibitors of cruzipain were initiated (Brak et al. 2008; Caffrey et al. 2000; Du et al. 2002). Structure-activity relationship studies (SARs) demonstrated that thiosemicrbazone and semicarbazone scaffolds are potent and selective cruzipain inhibitors (Du et al. 2002; Guido et al. 2008). Moreover, several other classes of non-peptidic inhibitors have been identified, including vinyl-sulphone containing macrocycles (Brak et al. 2008), aryl oxymethylketone inhibitors (Brak et al. 2008) and quinoxaline-N-acyl hydrazone inhibitors (Romeiro et al. 2009). Thus, cruzipain is a confirmed drug target, and research for the identification of a good cruzipain inhibitor suitable for future drug development is ongoing.

Overall, parasitic proteases are enzymes with a potential to be good drug target candidates and thus further investigation on their role in the parasitic life-cycle, essentiality and druggability is required.

4.6.3 Kinases

In view of the success in targeting eukaryotic protein kinases (PKs) in other disease contexts (notably cancer), parasitic kinases are considered attractive drug targets. Moreover, additional reasons exist that justify the selection of parasitic kinases as ideal drug targets. These include the fundamental role of parasitic kinases and phosphorylation cascades in critical parasite pathways for survival and virulence, such

as cell-cycle progression, differentiation and others and the divergence between the kinomes of the parasites and of their human hosts, which allows the design of drugs that may specifically target the parasitic kinase (Naula et al. 2005). Despite the pharmaceutical interest on host protein kinases, protozoan PKs remain largely underexplored as targets for neglected diseases. Interestingly, tryapanosomatids have a significant number of PK genes, with 179 genes present in *L. major*, 156 in *T. brucei* and 171 in *T. cruzi* (Naula et al. 2005). Several of these kinases, notably members of the CMGC group of kinases, have already been validated as drug targets for the treatment of parasitic diseases. Amongst these are the cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MPK) and glycogen synthase kinase 3(GSK-3) (Grant et al. 1998; Hassan et al. 2001; Tu and Wang 2004; Wang et al. 1998, 2005; Wiese 1998, 2007; Ojo et al. 2008; Xingi et al. 2009).

The number of MPK homologues in *Leishmania* is rather large, with 15 putative mitogen activated protein kinases (MPK) identified, along with 13 in *T. brucei*, several of which are two-copy genes (Wiese 2007). Several of these kinases are characterized, including *Tb*ECK1 (the homologue of *Lmx*MPK6) (Ellis et al. 2004), *Lmx*MPK11 and *Lmx*MPK13 (Marshall and Rosenbaum 2001; Bengs et al. 2005), with no evidence up to date that they could serve as molecular targets for the treatment of trypanosomatid diseases. On the other hand, MPK5 is a validated drug target, since a null *Lmx*MPK5 mutant displayed an impaired ability to cause lesions in BALB/c mice infected with *L. mexicana* (Wiese 2007). The *Lmx*MPK5 homologue in *T. brucei*,*Tb*MPK5, has been found to be involved in the differentiation of bloodstream forms to stumpy forms, and a null mutant of this kinase displayed a massive reduction of parasitemia (16-fold lower) in immunosuppressed mice (Domenicali Pfister et al. 2006). Moreover, a null *Lmx*MPK5 mutant displayed an impaired ability to cause lesions in BALB/c mice infected with *L. mexicana*. Thus, these data suggest that trypanosomatid MPK5 might be exploitable as a target for chemotherapy against human sleeping sickness and leishmaniases. Another MPK, *Lmx*MPK1, has been shown to be essential for cell-cycle progression and survival in *L. mexicana* amastigotes in a murine model of leishmaniasis, representing thus a functionally validated drug target (Wiese 1998). In addition, the related kinase *Lmx*MPK2 also represents a putative drug target, as it is shown that it is required for the establishment of infection and in important processes in the mammalian amastigote stage (Wiese 2007). One of the most studied and validated target that belongs to this group of kinases is *Lmx*MPK4. This enzyme is essential for viability in *L. mexicana*, as *Lmx*MPK4 null mutants could not be generated (Wang et al. 2005). The same kinase displays enhanced activity upon exposure to pH 5.5 and 37°C in *L. major* promastigotes and *L. donovani* axenic amastigotes, the natural stress signals that occur during the differentiation of the parasite from the insect to the mammalian stage (Morales et al. 2007). In addition, a new *E. coli* based expression system was generated for *Lmx*MPKK5 that included a STE7-like protein kinase from *L. mexicana*, required for its activation. This effort is the basis for the development of drug screening assays of *Lmx*MPK4 (John von Freyend et al. 2010).

