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Drug target identification in protozoan parasites

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Abstract

Introduction—Despite the fact that diseases caused by protozoan parasites represent serious challenges for public health, animal production and welfare, only a limited panel of drugs has been marketed for clinical applications.

Areas covered—Herein, the authors investigate two strategies, namely whole organism screening and target-based drug design. The present pharmacopoeia has resulted from whole organism screening, and the mode of action and targets of selected drugs are discussed. However, the more recent extensive genome sequencing efforts and the development of dry and wet lab genomics and proteomics that allow high-throughput screening of interactions between micromolecules and recombinant proteins has resulted in target-based drug design as the predominant focus in anti-parasitic drug development. Selected examples of target-based drug design studies are presented, and calcium-dependent protein kinases, important drug targets in apicomplexan parasites, are discussed in more detail.

Expert opinion—Despite the enormous efforts in target-based drug development, this approach has not yet generated market-ready antiprotozoal drugs. However, whole-organism screening approaches, comprising of both *in vitro* and *in vivo* investigations, should not be disregarded. The repurposing of already approved and marketed drugs could be a suitable strategy to avoid fastidious approval procedures, especially in the case of neglected or veterinary parasitoses.

Keywords

Apicomplexa; drug design; Kinetoplastida; mode of action; repurposing; target

1. Introduction

Novel anti-infective drugs are of crucial importance because of the steady increase in resistance development against well-established antibiotics, and due to the emergence of novel, previously unnoticed infectious diseases. This also holds true for protozoan parasites, which have been a threat to human and veterinarian health since the dawn of mankind. Examples include, among others, apicomplexan parasites (e.g. *Plasmodium*, *Toxoplasma, Cryptosporidium* and many more), diplomonadids (*Giardia* sp.), axostylata (*Trichomonas*

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sp.), kinetoplastids (*Leishmania* sp., *Trypanosoma* sp.) and amoebazoans (e.g. *Entamoeba histolytica*). Since only a small number of drugs is on the market to confront a plethora of parasites, the overall supply of alternative treatment options is limited [1], and this is a critical issue especially in cases where resistance development has taken place. Therefore, novel and more specific drugs are needed to supplement the current supply of antiparasitic compounds.

Currently, two strategies of antiparasitic drug development are being followed. The first strategy is based on the initial screening of compound libraries employing suitable *in vitro* culture models, followed by studies in a suitable *in vivo* model. Investigations on potential targets are carried out after the efficacy of a given compound has been confirmed. Currently, all antiparasitic drugs that are available on the market have been identified through this approach, thus this strategy still provides a major source of drug candidates [2-4]. In the case of neglected diseases, where a specific drug development approach has not been regarded as affordable since there is no market return for the enormous investments, an elegant variation of this strategy is the repurposing of available drugs or drug candidates [5]. A good example for drug repurposing is the classical aminoglycoside paromomycin recently approved against leishmaniasis in India [6]. In the post-genomic era, a second strategy has come into the focus, namely target-based drug design [7], in which the identification of potential drug targets occurs by *in silico* methods including genome and proteome data base mining prior to any whole-organism-based tests. From a theoretical point of view, this strategy is attractive, but in reality it has so far not led to the expected breakthrough in drug development, neither in antiparasitic drug development nor in drug development in general [8]. In this review, we present recent efforts concerning the identification of drug targets in protozoan parasites by both strategies.

2. Targets identified through whole organism screening-based antiprotozoal drug development

2.1. General remarks

Up to date, all drugs available for antiparasitic chemotherapy (see examples listed in Table 1) have been derived from whole organism screening approaches, which are initially performed using *in vitro* culture systems. During the first steps of such a screening, potentially interesting drug candidates are identified via determination of proliferation inhibition constants (EC_{50}), and the therapeutic indexes are assessed by exposure of selected mammalian cell lines. Then, the most promising candidates are tested in (a) suitable *in vivo* model(s) based on experimentally infected rodents in most instances. For many veterinary applications, however, promising drug candidates are often directly assessed in the relevant target host. Next steps in this classical drug development approach are preclinical or “translational” studies including ADME, pharmacokinetics, pharmacodynamics, and extensive safety and toxicology studies in two animal species. These preclinical studies constitute a major hurdle in drug development, and a majority of promising lead compounds fail at this stage simply due to the fac that they exhibit properties such as restricted absorbtion and bioavailability, unfavourable pharmacokinetics, insufficient exposure at the anticipated target site(s), and toxicity values that indicate adverse side effects already at this

