VECTOR-BORNE DISEASES

Anti-trypanosomatid drug discovery: an ongoing challenge and a continuing need

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Abstract | The WHO recognizes human African trypanosomiasis, Chagas disease and the leishmaniases as neglected tropical diseases. These diseases are caused by parasitic trypanosomatids and range in severity from mild and self-curing to near invariably fatal. Public health advances have substantially decreased the effect of these diseases in recent decades but alone will not eliminate them. In this Review, we discuss why new drugs against trypanosomatids are required, approaches that are under investigation to develop new drugs and why the drug discovery pipeline remains essentially unfilled. In addition, we consider the important challenges to drug discovery strategies and the new technologies that can address them. The combination of new drugs, new technologies and public health initiatives is essential for the management, and hopefully eventual elimination, of trypanosomatid diseases from the human population.

Trypanosomatid parasites cause several neglected diseases in humans and animals, which range in severity from comparatively mild to near invariably fatal[1,2]. The organisms that are responsible for human diseases are *Trypanosoma brucei* subsp., which cause human African trypanosomiasis (HAT), *Trypanosoma cruzi*, which causes Chagas disease, and *Leishmania* spp., which cause leishmaniasis. Together, these insect-transmitted parasites threaten millions of people. All of these organisms have complex life cycles, with substantial differences in morphology, cell biology and biochemistry between life cycle stages, and, in some cases, between species (BOX 1).

The control of trypanosomatid diseases has had a mixed history, although public health campaigns are showing success in many instances. For example, the Southern Cone and Andean initiatives are tackling Chagas disease using a combination of insecticide spraying of dwellings, improved housing, screening of people in endemic zones and blood bank monitoring[3]. However, in South America there is a substantial number of individuals who are infected with *T. cruzi* and many infected individuals have migrated to North America and Europe, where the disease is non-endemic. In the case of leishmaniasis, co-infection with *Leishmania* spp. and HIV can increase disease burden and severity, and recent refugee movements from the Middle East into Europe are likely to increase the prevalence of leishmaniasis in Europe. In the immediate post-colonial period, HAT resurged, but vector control, active case-finding and treatment have all helped to control the disease[4]. However, many trypanosomatid diseases are zoonotic, which makes eradication extremely unlikely. The current target is elimination, which is still an ambitious goal. Despite progress, trypanosomatid diseases remain a substantial public health problem and there is an urgent need for new drugs to tackle them.

None of the available drugs for the treatment of trypanosomatid diseases (TABLE 1) is satisfactory and new drugs are required, especially those that are suitable for rural health systems that have limited resources. The current standard of care is monotherapy, with the exception of nifurtimox–eflornithine combination therapy (NECT) for HAT, although various drug combinations are in clinical trials. Importantly, many of the current treatments require parenteral administration[5] and also have poor efficacy, major side effects and increasing levels of resistance[6–8]. Most of the drugs that are in use probably have several modes of action, as they act on multiple parasite targets[9]. Goals for drug discovery include the development of completely new classes of therapeutic, reduced host toxicity, improved administration regimens and the development of combination therapies.

Vaccine development is a powerful approach to disease management but remains challenging in trypanosomatid diseases, owing to efficient immune-evasion mechanisms, such as antigenic variation in African trypanosomes, and the intracellular locations of *T. cruzi* and *Leishmania* spp. in the human host. Progress towards

Trypanosomatid
A member of the order Kinetoplastida (suborder Trypanosomatida), a group of protozoan flagellates that includes many pathogenic species. Trypanosomatid is frequently used interchangeably with kinetoplastid.

Eradication
The permanent reduction of the global incidence of infection or disease to zero.

Elimination
Zero incidence of infection or disease in a defined geographical area.

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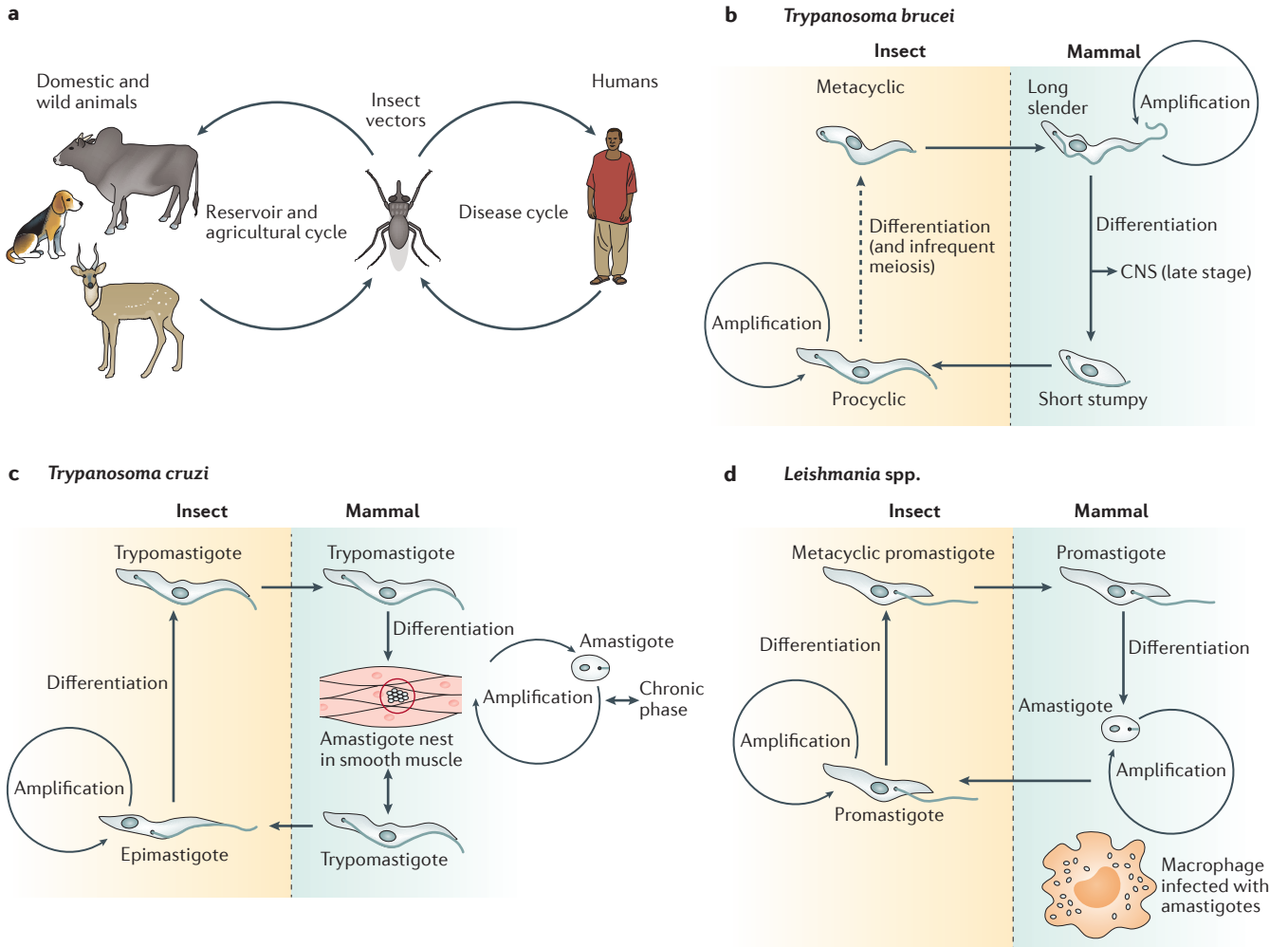
Box 1 | **The life cycles of trypanosomatid parasites**

Trypanosomatid parasites have several different hosts and are transmitted by insect vectors to humans (see the figure, part **a**). *Trypanosoma brucei* subsp. are transmitted by the tsetse fly (see the figure, part **b**). Following infection at the site of the insect bite, the parasites circulate freely in the bloodstream and may also accumulate in tissues, such as adipose tissue[145] and the skin[146]; symptoms during the early stages of human African trypanosomiasis (HAT) are non-specific and include fever, headache, fatigue, muscle pain, anaemia and swollen lymph nodes. During the second stage of disease, trypanosomes invade the central nervous system (CNS), which causes various neurological symptoms that culminate in coma and death. Diagnosis is frequently only made at this late stage when treatment options are limited, as first-stage drugs do not cross the blood–brain barrier. Closely related species (in particular *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma evansi*) also infect domestic and wild animals, causing nagana, which is a wasting disease that has a major effect on agricultural animals in Africa, Asia and parts of South America[147–149].

