Anti-trypanosomatid drug discovery
De Rycker, Manu; Wyllie, Susan; Horn, David; Read, Kevin D.; Gilbert, Ian H.

Published in:
Nature Reviews Microbiology

DOI:
10.1038/s41579-022-00777-y

Publication date:
2023

Citation for published version (APA):
De Rycker, M., Wyllie, S., Horn, D., Read, K. D., & Gilbert, I. H. (2023). Anti-trypanosomatid drug discovery: progress and challenges. Nature Reviews Microbiology, 21, 35-50. https://doi.org/10.1038/s41579-022-00777-y
Anti-trypanosomatid drug discovery: progress and challenges

Manu De Rycker, Susan Wyllie, David Horn, Kevin D. Read, Ian H. Gilbert

Wellcome Centre for Anti-Infectives Research, Division of Biological Chemistry and Drug Discovery, University of Dundee, Dundee, DD1 5EH, UK

Abstract
Leishmaniasis (visceral and cutaneous), Chagas’ disease and human African trypanosomiasis cause significant mortality and morbidity, particularly in Low and Middle Income Countries. Whilst the situation has improved for human African trypanosomiasis, there remains an urgent need for new medicines to treat leishmaniasis and Chagas’ disease; the clinical development pipeline is particularly sparse for Chagas’ disease. Here, we describe recent advances in our understanding of the biology of these pathogens, particularly from the drug discovery perspective. The challenges in developing new clinical candidates and potential solutions under investigation are highlighted. The substantial progress made in the development of new drug candidates and identification of promising molecular targets is also discussed. We conclude with thoughts around future developments and prospects.
1. Introduction
Kinetoplastids are protozoa, characteristically defined as having a kinetoplast, a dense network of concatenated DNA within the mitochondrion. Some of these kinetoplastids give rise to parasitic infections in humans. Human African trypanosomiasis (HAT, also known as sleeping sickness) is caused by *Trypanosoma brucei gambiense* and *T. b. rhodesiense*. This disease has two phases, a peripheral phase, with generally mild symptoms and a second phase when the parasites enter the brain and give rise to the neurological symptoms, causing death unless treated. Leishmaniasis is caused by various *Leishmania* species and refers to several different diseases: visceral leishmaniasis (VL, also known as kala azar), a systemic infection which is almost invariably fatal unless treated, caused by *L. donovani* and *L. infantum*; cutaneous leishmaniasis (CL), which causes skin lesions which are very slow to heal, caused by multiple species and subspecies of *Leishmania*; mucocutaneous leishmaniasis which causes destruction of the nasal and buccal cavities, caused by multiple species and subspecies of *Leishmania*; and post kala azar dermal leishmaniasis (PKDL), which is a rash that can appear sometime after a patient has recovered from VL. Chagas’ disease (CD) is caused by *T. cruzi*. In CD, there is an initial acute infection, which has vague symptoms and is often diagnosed. Then patients remain asymptomatic for many years, when it is very difficult to detect the parasites. A subset of patients will progress to symptomatic disease, which usually manifests itself as heart or digestive tract disease. Chronic disease pathogenesis is complex and thought to result from complex interplay between the immune system and the parasites. These diseases are primarily transmitted by various insect vectors. CD can also be transmitted congenitally, through contaminated blood and through contaminated food and drinks. These parasitic diseases have been discussed in detail by ourselves and others. Key facts about the diseases are shown in Table 1, including the causative pathogens, endemicity, and current treatment options. It must be noted that obtaining this epidemiological data is very challenging, due to lack of diagnosis of patients and reporting.

Here we review recent progress in drug discovery for kinetoplastid diseases and how an improved understanding of parasite biology is impacting the drug discovery process. Recently, there has been significant progress in the development of new therapeutics for HAT, with one new drug registered and another in advanced, late-stage clinical trials. There is a promising pipeline developing for VL, with a number of new compounds with novel modes of action in early clinical development. However, there remains an urgent need for new drugs to treat CD, CL, mucocutaneous leishmaniasis, and PKDL, where there are very poor pipelines in drug discovery and development. New drugs will be required for leishmaniasis and CD to achieve the WHO road map for Neglected Tropical Diseases (2021-2030) which aims to fulfil the United Nations Sustainable Development Goals.