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preclinical stage. Those compounds that pass these preclinical trials will then undergo clinical trials (phases I to III) in healthy and diseased patients. The correct management of *in vitro* (see e.g. [9]) and *in vivo* test systems is thus of paramount importance.

2.2. Screening systems

In the case of extracellular parasites, e.g. trophozoites of *G. lamblia*, or the blood stages of *Plasmodium falciparum* or *Trypanosoma* sp., the set-up of a screening system is easy since the effect of candidate compounds can be monitored simply by measuring the vitality of the parasites through counting or by a quantitative vitality tests [1]. Care must be taken, however, that the screens are performed on parasite stages that are relevant for the disease. For instance, antimalarial compounds have been almost exclusively assessed against the erythrocytic stage of *P. falciparum*. However, for the eradication of other *Plasmodium* species, compounds should ideally also be effective against the persisting liver stage. To test for effectiveness against the liver stage, *in vitro* and *in vivo* models have been established, which are based on *P. berghei* infecting mouse hepatocytes *in vitro* and *in vivo* [10] and on *P. vivax* infecting human liver chimeric mice [11]. These models were shown to be suitable for screen-based antimalarial drug discovery [12]. For high-throughput screenings of compounds against extracellular parasites such as helminths [13], the evaluation of motility by image analysis is a suitable tool. Similar methods have been employed for screenings with *T. cruzi* in a myoblast cell line [14], *L. donovani* in human macrophages, and has been suggested for screenings with *Plasmodium* sporozoites [15]. Transgenic strains expressing reporter genes such as beta-galactosidase in the case of *T. gondii* [2] or *N. caninum* [16] or YFP in the case of *T. gondii* [17] have been established as versatile tools for high-throughput screenings of compounds effective against intracellular parasites [18]. However, only the proliferative tachyzoite stage of these parasites can be assessed *in vitro*, and in order to determine the effects of drugs against the cyst-forming bradyzoites, *in vivo* studies are required.

An example for a successful novel drug discovered via a screening approach is the antileishmanial miltefosine. Discovered in the early 1990s [19], this phospholipid has been approved for treatment of human leishmaniasis in 2004 in the EU and a successful phase 4 trial has been conducted in India [20]. Inspired by the success of artemisinin and its derivatives against malaria, the screening of natural compound libraries is also regarded as a valuable tool for antiprotozoal drug discovery. This is exemplified by the establishment of high-throughput screening platforms for natural product-based drug discovery against human African trypanosomiasis, leishmaniasis, and Chagas disease [21].

2.3. Identification of drug targets – selected examples

An inherently difficult aspect of whole organism screening-based drug development is the identification of drug target(s) and the elucidation of the mode of action of a given compound. To identify targets, biochemical methods (e.g. pull-down studies with immobilized drugs) and genetic approaches (e.g. analysis of resistant parasite strains) are employed, and these have been recently extensively reviewed in [1]. Different approaches can produce contradictory results. Some selected examples are presented in Table 1 and discussed in the following section.