Chagas disease is endemic in South America and Central America[150], but migration has spread cases of infection to North America, Europe, Japan and Australia[151]. *Trypanosoma cruzi* is transmitted by triatomine bugs; following a blood meal, infective parasites in the faeces of the vector can enter at the site of the bite or through transfer to mucous membranes of the eye, nose or mouth (see the figure, part **c**). Alternative transmission routes include blood transfusion, transplantation, ingestion of contaminated food or drink, and maternal vertical transmission. Parasites are predominantly intracellular in mammalian hosts and invade several cell types. Chagas disease has acute and chronic stages; the acute stage has a high mortality rate in children, but in adults frequently presents with non-specific symptoms that resolve. Parasites are detectable microscopically in the bloodstream during the acute stage but are generally absent after progression to the chronic stage, when diagnosis by microscopy is difficult, although xenodiagnostic and serological tests are effective. The infection may remain asymptomatic for life (the indeterminant phase), but, in a subset of cases, the disease progresses to involve the heart or gastrointestinal tract. Patients often only present when they have symptoms, such as cardiac dysfunction, difficulty in swallowing (megaoesophagus) or in defecation (megacolon). Pathology is thought to be either a consequence of the immune response to the ongoing low-grade infection or of an autoimmune response[152]. Differences in disease manifestation are probably due to both genetic variation between *T. cruzi* strains[153] and host factors[154].

Leishmania spp. cause a set of diseases that have varying severity, which is dependent on the species[155]. The parasites are transmitted in the saliva of sandflies and then invade monocytes and macrophages in the host, in which they replicate in parasitophorous vacuoles (see figure, part **d**). Visceral leishmaniasis is a systemic infection that is predominantly caused by *Leishmania donovani* and *Leishmania infantum*, and affects the liver, spleen and bone marrow. It is associated with progressive wasting, anaemia and hepatosplenomegaly, and has a high mortality rate unless treated. Mucocutaneous and cutaneous leishmaniasis are characterized by skin and mucosal lesions of varying severity. Co-infection by *L. donovani* or *L. infantum* and HIV is an increasing concern in Europe.



Parts **a** and **b** are adapted from REF. 14, Macmillan Publishers Limited.

Parenteral administration
Drug administration by routes other than through the gastrointestinal tract, generally by injection.

Insect vectors
Pathogenic trypanosomatids are commonly transmitted by insect species that are specific for the respective parasite. The geographical distribution of these insects restricts the range of parasite transmission.

Chemical series
A series of chemicals that have closely related chemical structures.

Suicide inhibitor
A compound that is activated by an enzyme to give a reactive intermediate that irreversibly inhibits the enzyme through covalent bonding.

human[10] and canine[11] *Leishmania* spp. vaccines, and the challenges in developing vaccines for HAT[12] and Chagas disease[13], have been reviewed recently and will not be discussed here.

In this Review, we discuss the potential for the development of new drug therapies against trypanosomatids. In addition, we highlight unique biological features of these parasites that suggest potential targets, methods that are used to identify bioactive compounds and consider some of the outcomes of recent campaigns. We encourage the reader to consider excellent reviews of the life cycles, genomes, pathogenesis and more general aspects of the biology of trypanosomatids that have been published elsewhere (see REFS 14–19).

Drug discovery

A successful drug discovery campaign typically takes 10–15 years (FIG. 1). High attrition rates, together with relatively few organizations working on drug discovery for trypanosomatid parasites, mean that the number of new compounds in clinical development is very low (FIG. 2) and unlikely to meet the clinical need. Ideally, the pipeline should contain several new agents that are suitable for combination therapy. The advantages of combination therapies are manifold: they can increase the clinical efficacy of treatments; they can decrease side effects by enabling lower dosing of individual agents; and they can decrease the risk of developing resistance. Decreasing resistance is crucial for safeguarding new medicines that emerge from the drug discovery pipeline.

Three broad approaches are used for drug discovery against trypanosomatids. First, there are target-based approaches, which involve screening for inhibitors against a purified protein (for example, an enzyme). Compounds identified through the screening (or structure-based) process are subsequently optimized to show efficacy in a cellular model. Second, there are phenotypic approaches, which involve screening for growth inhibitors directly against an intact parasite, usually in an *in vitro* culture. Last, there is compound re-positioning, which is the re-deployment of compounds that were previously developed for an alternative use as new anti-trypanosomatid therapies.

The drug discovery process is ideally driven by target product profiles (TPPs), which define the properties of a drug that are required for clinical application[20–22]. Such factors include the route of administration (for example, oral, inhalation or intravenous), acceptable dosing regimen and course of treatment, acceptable safety and tolerability levels, cost and shelf-life. TPPs enable the development of compound progression criteria, which define parameters for compounds at each stage of the drug discovery process (for example, hit, validated hit, lead and preclinical candidate; see FIG. 1). Progression criteria include assessment of the physicochemical properties (such as solubility in physiological media, lipophilicity, molecular weight, hydrogen bond donors and acceptors), potency (against the molecular target and intact organism), selectivity, chemical and metabolic stability, pharmacokinetics, efficacy and safety. Additional criteria for parasitic infections can include factors such as cytocidal activity and the rate of parasite killing. The Drugs for Neglected Diseases Initiative (DND*i*) is a public–private partnership that focuses on drug discovery and clinical development for these organisms. It has developed TPPs and compound progression criteria for trypanosomatid diseases (see the DND*i* website for more information)[21].

Target-based approaches

For target-based approaches, the key is careful selection of the most promising molecular targets. A recent review highlights some examples of target-based drug discovery against trypanosomatids[23]. For neglected diseases in general, including the trypanosomatid diseases, there has been very limited success from target-based approaches. This is often due to a lack of translation from inhibition of the target (enzyme) in a purified cell-free context to inhibition of proliferation of the parasite and/or subsequent activity in an animal disease model. In part, this reflects the absence of robustly validated targets (for example, enzymes that have essential activities for the parasite) and highlights the need for fundamental research into trypanosomatid biology and for thorough genetic and chemical validation of potential targets[24]. However, this is only part of the problem. As we discuss below, an improved understanding of how to translate compounds that are active *in vitro* into therapeutics is required, which includes better defining the cellular and animal models (BOX 2) that predict clinical efficacy in humans.

We have published some criteria to aid in the selection of molecular targets[9,20,24] (BOX 3). Many target-based drug discovery programmes can be initially viewed as target validation[25]. Therefore, it is vital to obtain proof-of-concept (POC) of anti-parasitic activity for new target-derived chemical series at the earliest possible stage, ideally both in cellular and animal models, to minimize the waste of resources if the target fails to progress.

Drug targets with the highest degree of validation. The best-validated drug target in *T. brucei* is ornithine decarboxylase, which is the target of eflornithine, a drug that is used clinically for the treatment of HAT. Eflornithine is a suicide inhibitor that was initially developed for the treatment of cancer, but was subsequently repurposed for HAT[26]. Selectivity is thought to arise from the more rapid turnover of human ornithine decarboxylase compared with the trypanosome enzyme[27], or due to the inhibition of the biosynthesis of trypanothione[28], which is a metabolite that is unique to trypanosomatids.