Despite the promising results that suggest that targeting MPKs in trypanosomatid parasites could provide a disease-modifying mechanism, another group of CMGC

kinases, namely the CDKs, has attracted more attention towards the validation of its members as potential drug targets. This family is well represented in trypanosomatids, with 11 members in *L. major* and *T. brucei* and 10 members in *T. cruzi* (Naula et al. 2005). Trypanosomatid CDKs contain cyclin-binding regions as anticipated (Naula et al. 2005), whereas the presence of a cyclin (CYC2, CYC3, CYC6 for *T. brucei*, and CYC1, CYCA and CYC6 for *Leishmania*) for the activity of the trypanosomatid homologue of mammalian CDK1 (CRK3) has been shown (Wang et al. 1998; Van Hellemond et al. 2000; Li and Wang 2003; Hammarton et al. 2003, 2004; Tu and Wang 2004; Banerjee et al. 2006; Gomes et al. 2010). The function of two kinases from *L. mexicana* and *T. brucei*, namely CRK1 and CRK3, have been analysed in detail. In *L. mexicana*, the activity of *Lmx*CRK1 was shown to be restricted to the promastigote stage, whereas its role in amastigotes has not been investigated in detail (Grant et al. 2004). In *T. brucei*, downregulation of CRK1 by RNAi in bloodstream and procyclic forms led to reduced growth and increase in the number of cells in the G1 phase of the cell-cycle, demonstrating the importance of this kinase in regulating the G1→S transition (Tu and Wang 2004). Despite these results, the validation of this kinase as a drug target in both *Leishmania* and *Trypanosoma* still remains an open issue.

On the other hand, CRK3 has been validated as a drug target in trypanosomatid parasites and has been studied in great detail compared to other parasitic CDK kinases. As mentioned above, CRK3 is considered to be the functional homologue of mammalian CDK1. Initially, the functional homology of the *L. major* CDK, CRK3 to *Schizosaccharomyces pombe* CDKs was demonstrated by the functional complementation of this kinase in a temperature-sensitive *S. pombe* cdc2/CDK1 null mutant (Wang et al. 1998). The gene for *L. mexicana* CRK3 was shown to be essential for viability, as it is a crucial regulator of cell division. CRK3 activity was found to peak in the G2/M phase of the cell cycle, and inhibition of CRK3 *in vivo* resulted in cell cycle arrest (Hassan et al. 2001). The same effect on cell-cycle progression was observed upon the downregulation of *Tb*CRK3 in *T. brucei* procyclics and bloodstream parasites (Tu and Wang 2004). Grant et al. previously described the screening of a diverse chemical library of antimitotic compounds for potential inhibitors of *Leishmania* CRK3 (Grant et al. 1998). However, despite the potent effect of these inhibitors on the leishmanial kinase, they failed to show selectivity over the mammalian CDK1–CYCB complex (Grant et al. 2004). Recent studies identified *Leishmania* CRK3–CYC6 inhibitors belonging to the class of azapurines, with micromolar potency, following a high-throughput screen with heterocyclic and kinase libraries (Walker et al. 2011). However, potent inhibitors of CRK3-CYC6 did not always display antiparasitic activity (Cleghorn et al. 2011). This suggests that CYC6 might not be the cyclin that *in vivo* is required for the function of CRK3 (Cleghorn et al. 2011). Further work is required to delineate the discrepancy between the drug inhibition of CRK3 and the lack of antiparasitic activity of certain inhibitors.