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One of the first anti-malarials, quinacrine, is generally considered to intercalate into DNA, and to thereby inhibit replication and transcription in various organisms including *E. coli* [22]. Another mode of action is the inhibition of adenosine uptake and of ATP-incorporation into RNA [23]. Other well-described drugs that target DNA are ethidium bromide, which has been used against trypanosomiasis in African cattle since the 1950s [24], and pentamidine and its derivatives, originally developed against trypanosomiasis [25-27]. Such amidine-containing compounds are DNA minor groove binders with a broad spectrum of activities against human and veterinary pathogens such as *Giardia* [28]), *Trypanosoma* and *Leishmania* [29], *Plasmodium* [30], *Toxoplasma* [31] and *Neospora* [32]. However, DNA is ubiquitously present and thus represents a rather unspecific target that could be affected in both, parasites and host cells. Interestingly, two essential organelles, namely the kinetoplast in trypanosomatids [25] and the apicoplast in apicomplexa [33], contain circular DNA, and this may increase the susceptibility to DNA intercalators at concentrations which do not affect host cells. In trypanosomes, *Leishmania* and related organisms, the mitochondrial kinetoplast DNA network typically contains minicircles that encode for guide RNAs that edit transcripts from the mitochondrial genome. Similar to the genomic DNA of *Plasmodium,* these DNA minicircles exhibit greater AT content (> 75%), with extensive, closely spaced, sequences that act as strong and selective binding sites for these dicationic compounds [34]. In trypanosomes, pentamidines and other DNA binding compounds induce the cleavage of DNA minicircles in a pattern similar to topoisomerase II inhibitors [25]. In *Plasmodium*, quinacrine resistant strains were shown to exhibit mutations in a transporter, suggesting that susceptible strains export quinacrine less effectively than resistant strains [35]. On the other hand, resistance formation in *T. brucei* against pentamidine was shown to be based on the downregulation of the expression of a parasite adenosine transporter, thereby inhibiting drug uptake [36]. Thus, differential uptake/excretion may also be a resistance factor for host cells. Furthermore, DNA may not be the unique target of this type of drugs, and other targets have been described for dicationic pentamidine derivatives, including microtubules, acidocalcisomes, and enzymes involved in lipid metabolism (reviewed in [34]).

The fluoroquinolone ciprofloxacin, effective against various prokaryotes through the inhibition of topoisomerase II [37], is also active against protozoan parasites such as *Giardia* [38] and some apicomplexans including *T. gondii* [39], most likely due to the presence of prokaryote-like enzymes in these protozoa. The presence of prokaryote-like structures in the translation machinery of protozoans renders protozoan parasites also susceptible to aminoglycoside antibiotics that bind to 16-S-rRNA of the small ribosomal subunit, as shown for *Giardia* [40] and for apicoplast-associated ribosomes of apicomplexans [39, 41]. The macrolide spiromycin [42] and the lincosamide clindamycin [39], both used against *T. gondii*, are examples for antibiotics targeting the large ribosomal subunit of apicoplast ribosomes.

Albendazole and other benzimidazoles have a broad spectrum of activity on evolutionary distant organisms including fungi, protozoans such as *Giardia*, and helminths (reviewed in [43]). Susceptibility correlates to the presence of specific alleles of the beta-tubulin gene [44], especially a Phe in position 200.

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The semiquinone buparvaquone has been used for the treatment of cattle suffering from tropical theileriosis caused by *Theileria annulata* and East Coast fever caused by *T. parva* since the early 80s [45], and has since then remained the only treatment option. Although single dose injections applied at the onset of infection have been shown to be effective, more recent reports have now documented the appearance of resistant strains with point mutations in the cytochrome bc gene, coding for the ubiquinone reductase of the respiratory chain [46, 47]. Recently, a completely different target for buparvaquone, namely a prolyl-isomerase involved in the suppression of host cell apoptosis, has been identified [48]. Since buparvaquone affects the viability of the intracellular schizont already after 2 hours of treatment [49], it is, however, unlikely that this prolyl-isomerase represents the primary target. More recently, buparvaquone was demonstrated to exhibit outstanding activity against *N. caninum* tachyzoites *in vitro* [50] and in a pregnant neosporosis mouse model [51]. Since both targets are present in the mammalian host cell as well, it is unclear why apicomplexans such as *T. annulata* or *N. caninum* are highly susceptible without any discernible host cell toxicity.