The enzyme *N*-myristoyltransferase (NMT) has also been well validated as a molecular target for HAT[29–31]. In a programme that was initiated with a high-throughput screen against NMT, a compound series was identified and subsequently optimized (typified by DDD85646; FIG. 3b) to be active in a mouse model of the first stage of HAT, which does not involve the central nervous system. There was strong evidence that the compounds inhibit NMT in cells and that this inhibition kills parasites, which validates both the target and the mode of action. NMT is also present in humans, but *T. brucei* is acutely

Table 1 | **Current drugs that are used to treat trypanosomatid diseases**

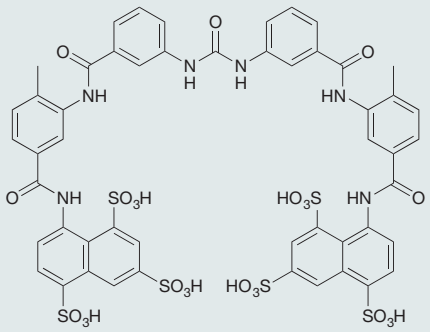
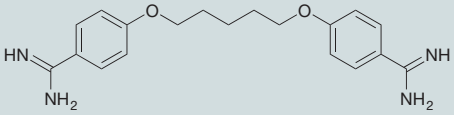
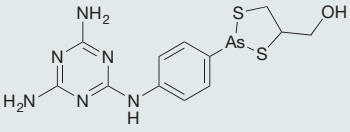
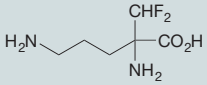
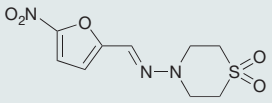
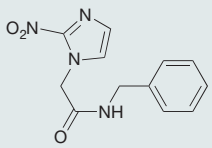
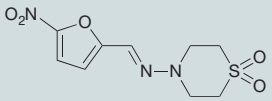
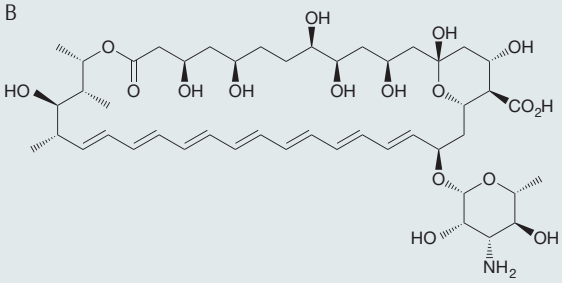
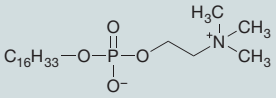
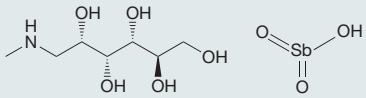
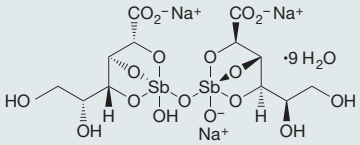
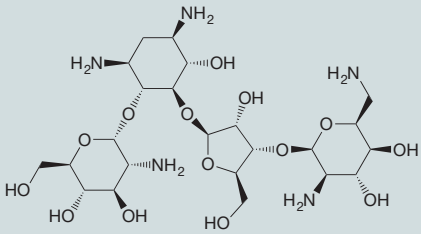
Drug	Structure	Comments
Human African trypanosomiasis		
Suramin		• Only suitable for first-stage infection with *Trypanosoma brucei rhodesiense* • Associated with toxicity • Given intravenously
Pentamidine		• Only suitable for first-stage infection with *T. b. gambiense* • Associated with toxicity • 7-day treatment • Given intramuscularly
Melarsoprol		• Suitable for second-stage disease • Highly toxic and causes substantial levels of drug-related mortality due to reactive encephalopathy • 10-day treatment • High levels of treatment failure reported in some regions • Given intravenously
Eflornithine		• Suitable for second-stage disease • High cost • Requires intravenous administration of large amounts of compound over extended periods of time • Septicaemia is a major adverse effect • Not efficacious against *T. b. rhodesiense* • Given by slow intravenous infusion
NECT (nifurtimox-eflornithine combination therapy)		• Suitable for second-stage disease • Same issues as eflornithine monotherapy, but reduced length of treatment and cost • Nifurtimox is given orally
Chagas disease		
Benznidazole		• Reasonably effective against the acute form of the disease • Problems with tolerability and patient compliance • A recent clinical trial indicates that once heart failure develops in chronic Chagas disease, treatment with benznidazole has no relevant effect[142]
Nifurtimox		• Reasonably effective against the acute form of the disease • Problems with tolerability and patient compliance
Visceral leishmaniasis		
Amphotericin B		• Very toxic in most formulations • Ambisome (a liposomal formulation) is the best tolerated formulation and is very effective in India. However, it is very expensive, requires intravenous administration and has low efficacy in East Africa

Table 1 (cont.) | **Current drugs that are used to treat trypanosomatid diseases**

Drug	Structure	Comments
Visceral leishmaniasis (cont.)		
Miltefosine		• Only oral treatment for visceral leishmaniasis • Teratogenic, which limits clinical use • Reports of increasing treatment failures
Pentavalent antimonials	 Meglumine antimoniate  Sodium stibogluconate	• Associated with toxicity • Two options available: sodium stibogluconate (pentostam) and meglumine antimoniate (glucantime) • High levels of resistance in Bihar State in India and the neighbouring region of Nepal • Up to 30-day treatment • First-line treatment in combination with paromomycin in Africa • Given intramuscularly
Paromomycin		• Good efficacy in India (although not used extensively there), but much less so in East Africa • 21-day treatment • Given intramuscularly • Pain at injection site • Ototoxicity

sensitive to NMT inhibition, probably because endocytosis, which occurs at a very high rate in *T. brucei*, is affected. NMT has also been validated as a target in a second-stage mouse model of HAT (K.D.R., unpublished observations). The challenge with the second-stage disease is that compounds need to penetrate the blood–brain barrier and achieve therapeutic concentrations in the central nervous system without causing host toxicity.

Very recently, the proteasome was shown to have great potential as a target in all three types of trypanosomatid[32]. This study used a phenotypic approach to develop a parasite-specific selective inhibitor (GNF6702) that does not inhibit the human proteasome. This is an excellent example of taking a phenotypic hit and subsequently deconvoluting the target. The initial experiments to determine the mode of action involved generating compound-resistant *T. cruzi* mutants followed by whole-genome sequencing, which revealed mutations in the β4 subunit of the proteasome. Various additional biochemical experiments have demonstrated that GNF6702 specifically inhibits the chymotrypsin-like activity of the parasite proteasome.

Biological features of trypanosomatids that might be targeted. Trypanosomatids are one of the most evolutionary divergent eukaryotic lineages from mammals, a feature that is reflected in their distinct biology (FIG. 3a). Conversely, there are many similarities between *T. brucei*, *T. cruzi* and *Leishmania* spp., and many molecular mechanisms are conserved between all three lineages. Trypanosomatid-specific metabolic and cellular pathways (discussed below) should represent excellent drug targets as specificity should be an easier criterion to control, but no candidate drugs have been developed that inhibit such targets. In fact, most potential trypanosome-specific targets remain unexplored for drug discovery and/or are of unknown druggability. Ironically, the best-validated targets in trypanosomatids are those repurposed from oncology (ornithine decarboxylase) and two pan-eukaryotic essential targets (NMT and the proteasome), which are discussed above.