Apart from the MPK and CDKs of the CMGC kinase family, as mentioned above, GSK-3 has attracted attention, constituting a model candidate for drug target selection. GSK-3 is a multifunctional serine/threonine kinase of the CMGC family,

found in all eukaryotes. This enzyme is known to play a key role in many cellular and physiological events, including Wnt[Int and Wg (wingless) in *Drosophila*] signaling, transcription, cell-cycle and differentiation, neuronal functions and circadian rhythm (Phukan et al. 2010). These functions of GSK-3 and its implication in many human diseases, such as Alzheimer's disease, non-insulin-dependent diabetes mellitus and cancer, have stimulated an active search for potent and selective GSK-3 inhibitors (Phukan et al. 2010). Two GSK-3 genes, GSK-3s (short) and GSK-3l (long), are encoded in the *T. brucei* and the leishmanial genome. Of the two isoforms, the short isoform has been analysed in both *T. brucei* and *L. donovani* (Ojo et al. 2008; Xingi et al. 2009). Previous studies demonstrated that leishmanial GSK-3s was essential for parasitic viability, and its inhibition causes cell-cycle defects and apoptosis-like death (Xingi et al. 2009). Moreover, RNAi experiments showed that *Tb*GSK-3s is essential for viability in bloodstream forms of *T. brucei* (Ojo et al. 2008). These results validate this kinase as a drug target for the treatment of leishmaniasis and HAT. Apart from the essentiality in the life-cycle of trypanosomatid parasites, parasitic GSK-3s has many desirable characteristics that justify its selection as a drug target. More importantly, inhibitor scaffolds and inhibitors of parasitic GSK-3 have been identified that also show good antiparasitic activity (Oduor et al. 2011; Ojo et al. 2011, 2008; Xingi et al. 2009). Indirubins, a class of bis-indole compounds known for over a century as a minor constituent of plant, animal and microorganism-derived indigo, represent inhibitory scaffolds targeting *Ld*GSK-3s (Xingi et al. 2009). More specifically, a series of 6- and 5- halogen substituted indirubins, were tested against promastigotes and intracellular amastigotes (Xingi et al. 2009). Four of these compounds displayed antileishmanial activity against *L. donovani* promastigotes and intracellular amastigotes. All of the analogues with antiparasitic activity inhibited both CRK3 and *Ld*GSK-3s. Of these, 6-Br-5methylindirubin-3′oxime inhibited 7 times more potently *Ld*GSK-3s (IC_{50} 90 nM) and killed effectively promastigotes and intracellular amastigotes ($IC_{50} < 1.2$ μM). Interestingly, although 6 bromo-substituted indirubins are powerful ATP competitive inhibitors of mammalian GSK-3 (Polychronopoulos et al. 2004), they displayed a high selectivity index because the inhibition of host kinase is tolerable in adult mammals (Henriksen et al. 2003; Kaidanovich-Beilin and Eldar-Finkelman 2006). Furthermore, since GSK-3 has also been pursued as a human drug target, a large number of inhibitors are available for screening against parasites. To this end, a collaborative industrial/academic partnership facilitated by the World Health Organization Tropical Diseases Research division (WHO TDR) was initiated, to stimulate research aimed at identifying new drugs for treating HAT. In this context, a subset of 16,000 inhibitors that target human GSK-3β from the Pfizer compound collection were screened, and potent and selective inhibitors of *Tb*GSK-3s were identified (Oduor et al. 2011).

The continuation of a drug discovery program by the synthesis of more potent and selective inhibitors of parasitic GSK-3s is possible because critical differences exist in the ATP-binding pocket between parasite and human GSK-3 (Ojo et al. 2011; Xingi et al. 2009). More specifically, the two major differences in the leishmanial enzyme that are conserved in *T. brucei* are the replacement of Gln185*h*GSK-3β

by His155*Ld*GSK-3s in the sugar-binding region, and the replacement of the "gatekeeper"Leu132*h*GSK-3β by Met100*Ld*GSK-3s (Xingi et al. 2009). In addition, the crystal structure of *L. major* GSK-3s is now available that allows more accurate structure-activity relationship studies for future drug design.

Thus, with respect to parasite essentiality, assayability for high throughput screening, the availability of crystal structure, the existence of differences in the active site in comparison to its mammalian homologues, the redundancy of mammalian GSK-3β function in the mammalian host and the tolerance of its inhibition, and finally the presence of a multitude of compounds known to inhibit parasitic GSK-3s, make this enzyme an excellent "model for drug target selection". Research into parasitic GSK-3s kinases provides an excellent opportunity to develop a targeted drug therapy for leishmaniases and HAT.