There is growing evidence of the existence of “persistor” forms in *T. cruzi* and *Leishmania* spp. These transiently dormant stages of parasites have serious implications for the development of therapies capable of curing kinetoplastid diseases. Other challenges include asymptomatic patients, almost impossible to identify, that may act as reservoirs of disease or may suffer clinical symptoms in the future (CD) and reservoirs of infection in animals.

Recently, a range of new tools have been developed that are beginning to significantly impact drug discovery for these diseases. These include new tools to facilitate studies of drug mechanisms of action, new tools that enable genetic manipulation of parasites; and refined cellular and animal models of infection that more closely mimic the human diseases.

2. Improved understanding of parasite biology
Unique features of parasite biology have been reviewed previously. Here we discuss some recent learnings which have the potential to affect drug discovery for these pathogens.

2.1 Persisters
Persisters forms are common in infectious diseases. Initially identified in bacteria, their role in antibiotic resistance has been studied extensively in recent years. They are phenotypically defined as a transient sub-population that is less susceptible to drug treatment (Figure 1). This recalcitrance is either due to differing degrees of metabolic quiescence, which makes them less susceptible to drugs, that target processes found in actively dividing and growing cells, or due to other physiological changes that reduce drug effect, rather than genetically encoded resistance. However, the precise
mechanisms of tolerance are not well understood. If the cell is not dividing, or only dividing very slowly, drugs which target DNA replication and cell division may not be effective. There is also indication of changes in regulation of transporters in some circumstances, which may affect drug levels in the pathogen, either by reducing uptake or efflux. Persister forms are not limited to prokaryotes and have been identified in eukaryotic protozoan parasites such as *Plasmodium vivax* (hypnozoites),19 and *Toxoplasma gondii* (bradyzoites)20 where they play a key role in disease persistence. Recently it has become apparent that persister forms also occur in trypanosomatids. While understanding of persisters in kinetoplastids is in its infancy, this emerging knowledge needs to be considered in drug discovery efforts. The small number of persisters in the parasite population and the potential interference of reagents used for their study20 pose significant challenges for the development of persister-specific drug discovery assays (see key challenges section).

In CD, both long-term persistence of parasites in patients and frequent treatment failure of standard therapies (benznidazole and nifurtimox)21,22 may at least partially be explained by a recently described small subpopulation of *T. cruzi* parasites that is able to survive drug treatments that kill the majority of the parasite population.12,23,24 Some persisters occur spontaneously, both in *vitro* and *in vivo*, and, as for other pathogens, the *T. cruzi* persister phenotype appears to be associated with quiescence.12 Whether they represent a discrete life-stage or are simply the slowest replicating parasites in a distribution of normal amastigotes remains to be understood. Mouse model data indicates that population level replication rates in chronically infected animals are lower than in acute stage animals,20 which may contribute to the lower efficacy of treatments in the chronic phase patients. Like many other single cell organisms *T. cruzi* is also able to regulate its growth rate in response to stresses such as nutrient limitation, inhibition of key energy pathways and drug treatment.20