A similar situation has been observed in the case of artemisinin and derivatives. The commonly accepted mode of action is the reduction of the peroxide bridge by mitochondrial reductases, namely xanthine reductase in apicomplexan parasites, but not or only to lesser extents in the host [52]. Moreover, as evidenced by studies in yeast, artemisinin derivatives also cause the blocking of calcium channels thereby interfering with intracellular signaling [53]. Artemisinin derivatives are not specific for apicomplexan parasites. Depending on their structure, they are also effective against helminths [54] and proliferating mammalian cells [55], thereby offering novel tools for anti-cancer chemotherapy .

One of the most widely used coccidiostats, monensin, is a polyether ionophore causing uncoupling of membrane gradients, and in no way specific for coccidian or other apicomplexan [56-58]. It is therefore likely, that the higher susceptibility of apicomplexans as compared to their host cells is due to an additional mode of action, such as the cell cycle arrest mediated by a mitochondrial DNA repair enzyme, as evidenced in *T. gondii* [57].

3. Target-based drug design for anti-protozoan chemotherapy

3.1. General remarks

As discussed above, the definition of the precise mechanism of action of a given compound identified through whole organism screening is difficult, especially in cases where multiple targets are hit by the same compound. If homologues of these drug targets are also expressed within the host tissues, adverse side effects are more likely to occur. The scientific community therefore favors a strategy, which is regarded as more rational, namely target-based drug design. An ideal drug target is expressed solely within the pathogen or has a low degree of similarity to homologous host proteins and carries out a functional activity that is essential for the survival of the parasite. The wide range of pathogen genomes that have been sequenced and are openly accessibly to date can be exploited for comparative genomics and transcriptomics analyses and enable researchers to identify parasite-specific targets. In fact, due to the evolutionary distance between protozoan and their mammalian hosts, both have highly diverging proteomes and harbor different biochemical pathways. If these biochemical

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pathways are essential for stages that are relevant for the pathogenicity, they should constitute ideal targets for chemotherapeutics [59].

Sequencing efforts have allowed identifying such diverging pathways as well as the key enzymes in a variety of protozoan parasites. The classical strategy that is followed in a target-based drug development approach is depicted in Table 2. Gene knock-out studies are most valuable tools for the identification of suitable targets, but can only be performed if suitable knock-out systems are available. Powered by bioinformatics tools and high-throughput screening methods including robotics, functional, structural and *in vitro* studies yield valuable information concerning the interaction of selected targets with ligands, in general functional inhibitors.

3.2. Selected examples

A classical paradigm for parasite specific pathways and thus potential targets is the apicoplast in apicomplexan parasites. The apicoplast is of secondary endosymbiotic origin with “plant-like” and bacterial characteristics, and contains a circular DNA genome of ~35kb that originates from an ancient cyanobacterium and a eukaryotic alga. This non-photosynthetic plastid is essential for parasite survival and codes for less than 50 proteins, most of which are for basic metabolic processes such as replication, transcription and translation. The apicoplast represents an evolutionary remnant, which is targeted by a variety of compounds that have been identified earlier [41] as detailed in the previous section.

During the last two decades, many studies following the strategy detailed above have been performed, mainly on targets expressed in *Plasmodium* sp. As shown in Table 3, most of the studies have been focusing on proteins with enzymatic functions. These include enzymes involved in energy metabolism such as glycolysis, intermediary metabolism (e.g. nucleoside biosynthesis), proteolytic enzymes, or enzymes involved in signal transduction such as calcium dependent kinases or phosphodiesterases (Table 3). These and other kinases as potential targets in protozoal parasites are extensively discussed elsewhere [60]. Interestingly, similar targets have been identified along the course of classical antiprotozoal drug development (as shown in Table 1), however with a notable exception: despite the fact that nucleic acids have been regarded as valuable targets for several classes of anti-infective drugs (see Table 1), target based studies based on these macromolecules are scarce (see e.g. [61]), most likely due to methodological difficulties.