5 Investigating Drug Repurposing Opportunities for Drug Target Identification and for Antiparasitic Drug Discovery

The process of finding new uses for existing drugs (marketed drugs and failed or idle compounds, or drugs whose mammalian targets are known) outside the scope of the original indication is variously referred to as repositioning, redirecting, repurposing, and reprofiling. Nowadays, as more and more pharmaceutical companies are exploring the existing arsenal of known drugs for repositioning candidates, the number of repositioning successful stories is steadily increasing (Padhy and Gupta 2011).

Thus, the exploitation of drugs whose targets have already been discovered in other systems can offer advantages for both drug target identification and antiparasitic drug discovery. For drug repositioning strategy, in order to facilitate the access of information concerning therapeutic targets, there are publicly accessible databases, such as DrugBank, Potential Drug Target Database, Therapeutic Target Database, and SuperTarget. These databases complement each other to provide target and drug profiles (Zhu et al. 2010). Moreover, it is now possible to reposition marketed drugs to novel targets and vice-versa. This can be achieved by a java-based software called IDMap (Ha et al. 2008). The next step is to gain access to compound libraries.

This step is challenging and the choice of a compound library is critical for the discovery of a potential drug target in protozoa. Despite the fact that genetic validation will be required for assessing the essentiality for viability or virulence of a potential drug target, this approach saves time and aids researchers to avoid laborious and high-cost techniques, like genetic manipulation (i.e. targeted gene deletion) over targets that are not essential for parasite viability (Fig. 2.1). This approach has been used to validate *Ld*GSK-3s as a drug target for the treatment of leishmaniasis by using known inhibitors of mammalian GSK-3. These inhibitors displayed both antiparasitic activity and activity against *Ld*GSK-3s. The inhibitory activity of *Ld*GSK-3s, as the leishmanicidal mechanism of action, was further validated by the over-expression of *Ld*GSK-3s in *L. donovani* promastigotes, and the demonstration

that *L. donovani* over-expressing *Ld*GSK-3s were protected from the leishmanicidal effects of indirubins (Xingi et al. 2009).

Drug repositioning can be also a good strategy once a target is selected (Fig. 2.1), for identifying compounds that represent potential attractive starting points for a drug discovery program. The *T. brucei* GSK-3s has provided an excellent example for this strategy and the screening of a compound library from the Pfizer compound collection as mentioned above, known to target mammalian GSK-3β,resulted in the identification of inhibitors that target better the parasitic *Tb*GSK-3s (Oduor et al. 2011).

Finally, drug repurposing offers an additional advantage for the choice of drugs that are marketed. For these drugs, information already exists concerning their clinical safety data, pharmacokinetics, and viable dose range are available at the start of a development project, and the risks associated with clinical development are significantly reduced with fewer failures in the later stages. One such example is amphotericin B, an antifungal agent that creates membrane leaks by binding to ergosterol used to treat thrush, and is used today as an antileishmanial agent (Hartsel and Bolard 1996). Commercial inhibitors of yeast CYP51, an enzyme that is also present in trypanosomatids, have proved to have antiparasitic effects. One such example is posaconazole (Noxafil) a broad spectrum antifungal, is also a prime candidate for clinical trials in patients with Chagas disease (Robertson and Renslo 2011).

6 Concluding Remarks

There are many opportunities for the selection and/or identification of novel drug targets for drug development against diseases caused by trypanosomatid parasites. These include methods, like systems biology and network analysis, which integrate biochemistry and cell biology with genetics, as well as bioinformatics and computational biology to obtain holistic descriptions at the organism level. A good drug target gives the opportunity to develop safer drugs at a lower cost. Today the process to predict and validate drug targets is faster than before. This is due to the advances in *in silico* drug target prediction, in the completion of the genome project of trypanosomatid pathogens, and in systems biology. Despite this, researchers that are new to the field have to have in mind that a multitude of trypanosomatid (potential) drug targets are already validated or close to validation. In addition to these molecular targets, a variety of attractive drugs that inhibit many of these enzymes exists, which could serve as promising scaffolds for the synthesis of more potent and selective antiparasitic agents. However, despite these advances, there seems to be little interest to make new drugs for the neglected diseases caused by trypanosomatid parasites. This cannot be attributed to a lack of scientific knowledge. It is more likely that the lack of interest to develop antiparasitic drugs is attributed to the low probability of pharmaceutical companies to have a profitable financial return. Thus, a targeted response is required for an adequately funded, needs-driven priority R&D agenda to combat these devastating diseases.

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