For CD the drug discovery community aims to achieve full cure (i.e. every single parasite in the patient is killed), as long-term low parasite burden is a hallmark of chronic disease and eventually leads to symptomatic disease in about 30% of people. Eliminating persister parasites is therefore likely to be critical. Broadly two approaches can be envisioned. One approach is to explore extended treatment durations. This option relies on natural dynamics of persister parasites, with all persisters eventually becoming susceptible to drug treatment. The challenges with this approach are that dynamics of spontaneous persister state entry and exit are not understood, and the potential existence of drug-induced persistence. Long term intermittent treatment may overcome these challenges; proof-of-concept for this approach in mice using benznidazole has been demonstrated.20,27 The Tarleton laboratory found that a higher dose (2.5-5 times the conventional dose) of benznidazole gave a more sustained lowering of parasite numbers. The higher dose was more effective in clearing the persister state, than the conventional dose. They investigated intermittent (weekly) testing and found that the duration (30 weeks) was important in obtaining parasitological cure, even at the higher doses. The authors suggest that this is due to extending treatment beyond the time period when the parasites remain "dormant" or as "persisters". The lower doses were unable to clear the persister forms at 30 weeks. They conclude that both the higher dose and the extended treatment regimens are required for cure. In order to ascertain that the parasites had been cleared, the authors used CUBIC (clear, unobstructed brain/body imaging cocktails and computational analysis) tissue clearing protocols followed by light sheet microscopy to check for parasite clearance and also looked at immunological markers. It remains to be seen if this high-dose intermittent treatment could be an approach in treating humans generally with drugs against CD. However, a pilot clinical study indicates that intermittent benznidazole treatment does not give an improved response relative to standard daily treatment.28 Nevertheless, this approach may provide a reduction in adverse effects, and longer intermittent dosing schemes may offer higher efficacy. The major drawback from a public health perspective is that shorter treatments are preferred over longer ones. A more direct approach that may allow shortening treatment duration is through identification of compounds that kill persister parasites. While persister parasites may resist treatment due to slower metabolism, they likely still maintain active pathways essential for their survival. Increased understanding of persister biology as well as large-scale phenotypic screens to identify compounds that target persisters are key for this approach to be successful. As an extension of this approach, combination therapy may offer the ability to overcome the persister challenge, in a similar way as applied in the tuberculosis field.29

Evidence for persistence also exists in leishmaniasis. PKDL occurs in up to 15% of apparently cured VL patients on the Indian subcontinent, and a similar relapse condition exists for cutaneous
leishmaniasis (leishmaniasis recidivans). These conditions may result from persister forms in leishmaniasis. Laboratory experiments support this and confirm the existence of slow dividing and potentially quiescent parasites in in vitro cell based systems as well as in animal models.

2.2 Parasite Distribution
In terms of understanding the disease biology, it is important to understand where the parasite is distributed within the host. This gives information around the interaction between the parasite and host, the pathology in the host and also from a drug discovery perspective, understanding on the required distribution of a therapeutic to different organs and tissues. Care has to be taken, as there may be differences in compound distribution between different hosts.

HAT. African trypanosomes can circulate in the host bloodstream and invade the central nervous system (CNS), causing the potentially fatal neurological disorders associated with sleeping sickness. Data from humans, other natural hosts, and experimental animal models indicate that these parasites are also found in interstitial spaces and at other extravascular sites including adipose tissue, skin, (cardiac) muscle, eyes, lungs, lymph, endocrine glands, and reproductive organs. Invasion of these tissues has been linked to weight loss, dermatological symptoms, heart disease, ocular symptoms, and respiratory symptoms. Complicating this picture further, tissue distribution may change during an infection and could differ between species and sub-species. This extensive tissue invasion was initially observed about 100 years ago, as summarised by Ikede and Losos, but the clinical significance has not perhaps been fully taken into account. Recent research in mouse models reveals that adhesion molecules can drive tissue tropism, and those parasites that occupy adipose tissue, for example, can adapt their metabolism to local conditions which may in turn impact drug susceptibility. Some sites may be immune privileged or may be sources of relapse infections after chemotherapy. Potentially further challenging elimination efforts, parasite reservoirs found in human skin in asymptomatic cases can contribute to transmission. In this regard, the development of a portable and non-invasive human skin test could be particularly useful.