A good paradigm for an enzyme from intermediary metabolism as a target is dihydroorotate dehydrogenase (DHOOD), a key enzyme of pyrimidine biosynthesis. Although pyrimidine biosysnthesis occurs ubiquitously, plasmodial and human DHOODs are divergent enough to allow the design of inhibitors specific for the plasmodial enzyme [62]. One inhibitor issuing from a target based screening program, DSM265, is highly selective for plasmodial vs. mammalian DHOODs, effective against blood and liver stages in vitro and in vivo, well tolerated in animal models and safe in first studies in humans [63].

Calcium dependent protein kinases containing calmodulin-like domains (calmodulin-like domain protein kinases; CDPKs) are present in many organisms. Due to their evolutionary origin, apicomplexan parasites contain CDPKs of a type commonly found in plants [64], but

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not in their mammalian host. *Toxoplasma* has more than 20 CDPKs, *Plasmodium* sp. and *Cryptosporidium* sp. have less than 10. Several of these CDPKs have been shown to play vital roles in protein secretion, invasion, and differentiation [65]. These kinases thus have the two features required for a successful target based drug development, i.e. they are essential for the parasite and absent in the host. The functions and structures of various Class I CDPKs have been analyzed. Based on these findings, a particular class of inhibitors, the bumped kinase inhibitors (BKIs) with bulky C3 aryl substituents entering a hydrophobic pocket in the ATP binding site thus acting as ATP competitive inhibitors, has been developed. First studies have shown that BKIs inhibit *T. gondii* CDPK1 at low nanomolecular levels and interfere with the infection of cells at early stages [66]. Based on these findings, a library of BKIs has been generated and successfully tested against *T. gondii* [67] and *C. parvum* [68]. BKIs selectively inhibit CDPK1 from apicomplexans in a good structure-activity-relationship [69, 70] but do not inhibit mammalian kinases because they have larger amino acid residues adjacent to the hydrophobic pocket, thereby blocking the entry of the bulky C3 aryl group. In *Plasmodium* sp., BKIs do not affect intra- and extraerythrocytic stages in humans, but inhibit the sexual stages, namely microgametocyte exflagellation, oocyst formation and sporozoite production, necessary for transmission to mammals, in mosquitoes [71]. CDPK1 is, however, essential for microneme secretion, host cell invasion, and egress of *T. gondii* [72] and thus constitutes a potential target in *T. gondii* and related apicomplexans such as *N. caninum, Cryptosporidium,* and – most recently – *B. bovis* [73]. Some BKIs, especially BKI-1294, exhibit good efficacy both against *N. caninum* CDPK1 in functional assays and against *N. caninum in vitro* [74]. Moreover, BKI-1294 is effective against acute neosporosis [74] and toxoplasmosis [75] *in vivo* and achieves a good protection against vertical transmission of *N. caninum* in a pregnant mouse model [76]. BKI-1294 may also constitute a suitable tool against cryptosporidiosis since it prevents shedding of *C. parvum* oocysts in artificially infected immune suppressed mice [77] and in calves [78].

4. Conclusion

In the previous sections, we have presented the two principal strategies of anti-protozoal drug development, namely whole organism based screenings and target based drug design. All anti-protozoal drugs currently on the market originate from whole organism screening approaches and target fundamental biological functions such as replication, transcription, translation, respiration, and in the case of apicomplexan parasites essential functions of the apicoplast. The case studies and the examples listed in Table 3 show that – when judiciously handled – target-based drug design represents a useful tool for the development of novel anti-parasitic drugs. The main line of targets currently studied is focused on proteins with enzymatic functions including metabolic enzymes, and protein kinases involved in intracellular signaling. Why does this not yield more success? The following section will give some explanations.