Uniquely, trypanosomatids package the first six or seven enzymes of glycolysis into the glycosome, which is a specialized form of peroxisome. Glycolysis is especially important for the bloodstream forms of African trypanosomes, which rely exclusively on this pathway for the production of ATP. The compartmentalization of glycolysis in trypanosomatids is accompanied by fundamental differences in allosteric regulation of the pathway compared with most other eukaryotes. Consequently, phosphofructokinase, for example, is being pursued as a target[33]. However, computational modelling of glycolysis suggests that there is little prospect of killing trypanosomes by suppressing glycolysis unless inhibition is irreversible or uncompetitive, owing to the enormous glycolytic flux through the system[34]. Metabolic compartmentalization requires the transport of substrates (glucose), negatively-charged metabolic intermediates (such as 3-phosphoglyceric acid, dihydroxyacetone phosphate and glycerol-3-phosphate) and products

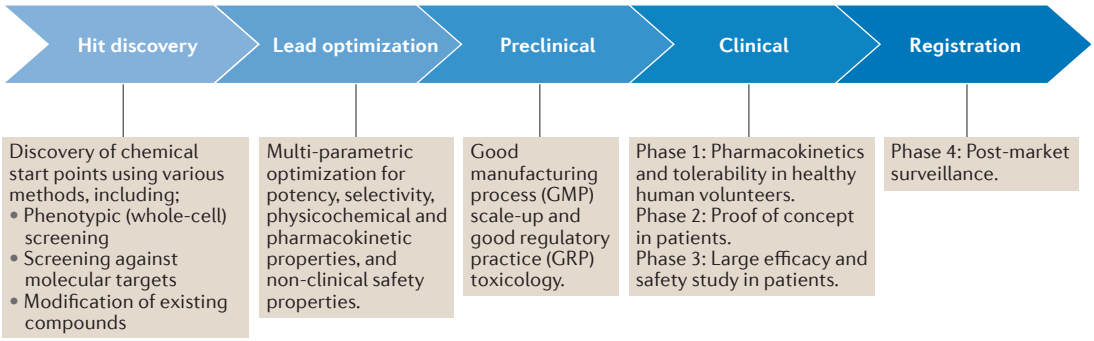


Figure 1 | **The drug discovery process.** Drug discovery progresses through several stages (hit discovery, lead optimization, preclinical, clinical and registration stages), each of which involves specific steps and regulations. The failure rate at each stage is high, which underscores the need for an active pipeline of drug discovery projects.

(such as pyruvate). The transporters and permeases for these molecules (and other larger charged metabolites and cofactors, such as nucleotide diphosphates and triphosphates, nucleotide sugars and NADH) remain elusive but could represent potential drug targets[18]. Similarly, the biogenesis of glycosomes might also have unique and druggable features.

With about 180 members, the kinomes of trypanosomatids are extensive but lack predicted receptor tyrosine kinases, or even general tyrosine kinases, and contain disproportionately high numbers of certain enzyme subtypes; for example, STE and NEK kinases[35]. Chemical biology has demonstrated distinct inhibition profiles for host and parasite kinases[36], which suggests that the selective inhibition of parasite kinases is feasible. Furthermore, both genome-wide and kinome-wide RNAi-knockdown screens indicate that several of these enzymes are essential[35,37]. However, although potent and selective inhibitors against essential protein kinases in cultured parasites have been developed[38–40], none was sufficiently active *in vivo*. The repurposing of mammalian kinase inhibitors has shown promise[41], with cure of HAT in an animal model reported for one kinase inhibitor[42]. However, so far, it is unknown which (if any) trypanosomatid kinases are being targeted by the repurposed mammalian kinase inhibitor, and both chemical and genetic validation of this approach are still required. The recent identification of a highly divergent kinetochore in trypanosomes[43] may provide new kinase targets in this class, but their druggability remains to be determined.

Trypanosomatids also have other divergent signalling pathways that could provide therapeutic opportunities. For example, whereas trypanosomatids lack identifiable G protein-coupled receptors, they have a large family of membrane-bound adenylate cyclases that modulate the immune response of the host[44] and are probably involved in parasite differentiation[45] through unconventional downstream cyclic AMP (cAMP) response proteins[46,47]. Similarly, a family of cAMP phosphodiesterases have attracted interest as potential targets[48,49].

The assembly and maintenance of the cell surface is crucial for organisms that interact with, and defend themselves against, their hosts and the immune system. Although the fundamentals of protein and membrane synthesis, transport and recycling are well conserved among eukaryotes, there is substantial specialization between species. For example, trypanosomatids have evolved divergent protein *N*-glycosylation[50,51] and glycosylphosphatidylinositol (GPI) membrane anchor biosynthetic pathways, the latter of which is a validated target for HAT[52]. Similarly, the machineries for the export of glycoproteins, and for endocytosis and recycling, are highly divergent in trypanosomes, with several canonical components being replaced by novel factors[53–55]. The major surface glycoproteins are also distinct, and although the functions of many of these glycoproteins remain unknown, they are probably crucial for survival in the host[56]. Furthermore, the endosomal apparatus contains some components that are important for defence against the innate immune response[57]. All of these peculiarities provide the potential for therapeutic exploitation.

Interestingly, endocytosis and transport mediated by transmembrane proteins are important for drug uptake by trypanosomatids. For example, aquaglyceroporin 2 from *T. brucei* is responsible for the uptake of melarsoprol and pentamidine, and the invariant surface glycoprotein 75 is responsible for the uptake of suramin[58–60].

Divergent gene expression might also be targeted. Transcription in trypanosomatids is almost exclusively polycistronic, and several chromatin modifiers are involved in determining the sites of transcription initiation and termination[61]. Bromodomain 'readers' in particular, which bind to acetylated histones, are potential targets[62], as are histone acetyltransferases (also known as 'writers' (REF. 63)) and deacetylases (also known as 'erasers' (REF. 64)). Novel transcription factors are also potentially druggable, such as class I transcription factor A[65], which is also, unusually, required for the transcription of genes that encode the major surface glycoproteins by RNA polymerase I (Pol1) in the African trypanosome; Pol1 is restricted to ribosomal RNA transcription in most other eukaryotes.

Protein-coding mRNAs require *trans*-splicing in trypanosomatids, which is distinct from the *cis*-splicing that is required to remove introns from the vast majority of mammalian mRNAs. Although the splicing mechanism for *cis*-splicing and *trans*-splicing is broadly similar,

Druggable
A protein that can be inhibited or its function modulated by a drug-like molecule.

Kinomes
All protein kinases in certain organisms.

Kinetochore
A protein complex that assembles at the centromeres of chromosomes and is important for chromosome segregation during cell division.

Polycistronic
Polycistronic transcription produces an mRNA that encodes several polypeptides in one molecule, which is then processed into individual polypeptide mRNAs.

trans-splicing
A process that is similar to *cis*-splicing but, in this case, two different transcripts are spliced together.

cis-splicing
A step in pre-mRNA maturation during which exons are spliced together and introns are removed.

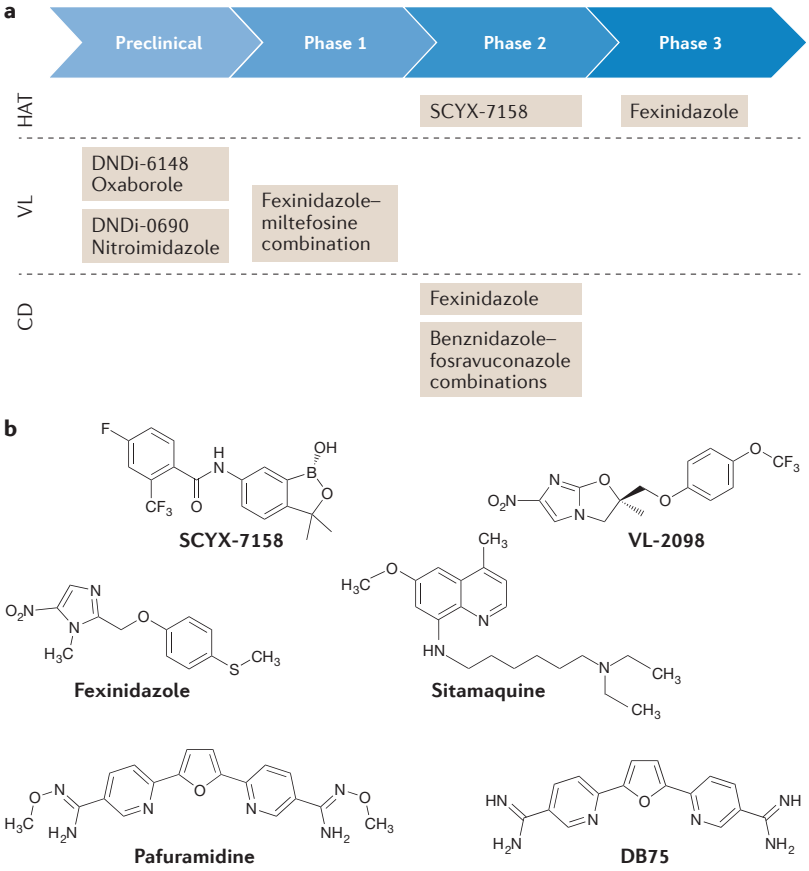


Figure 2 | **Anti-trypanosomatid compounds currently in preclinical and clinical development. a** | Several compounds are currently in preclinical and clinical development for human African trypanosomiasis (HAT), visceral leishmaniasis (VL) and Chagas disease (CD). **b** | Anti-trypanosomatid compounds that were identified through phenotypic approaches that have been progressed into clinical trials.

there are substantial differences in the splicing machinery[66]. Polycistronic transcription relies on the post-transcriptional control of gene expression and, consistent with this, numerous trypanosomal RNA-binding proteins have key roles in mRNA maturation, stability and translational control[67]. The process of translation itself also presents novel targets at the level of the ribosome[68] and aminoacyl-tRNA synthetases[69].