5. Expert opinion

Target-based design of antiprotozoal compounds is based on the following hypotheses: (a) the pathogen contains specific proteins or organelles that (i) are absent in the host, or (ii)

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homologs are present in the host but in a more vulnerable location in the parasite, or (iii) or homologs are present in the host but with higher expression levels or a greater redundancy; (b) these targets are essential; (c) by structural analysis of the targets, anti-target compounds can be designed; (d) anti-target compounds are effective against the pathogen *in vitro*, and the effectivity is target-dependent; (e) *in vivo,* anti-target compounds reach their targets without being metabolically inactivated; (f) anti-target compounds (or their *in vivo* metabolites) have no, or negligible, off-target effects on the host.

Due to the availability of powerful *in silico* tools, and the significant advances in molecular and biochemical and structural analysis, the points (a - d) are verified in most of the target-based antiprotozoal drug development studies, as exemplified in Table 3. One should, however, not forget that *in silico* methods based on sequence comparison of homologous proteins bear the danger that small differences in the primary structure may have dramatic consequences on the functions of respective proteins. *G. lamblia* trophozoites, for instance, express two homologous nitroreductases with a high degree of similarity, but apparently differing functional activities: one is activating nitro drugs such as metronidazole, the other one inactivating them, as recently demonstrated by suitable *in vitro* models [79]. In addition, target-based screening is focused on one enzymatic activity, and compounds that would potentially be useful for chemotherapeutical applications are not detected when using this approach, simply due to the fact that they might interact with a different target.

More difficulties arise with respect to points (e) and (f). *In vivo* studies are expensive, subjected to strict regulations, and need specific personal skills. In order to validate a target based-strategy, as well as for candidate drugs derived from whole organism screening approaches, animal experimentation using appropriate models is necessary. The compound pharmacokinetic properties and bioavailability can only be established *in vivo.* Moreover, off-target effects that have been invisible during the preceding *in vitro* studies can be evidenced. These off-target effects can be due to the presence of target-related macromolecules in cell types for which suitable *in vitro* tests do not exist or do not have been carried out, or due to effects of metabolites of the target-specific inhibitor that has been tested.

Taken together, the “theoretical” advantages of target-based antiprotozoal drug design over the classical whole organism screening are diminishing when the two approaches are being put to the test in more realistic situations. On the other hand, due to recent developments in candidate drug library design (see e. g. fragment based screening [80] and the probe-like compounds in the MMV malaria box; www.mmv.org), *in vitro* test systems [9] together with QSAR analysis as first steps, and appropriate biochemical tools for subsequent target identification, whole organism screening is far from being discontinued as a drug design strategy, and this not only in the case of anti-infective agents. Especially in the case of neglected diseases that challenge human and veterinarian health, where *de novo* drug development would be too fastidious, drug repurposing combined with whole organism screening is a suitable way to rapidly identify novel active compounds, as exemplified by a high-throughput screening of natural compounds against neglected tropical diseases [21]. The screening of natural compounds includes, however, the subsequent need to identify the

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active ingredient and resynthesize the product, and is therefore not a straightforward way to novel anti-protozoal compounds.

Moreover, many antiprotozoal drugs do not target specific parasite organelles such as the apicoplast, but the mitochondrion (see Table 1). The question is why host mitochondria are not, or less, affected by these compounds. The answer to this may pave the way to novel antiprotozoal compounds that interfere in mitochondrial activity.

Last but not least, the highest hurdle in anti-parasitic drug development is not the choice of the approach that is used for identifying active drug candidates, but economic aspects concerning the development of these candidates into market-ready drugs. These aspects represent a major driving force in our society. The current hurdles set by regulatory agencies for approval of novel drugs are incredibly high, which leaves pharmaceutical companies to focus on the development of drugs that promise a high market return on the investments. However, for parasitic diseases, especially tropical neglected diseases, there is no, or only little, market return on these investments. As a consequence, even an ideal drug candidate that affects an ideal target in a parasitic organism is worthless, if the investments for developing this compound for the market are not provided. Collaborative efforts between national, supra-national and international health agencies, regulators, academic institutions, financing institutions and interested pharmaceutical companies could provide a solution for this issue.