Examples of target-based drug discovery programmes. There are several examples of trypanosomatid-specific targets that have been investigated. One example involves redox metabolism; trypanosomes have a unique di-thiol called trypanothione. Several enzymes that are involved in the synthesis and modulation of the trypanothione redox system, including trypanothione reductase (TryR)[70] and synthetase (TryS)[71,72], are essential for the survival of the parasite. Numerous attempts have been made to discover drug-like inhibitors of TryR[73,74]. Multiple series have been identified from several large-scale and medium-scale screens of synthetic libraries and natural products, some of which have been used in structure-based drug design. Unfortunately, so far, none has delivered compounds that are suitable for clinical development. A key reason seems to be the large hydrophobic active site of TryR[70], which is difficult to inhibit with a small drug-like molecule. Active compounds have also been designed against the companion biosynthetic enzyme TryS[75].

In Chagas disease, the biosynthesis of sterols has been the focus of several drug discovery programmes. Several molecular targets have been investigated, including sterol 14α-demethylase (CYP51)[76] and squalene synthase[77]. Much of this effort has involved repurposing compounds that were developed as antifungals or as cholesterol-lowering agents. Clinical trials have tested two CYP51 inhibitors, posaconazole[78] and fosravuconazole (also known as E1224; a prodrug of ravuconazole; FIG. 3b). Although there was an initial clearance of parasites with posaconazole and fosravuconazole, disease recurred after treatment ceased, which indicates that neither agent is suitable for treatment, at least as a monotherapy. The reasons for these failures are not fully understood but they highlight the need for animal models (BOX 2) that can distinguish between compounds that are efficacious in humans and those, such as posaconazole, that are not[79].

Another substantially progressed target for Chagas disease is cruzipain, which is a protease that has similarities to cathepsin L. A vinyl sulfone irreversible inhibitor of cruzipain (K777) was advanced to preclinical development[80,81] but was abandoned owing to poor tolerability in primates and dogs, even at a low dose.

Folate metabolism has also been the subject of extensive drug discovery programmes, in particular the enzymes dihydrofolate reductase and a trypanosome-specific target, pteridine reductase 1 (PTR1). Both of these enzymes are thought to be essential, at least in *T. brucei*[82,83]. There are similarities between the substrates for these enzymes, and inhibitors have been identified that inhibit both enzymes[84]. Despite extensive work in this area, for reasons that are not fully understood, there is little correlation between activity against the enzyme and activity against the parasite[85]. As far as we understand, no inhibitors for these targets have progressed to preclinical development.

Trypanosomatids lack purine biosynthetic pathways and take up purines from the host. In leishmaniasis, this dependence on external purines has been targeted with allopurinol. Allopurinol is taken up by the parasites and is then phosphoribosylated to the corresponding nucleotide, which then acts as a cellular poison[86]. It is used for the treatment of leishmaniasis in dogs and has been in clinical trials in humans but has not progressed.

Phenotypic approaches

To circumvent the challenges of target-based drug discovery, phenotypic approaches have been widely used for most neglected disease agents, including for the trypanosomatids[87]. In this regard, the key requirements are appropriate chemical libraries for screening[5,88], robust assays and appropriate screening cascades.

Screening cascades. Many different cellular assays are available for the analysis of trypanosome responses to compounds (FIG. 4). It is especially important to establish that compounds are effective against the

Structure-based drug design
The use of 3D structures of the inhibitors or modulators that are bound to a target protein (derived from X-ray crystallography or NMR) and computational chemistry to aid the design and optimization of lead compounds.

Drug-like molecule
A molecule that has the potential to be an oral drug. Such a molecule will generally follow Lipinski's rule of five: have a molecular weight of less than 500, a cLogP (measure of hydrophobicity) value of less than 5, less than 5 hydrogen bond donors and less than 10 hydrogen bond acceptors.

Box 2 | **Animal models**

Currently, human African trypanosomiasis (HAT) is the trypanosomatid disease that has the best-evaluated animal models. Peripheral disease (first-stage disease) is studied in mice that are infected with *Trypanosoma brucei brucei* S427 (infective to animals) or *T. b. rhodesiense* STIB900 (infective to animals and humans); cure is defined as no parasites present in the blood and survival after 30 days. Recently, bioluminescence imaging with transgenic parasites that express luciferase has been developed[156,157]. This greatly decreases the number of animals that are required for monitoring and provides improved longitudinal insight into tissue tropisms and parasite population dynamics in the same mouse; this advance is likely to substantially improve *in vivo* models of HAT. Although most patients who have HAT are infected by *T. b. gambiense*, models for this parasite are more challenging[158].

For central nervous system (CNS) disease (second-stage disease), the standard model is infection with *T. b. brucei* GVR35 (REF. 159), which infects the CNS after ~21 days[160]. As relapse is common, the major issue with this model is the length of time that is required before cure can be declared (180 days). Bioluminescence imaging may shorten this time frame[160].

Chagas disease has both an acute stage and a chronic stage. There are several animal models for the acute stage of infection. Early mouse models of acute Chagas disease used a decrease in parasitaemia or mean survival time as a measure of efficacy[161,162]. More recent models are also using bioluminescence[163–166]. However, treatment does not always cause complete cure and parasite levels rebound after immunosuppression with cyclophosphamide, which indicates that a treatment-refractory reservoir exists[164]. Sterile cure is likely to depend on many factors, including the compound that is used, the treatment regimen and the strain of *Trypanosoma cruzi*. An animal model that can predict efficacy in humans will be key to avoid failures such as those experienced in the recent clinical trial of posaconazole[166].

Although there are several long-term mouse models for Chagas disease, it is unclear whether they accurately reflect the human chronic stage, and confirmation of complete cure is difficult as parasites can rarely be detected in the blood. Quantitative PCR is problematic as parasites can be found in different tissues, which requires the examination of several tissues and multiple sampling to minimize false-negative results. The new bioluminescent models provide an alternative strategy, which is more direct and only detects live cells. Interestingly, in the bioluminescent models of chronic infection in mice, parasites were mainly detected in the gastrointestinal tract (principally the colon and stomach)[163], and essentially no parasites were detected in the heart. Whether these tissue tropisms apply to all strains of *T. cruzi* is not known.

Mice and hamsters are the most common animal models for visceral leishmaniasis, although other species, such as dogs, are sometimes used[167]. In the typical mouse model, animals are infected intravenously with amastigotes that are derived from a hamster spleen and treatment is started seven days after infection and is usually continued for five days. Animals are euthanized three days after the completion of treatment and liver smears are taken.

clinically relevant life cycle stages, which can be problematic for the intracellular life cycle stages of *Leishmania* spp. and *T. cruzi*. Compounds must cross multiple membranes to reach targets with the parasite in cellular assays; three in the case of *Leishmania* spp. amastigotes that reside inside acidic (pH ~5.5) parasitophorous vacuoles within macrophages. In animal models the situation is more complex still, as there are additional barriers to cross.