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Article highlights Box

- Only a limited panel of drugs against protozoal parasites is on the market.
- These drugs were discovered by empirical studies on fundamental aspects of parasite biology, or by whole organism screening approaches.
- Many of these drugs target cellular processes such as replication, transcription, translation, respiration, and are therefore prone to side effects.
- By focusing on proteins that are specifically encoded by protozoan parasites, target based drug design aims to provide compounds with good efficacy and large therapeutical indexes.
- Despite the enormous efforts in target-based drug development, this approach has not yet generated market-ready antiprotozoal drugs. However, whole-organism screening approaches should not yet be disregarded.

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Table 1

Selected examples of antiprotozoal compounds identified by whole organism screening approaches. BS, biosynthesis.

Target	Compound	Mode of action	Organism	Refs
Replication and transcription	Quinacrine	Intercalation into DNA	*Plasmodium* sp., *G. lamblia.*, bacteria	[22, 81]
	Ethidium bromide	Intercalation into DNA	*Trypanosoma* sp.	[24]
	Fluoroquinolones	Inhibition of gyrases and topoisomerases	*G. lamblia.*	[38]
			T. gondii	[39]
	Apicidin and derivatives	Inhibition of histone deacetylase	*P. falciparum, T. gondii*	[82]
			Cryptosporidium sp., Eimeria sp., Plasmodium sp., T. gondii	[83]
	Cyclic tetrapeptides	Inhibition of histone deacetylase	*N. caninum, P. falciparum, T. gondii*	[84]
Translation	Aminoglycosides	Inhibition by binding to 16S-rRNA	*G. lamblia*	[40, 85]
			C. parvum	[86]
			Leishmania sp.	[87]
	Macrolides	Inhibition by binding to 23S-rRNA	*T. gondii*	[88, 89]
Cytoskeleton	Benzimidazoles	Inhibition of tubulin polymerization by binding to monomers of specific beta-tubulin alleles	Nematodes	[44]
			Encephalitozoon sp., G. lamblia, T. vaginalis	[90]
	Taxanes	Stabilization of microtubules	*Leishmania* sp.	[91-93]
Membranes	Fatty acids	Uncoupling by proton channeling	*G. lamblia*	[94]
	Miltefosine	Induction of cell death by affecting membrane integrity	*Leishmania* sp., tumours	[95-97]
	Monensin	Ionophore	various, including host cells	[56, 98, 99]
Fatty acid BS	Thiolactomycin	Inhibition of plastid acyl carrier protein	*P. falciparum*	[100]
Folic acid BS	Sulfonamides	Inhibition of H2-pteroate synthase	*T. gondii*	[42]
			T. brucei	[101]
Pyrimidine BS	Toltrazuril	Inhibition of dihydroorotate synthase	*Eimeria* sp.	[102]
Respiration	Atovaquone	Inhibition of cytochrome bc1	*T. gondii*	[103]
	Buparvaquone	Inhibition of cytochrome bc1	*T. annulata*	[46, 47]
	Decoquinate	Inhibition of cytochrome bc1	*T. gondii*	[103]
			Plasmodium sp.	[104, 105]
various	Metronidazole	Reduction to toxic intermediates under anaerobic conditions by nitroreductases	*G. lamblia, T. vaginalis*	[106]
	Artemisinin and derivatives	Reduction to toxic intermediates under aerobic conditions by xanthine oxidase in mitochondria	*Plasmodium* sp.	[52, 53]

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Table 2

Target based design of compounds active against protozoan parasites.