To identify molecules that are suitable for drug discovery, it is essential to use an appropriate combination of assays to build confidence in the chemical start points (referred to as hits). For example, initial hit finding generally requires a high-throughput assay to access chemical diversity, followed by confirmation by more physiologically relevant (but lower throughput) assays (FIG. 4). Additional cellular assays that are representative of the *in vivo* situation are important to support combined pharmacokinetic and pharmacodynamic analyses in animal models. These provide an indication of the concentration and exposure time of a compound that are required to kill the parasites in animals and to predict the situation in humans. The best combination and the optimal order of phenotypic assays depend on the parasite in question.

For *T. brucei*, typical high-throughput screens identify not only favoured cytocidal compounds but also proliferation-slowing and cytostatic compounds. Therefore, a secondary assay is required to select those hits that are cytocidal, either using washout experiments to demonstrate a lack of reversibility[29,89,90] or direct cell viability assays[91]. A further issue for HAT is that compounds need to penetrate the blood–brain barrier to be active against second-stage disease. Currently, there are no reliable *in vitro* (cell-based) assays for predicting penetration of the blood–brain barrier. However, the physicochemical properties of compounds that are likely to penetrate this barrier have been analysed[92–94], which can assist in the selection of compounds for screening.

T. cruzi usually replicates well in intracellular amastigote assays[95], which enables the identification of both cidal and static compounds. However, as *T. cruzi* evades the immune system during chronic infection, cidal compounds are probably essential for cure. Therefore, hits need to be followed up in a cidality assay. There is now also a drive to remove compounds that target CYP51 (see above), and assays that directly assess activity against CYP51 (REFS 78,96) need to be added to the screening cascade.

For *Leishmania* spp., many of the intracellular assays only report cytocidal compounds, as intracellular amastigotes replicate relatively slowly[97,98]. Although this eliminates the need for further cidality assays, the hit rates are low[21] and throughput can be relatively poor. Furthermore, it is challenging to identify potentially valuable but weak or poorly selective hits. One solution to the low throughput is to use an axenic (free-growing) amastigote assay as the primary screen. Axenic amastigotes do not occur naturally, so care must be taken in interpreting the data. Such assays also need to be designed to only identify cytocidal compounds to prevent false-positives, as we have recently reported[99]. Hits can then be confirmed in an intracellular assay.

For all trypanosomatids, as with other areas of anti-infective drug discovery, it is also crucial to measure activity against a panel of clinical isolates before progressing compound series too far, to be sure that activity is not specific to laboratory strains. For all cell-based assays, replication rate, starting density and rate of killing are key factors that are required to correctly interpret compound potency; it is important to define these parameters as clearly as possible before interpreting data on new hits.

To date, phenotypic approaches have been more successful in discovering new developable series than target-based screens. In the case of HAT, the two compounds that are currently in clinical trials, fexinidazole and the oxaborole SCYX-7158, were both derived from phenotypic approaches (FIG. 2b).

Amastigotes
The forms of *Trypanosoma cruzi* and *Leishmania* spp. that resides within cells of a human host. Amastigotes are rounded and lack a free flagellum.

Pharmacokinetic
Relating to the effect that the body has on a drug.

Pharmacodynamic
Relating to the effects of drugs in the body.

Box 3 | **Proposed criteria for target selection**

- Genetic and chemical validation of the target (essentiality)
- Whether the target can be inhibited by drug-like molecules (druggability)
- Whether it is possible to establish a high-throughput assay (assayability)
- The potential for resistance to emerge against the target
- The potential for toxicity by inhibition of human homologues (selectivity)
- The availability of structural information of the target

Recognizing that nitroheterocycles have anti-trypanosomal activity, DND*i* sourced and screened numerous nitroheterocycles and re-discovered fexinidazole, a compound that had been investigated preclinically by Hoechst but was then abandoned[100]. Nitroheterocycles can be genotoxic; therefore, counter-screening for genotoxicity at an early stage was a key selection criterion[101]. Fexinidazole, similarly to nifurtimox, is a prodrug that requires activation by a nitroreductase[102]. Fexinidazole has also been shown to have potential for the treatment of Chagas disease[103] and leishmaniasis. Sulfoxide and the sulfone metabolites of fexinidazole, rather than the parent drug, are the active compounds against the intra-macrophage form of *Leishmania* spp.[104]. Results of a phase II proof-of-concept clinical trial against visceral leishmaniasis are expected soon. For Chagas disease, the metabolites are more active than the parent compound[105] and a phase II trial was initiated. Unfortunately, the doses that were used in this trial caused safety and tolerability issues and the trial was stopped.

Another nitroheterocycle, DNDi-VL-2098, showed activity in animal models of leishmaniasis[106] and was selected for further development from a series of nitroimidazooxazoles being investigated preclinically by DND*i*. Unfortunately, toxic effects were noted and the progression of the compound was stopped. A backup for this compound (DNDi-0690) has now been selected and is in preclinical development (FIG. 2a). The antitubercular drug delamanid, which belongs to the same chemical class, has also been proposed as a possible candidate[107]. A novel nitroreductase (NTR2) has been identified as the activating enzyme for these bicyclic nitroheterocycles in *Leishmania* spp.[108].

From a library of oxaboroles, the benzoxaborole 6-carboxamides were particularly active against *T. brucei* and, following a lead optimization programme, SCYX-7158 was selected as a clinical candidate for HAT[109]. The mode of action of oxaboroles against HAT is still not understood, but may include polypharmacology[110]. Another oxaborole, DNDi-6148, has recently been moved into preclinical development with DND*i* for visceral leishmaniasis.

A series of diamidines showed potent activity against HAT, one of which (pafuramidine) was taken into clinical trials. Pafuramidine is a prodrug that is metabolized by the host into the active compound diamidine DB75. Although the precise mode of action is unknown, similar to other diamidines, the drug is selectively concentrated within parasites[111]. However, clinical trials were unsuccessful and were stopped owing to safety concerns[112].

Sitamaquine, which is an orally bioavailable 8-aminoquinoline, was discovered by the Walter Reed Army Institute of Research and has progressed into clinical trials for the treatment of visceral leishmaniasis by GlaxoSmithKline[113,114]. The mechanism of its action is not fully understood[115].

Importantly, the modes of action of all of the aforementioned compound series were unknown during the drug discovery process and up to candidate selection, and indeed, remain at best incompletely characterized. Thus, although the absence of a clear understanding of the mode of action does not preclude clinical development, it does represent a major gap in knowledge that can hinder the further optimization and development of back-up series.

Compound repositioning

Recently, there has been considerable interest in repurposing or repositioning drugs and drug-leads for many diseases[116–122]. However, the concept is not new and many drugs that are currently used for the treatment of neglected tropical diseases were 'repositioned' from anticancer, antibacterial, antifungal and anti-helminthic indications. These include the antifungal amphotericin B, the anticancer agent miltefosine and the antibiotic paromomycin, all of which were repurposed for the treatment of visceral leishmaniasis[123]. Other examples have already been mentioned above. More recently, the nitrofuran drug nifurtimox, which was originally developed in the 1960s for the treatment of Chagas disease, was repositioned as a combination therapy with eflornithine (NECT; mentioned previously) to decrease the cost and duration of treatment of late-stage HAT[124]. Unfortunately, not all repurposing efforts have been successful; for example, the CYP51 inhibitors against *T. cruzi*.

Drug repurposing is not without its drawbacks. For example, the drugs may have been optimized for a different human disease and the initial therapeutic activity may become an undesirable side effect that needs to be reduced or eliminated. A second problem is that repurposed drugs often do not fit the TPP for neglected diseases and many are not fit for purpose in resource-poor settings. High cost, marginal safety windows, the need for hospitalization or prolonged treatment, poor stability in conditions of high temperature and high humidity, and lack of oral bioavailability are just some of the issues that must be addressed. Nonetheless, the adage of Sir James Black, "the most fruitful basis for the discovery of a new drug is to start with an old drug", still has substantial value, as the success with NECT, amphotericin B, paromomycin and other drugs attest[125].

Target deconvolution

Phenotypic screening of chemical libraries and existing drugs has produced many chemical start points, particularly for the treatment of infections with *T. brucei* and *T. cruzi*, and, to a lesser extent, for *Leishmania* spp.[21]. However, chemical optimization of these phenotypic start points can be challenging owing, for example, to pharmacokinetic issues, insufficient potency or off-target toxicity. Without target deconvolution (that is, the identification

Polypharmacology
Drugs that act through the inhibition or modulation of more than one molecular target or disease pathway.

Phenotypic screening
An approach that uses a whole-cell screen that is designed to identify the effects of a compound on a target cell or pathogen without a need to understand the underlying mode of action. Through the use of high-content screening, several phenotypes can be detected simultaneously; for example, the effects on intracellular parasite viability and host cell viability (that is, toxicity).

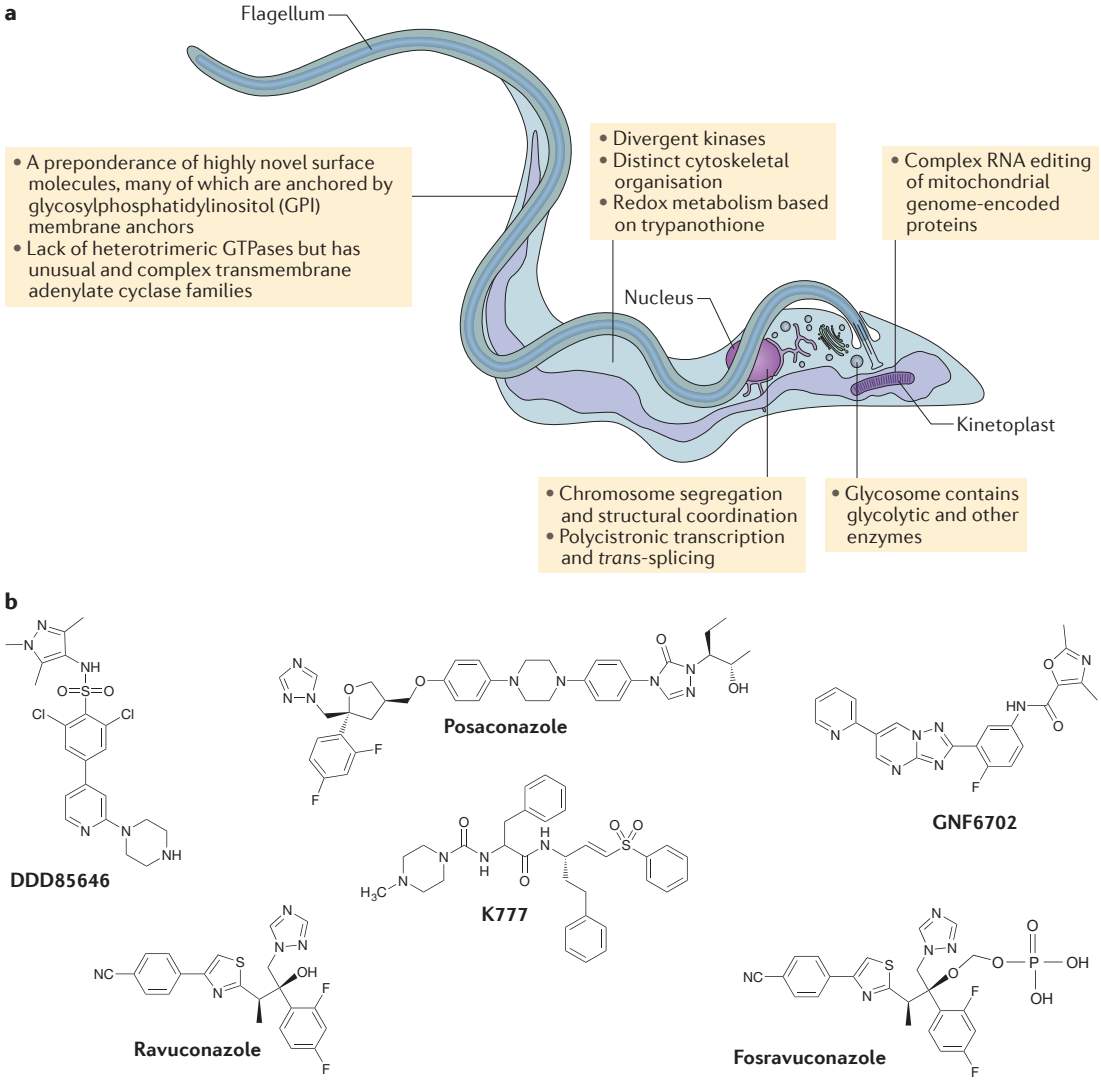


Figure 3 | **Molecular targets in trypanosomatids. a** | Trypanosomatids have unique metabolic pathways and cellular functions that are attractive for drug discovery. Many of the enzymes they produce are divergent from other eukaryotes, and they have unique or highly specialized organelles such as the kinetoplast and the glycosome, respectively. **b** | For some anti-trypanosomatid compounds the molecular targets are known. DDD85646 targets *N*-myristoyltransferase (NMT), posaconazole and ravuconazole are CYP51 inhibitors, K777 irreversibly inhibits the cysteine protease cruzipain, and GNF6702 selectively inhibits the trypanosomatid proteasome.

of the molecular target), target-based screening cannot be used to find alternative chemical scaffolds that might overcome these issues, and structure-based drug design cannot be used for compound optimization[126]. In addition, although not essential, knowledge of the mode of action can facilitate the design of combination therapies, surveillance for the emergence and spread of resistance, and assessment of the risk of resistance.

Target deconvolution has proved very successful in many therapeutic areas[127], in particular for malaria, for which several new targets have been identified recently from phenotypic hits, including *Pf*ATP4 (REF. 128), *Pf*PI4K[129], *Pf*eEF2 (REF. 130), *Pf*CARL[131] and *Pf*PheRS[132]. Another recent example of validating a trypanosomatid target (the proteasome) through deconvolution of an optimized phenotypic hit was discussed above[32].

Although several approaches to target deconvolution exist[133], further development is required for the trypanosomatids. Small molecules have many potential cellular targets, and unbiased screening approaches can be extremely powerful in identifying genetic, biochemical or metabolic associations with their modes of action. Genetic screens perturb gene expression by knockdown, knockout or overexpression. A particularly powerful approach for *T. brucei* is RNA interference target sequencing (RIT-seq)[37], which has successfully identified genes that contribute to anti-trypanosomal drug action[58]. The CRISPR–Cas9 genome-editing approach is established as a powerful alternative to RNAi for genome-scale loss-of-function screening[134] and is functional in *T. cruzi*[135,136] and in *Leishmania* spp.[137,138]. Gain-of-function screens have also been used for drug

CRISPR–Cas9
A prokaryotic immune system that has been repurposed for genome editing in eukaryotic cells; in prokaryotes, the system comprises clustered regularly interspaced short palindromic repeats and the programmable Cas9 nuclease.