Step	Input	Output	Tools
Identification of suitable targets by *in silico* approaches.	Sequenced genomes of host and parasite.	List of coding sequences of essential proteins (or RNA) from the parasite.	Genome libraries, sequence alignment software. *In silico*
Expression of recombinant targets in suitable systems (e.g. *E. coli*).	Coding sequences.	Recombinant proteins.	Molecular cloning, protein biochemistry.
Structural analysis and design of compounds binding to active sites.	Crystallized proteins.	Structure of active center and of suitable ligands.	Structure biology. Synthetic medical chemistry.
Functional assays with a library of potential ligands.	Recombinant target, compound library.	Quantitative structure activity relationship (QSAR) in a functional assay system.	High-throughput functional assay system, robotics, tools for statistics.
In vitro tests	Compounds with the most promising QSAR.	Series of compounds suitable for *in vivo* studies, i. e. with the greatest therapeutical indexes. (TI).	Cell based in vitro test systems. Tools for the determination of inhibition constants and TI. Overexpression, downregulation of targets in transgenic parasites (if possible).
In vivo tests	Compounds with good TI values	Toxicity *in vivo,* curing rates.	Animal experimentation skills.

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Table 3

Selected targets for target-based development of antiprotozoal drugs (alphabetical order). *In vitro* refers to experiments in culture systems (see also [9]), *in vivo* to experiments in animal models.

Molecular target	Organism	Type of study	References
Calcium dependent protein kinases	Different apicomplexans	Overview	[59, 64]
	Plasmodium sp.	*In vitro*: inhibition of transmission	[71]
	T. gondii	Development of inhibitors, tests *in vitro*	[66, 67]
	N. caninum	Efficacy *in vitro* and in *vivo*	[74, 76]
Cysteine protease	*P. falciparum*	Structure-based virtual screening	[107]
Cyclic nucleotide phosphodiesterases	*Leishmania* sp, *Trypanosoma* sp.	Conceptual	[108]
	P. falciparum, T. gondii	Repurposing	[109]
	T. brucei	Screening of molecule fragments in functional assay; *in vitro* on various protozoan parasites	[80]
Dihydroorotate dehydrogenase	*P. falciparum*	Functional assays with inhibitor screening	[62]
		virtual screening, *in vitro*	[110]
DNA (specific sequences)	Various, including *Plasmodium* sp.	Ligand discovery by REPSA	[61]
Glucose phosphate isomerase	*T. gondii*	Conceptual	[111]
	T. brucei	Functional assays with arabinose derivatives	[112]
	Eimeria tenella	*In silico*	[113]
Helicases	*Plasmodium* sp.	Functional assays	[114]
Hexose transporter	*Plasmodium* sp.	Functional assays with glucose derivatives	[115-117]
	Babesia bovis	Functional assays with glucose derivatives	[118]
Inosine monophosphate dehydrogenase	*C. parvum*	*In vitro* screening with transgenic *T. gondii*	[119]
Myosine A tail domain interacting protein	*P. falciparum*	Structure analysis, interaction screening with molecular fragments	[120]
N-Myristoyltransferase	*Plasmodium* sp.	Functional assays, SAR	[121]
		Functional assays, *in vitro* tests	[122, 123]
	Plasmodium sp., *Leishmania* sp.	Structure analysis	[124]
Oligopeptidase B	*L. amazonensis*	*In silico,* functional assays, *in vitro, in vivo*	[125]
Plasmepsins			
Pl II (hemoglobin degrading)	*P. falciparum*	QSAR, ADMET of novel compounds	[126]
Pl II, IV	*P. falciparum*	virtual screening, verification *in vitro*	[127]
Pl I, II, IV	*P. falciparum*	NMR-based fragment screening, *in vitro*	[128]

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Molecular target	Organism	Type of study	References
Protein phosphatases	*P. berghei*	Proteomics, reverse genetics	[129]
Purine nucleoside phosphorylase	*P. falciparum*	Structure/function, *in vivo*, in patients	[130]
Serine protease	*L. donovani*	Functional assays with secretory protease, *in vitro*	[131]
	P. falciparum	screening of carbamoyl triazoles, *in vitro*	[132]
Sterol-14-alpha-demethylase	*Trypanosoma* sp.	Structure/function analysis of inhibitor, *in vitro, in vivo*	[133]
	T. cruzi	molecular docking study, *in vitro*	[134]
Threonine peptidase	*P. falciparum*	Review	[135]
Topoisomerase 1B	*Leishmania* sp.	Review	[136]