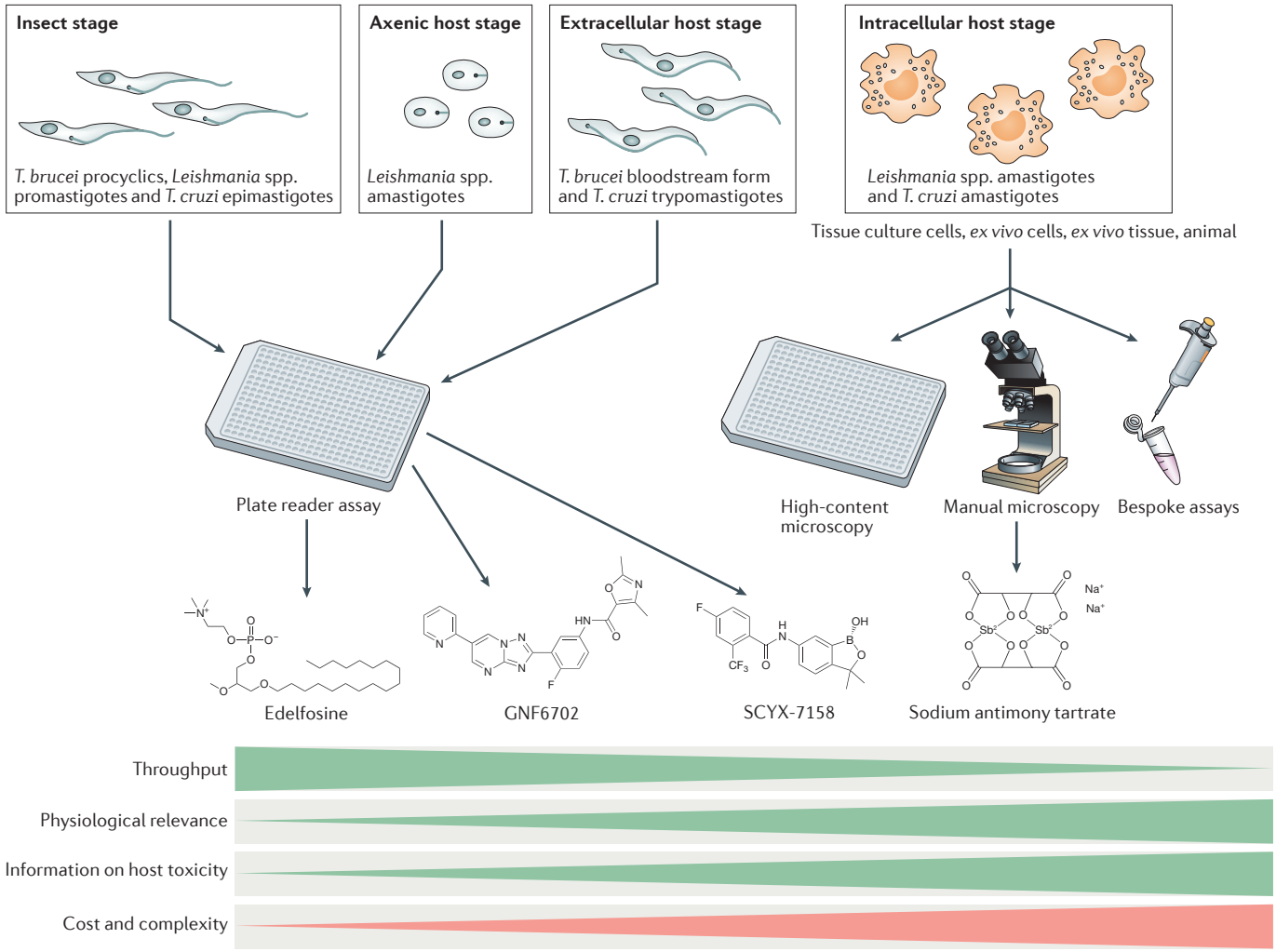


Figure 4 | **Phenotypic approaches to discover anti-trypanosomatid compounds.** Various life cycle stages can be used for the purpose of hit discovery that range from insect forms to host-stage forms in animal models. The different technologies that can be used for phenotypic assays depend on the parasite form and stage, and have specific advantages and disadvantages. Examples of compounds the anti-trypanosomal activities of which were detected using insect forms, *in vitro* host-stage forms and animal models are shown (edelfosine[168], GNF6702 (REF. 32), SCYX-7158 (REF. 109) and sodium antimony tartrate[169]). *T. brucei*, *Trypanosoma brucei*; *T. cruzi*, *Trypanosoma cruzi*.

target identification in *T. brucei*[139] and *Leishmania* spp.[140], and similar technology is available for *T. cruzi*[141], but these approaches have not yet been widely applied to target deconvolution.

Chemical proteomics is also useful for target deconvolution. Essentially, proteins from a cell extract are isolated based on their affinity for immobilized small-molecule drug leads and are then identified by mass spectrometry[142], an approach that has been used to identify potential target kinases in *T. brucei*[36]. Other approaches, such as the cellular thermal shift assay, also use chemical proteomic profiling but do not require immobilization of the inhibitor on beads[143], which can be problematic for maintaining binding to the target protein. In addition, metabolomics can detect the depletion of metabolic products and the accumulation of substrates, which can indicate specific target enzymes[144]. Cellular approaches can also contribute to target deconvolution by revealing morphological defects in the cellular compartments that are primarily affected by a drug lead. Similarly, computational approaches may be used for structure-based target prediction.

A combination of largely unbiased orthogonal approaches to target deconvolution (from those outlined above) represents a powerful new strategy to alleviate current bottlenecks in the progression of compounds that are developed from trypanosomatid phenotypic screens.

Perspectives

In the past decade, drug discovery efforts against neglected tropical diseases have increased. Importantly, some pharmaceutical companies have become more engaged during this time period and several academic centres have established powerful drug discovery capabilities. Public–private partnerships, such as DND*i* and various charitable and government funding agencies,

Scaffold hopping
The modification of the essential core of a molecule to produce a new core molecule that has broadly similar, but slightly different, properties. This is an approach that is generally used to optimize a hit or lead, improving features such as biological activity, solubility or metabolic stability.

Vector optimization
The modification of substituents on the core of a molecule (vectors) to improve a property, or properties, of a lead molecule (for example biological activity or solubility). It may also encompass optimizing the position on the core scaffold at which a substituent is placed.

have made major financial and other contributions to enable activities to proceed on the scale that is required for drug discovery. It is exciting that new compounds are undergoing clinical trials for HAT, although attrition in the drug discovery process suggests that there is no room for complacency. However, there are currently no new classes of drug in the clinical development pipeline for leishmaniasis or Chagas disease, and there is still a great need for new (ideally oral) drugs to treat each trypanosomatid disease. By definition, combination therapies to improve efficacy and decrease the risk of resistance require two or more drugs, preferably that have distinct modes of action, and place even more pressure on the development pipeline. Hence, more work is still required.

There are several reasons why the drug discovery process has not yet yielded new drugs for trypanosomatid diseases. There is a lack of well-validated molecular targets in the trypanosomatids, which has hampered traditional target-based approaches. Target-based assays have been replaced by more successful phenotypic screens. However, phenotypic screens have their own challenges. For HAT, compounds need to penetrate the blood–brain barrier to treat second-stage disease, which limits the compounds that should be screened or progressed. For Chagas disease, many of the hits target CYP51, which is a very promiscuous target and was unsuccessful in the clinical trials of posaconazole and fosravuconazole. For leishmaniasis, there is a very low hit-rate against the clinically relevant intra-macrophage form, for reasons that are not well understood.

One of the key challenges of phenotypic drug discovery is how to address issues, such as potency, toxicity and pharmacokinetic problems, that arise during the hit optimization process. Scaffold hopping and vector optimization become more problematic without knowledge of the molecular target. The identification of the targets of phenotypic hits should facilitate progression of these compounds and also enable more high-value target-based drug discovery in the future.

Another major challenge is defining the relevant cellular and animal models (BOX 2) that closely mimic human clinical conditions. This is problematic for trypanosomatid diseases, as there are very few clinically active compounds that can be used to define these models and many of the clinically active compounds are unconventional: they are reactive (for example, nitro drugs); they are selective as a result of active transport (for example, melarsoprol and pentamidine); they are active through polypharmacological actions (for example, arsenicals and antimonials); or they are covalent inhibitors (for example, eflornithine). It is possible that *in vitro* cellular assays, which more closely mimic animal and human leishmanial infections, could have a higher hit rate in phenotypic screening than current assays. For Chagas disease, we need cellular and animal models that can distinguish between compounds that are active in humans (for example, benznidazole) and those that are not (for example, posaconazole). Each new compound that is taken into the clinic can provide valuable pharmacodynamic insights, which should be fed back into the drug discovery process to refine all of these models.

Despite the aforementioned challenges, the development of new *in vivo* and *in vitro* technologies, and superior methods for genetic manipulation of parasites, and increased collaborations between the pharmaceutical industry, academic laboratories, charities and other non-government organizations will start to fill the drug pipeline against these devastating and global diseases.

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Competing interests statement
The authors declare no competing interests.

FURTHER INFORMATION
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