Leishmania spp. In their mammalian host, Leishmania parasites live and multiply intracellularly in phagocytic cells within phagolysosomes. Different species and sub-species of Leishmania cause different pathologies and different diseases as summarised in Table 1. In CL, the parasites infect macrophages resident in the skin. CL occurs in three different forms, localised cutaneous leishmaniasis (LCL), diffuse cutaneous leishmaniasis (DCL) and mucocutaneous leishmaniasis (MCL). LCL is the most common form and characterised by skin lesions and ulcers on exposed parts of the body, leaving permanent scars. In DCL, parasites disseminate through the skin and cause numerous non-ulcerated nodules and in MCL, after the initial skin lesion has healed, the disease spreads to the mucous membranes of the nose, mouth and throat. Subsequently, the mucosal ulcers cause destruction of the nasal septum, lips and palate leading to extensive facial disfiguring. In all forms of CL, the leishmania amastigotes are situated in the phagolysosome of macrophages located in the dermal layer of the skin at the borders of the lesion close to the inflammatory cells. When the host cell is full of parasites, it bursts and the released amastigotes will infect neighbouring macrophages in the skin. In VL, however, the released amastigotes are spread by the blood circulation and infect cells of the mononuclear phagocyte system (reticuloendothelial system) of liver, spleen, bone marrow, lymph nodes and the intestine. With the introduction of bioluminescent mouse models of VL it has become easier to track infection; this could potentially be used longitudinally to track location of parasites as the disease develops. For example, Dominguez-Asenjo and co-workers report that the thymus, a lymphatic organ, appears infected from the beginning until the late phases of the infection.

CL and VL clearly pose differing drug distributional challenges, with good dermal distribution through to more extensive tissue distribution requirements respectively. This aside, delivering compounds across multiple membrane barriers with a significant pH barrier in order to reach the intracellular amastigotes in the phagolysosomes of predominantly macrophages remains a fundamental pre-requisite for both CL and VL treatment.

CD. In cell culture systems T. cruzi is able to propagate in essentially any nucleated mammalian cell. This promiscuity is reflected in the wide tissue distribution seen in patients and animal models. In acute disease, parasites can be readily detected in patient blood, and autopsy reports indicate widespread infection of internal organs. A similar pattern is observed in mouse models, where parasites are found in essentially all tissues. Chronic disease is characterised by
low parasite levels, and frequently no detectable parasitaemia (i.e. no detectable parasites in blood). Human autopsy studies have detected parasites in hearts and other organs such as liver and kidney as well as smooth muscle and adipose tissue. Mouse models of chronic Chagas disease replicate this, with widespread distribution of parasites. Interestingly, live animal studies using bioluminescence to detect parasites in real-time, reveal a complex and spatiotemporally dynamic picture, with parasites detected in multiple organs and tissues. In these studies, the GI tract, in particular the stomach and colon, were identified as main sites of parasite persistence. The wide-ranging tissue distribution of *T. cruzi* poses a substantial challenge for drug discovery efforts as it is far from straightforward to develop compounds that achieve the extensive tissue distribution required to reach all parasites.

3. Compounds and targets

3.1 New Chemical Entities in Development

There has been very significant progress in drug discovery and development for kinetoplastids, since we reviewed this area in 2017. In particular, fexinidazole has been registered for the treatment of HAT caused by *T. b. gambiense* (2021) and is in clinical trials for treatment of *T. b. rhodesiense* (NCT03974178); the oxaborole, acoziborole, is in late-stage clinical trials for HAT (NCT05256017); and a number of new chemical entities have been developed and have progressed into phase 1 clinical development for VL (see below). This marks a major step forwards, although there is still a long way to go with the compounds for VL and there is little in the pipeline for CD. The majority of compounds in later stages of drug discovery and clinical development are being developed/co-developed with Drugs for Neglected Diseases Initiative (DNDi) (current portfolio: [https://dndi.org/research-development](https://dndi.org/research-development)). Of particular note is the development of new chemical entities (NCEs) (Figure 2). There are several reasons for the marked improvement in this situation. Firstly, there has been substantial investment in drug discovery through organisations such as DNDi, Wellcome and the Global Health Innovative Technology Fund. Secondly, more integrated, industry-standard approaches have been adopted to discovery, with several pharmaceutical companies (notably GSK, Novartis, Eisai, Takeda and Daichii Sankyo) now involved in discovery and development, along with a substantial number of academic groups. This has attracted expertise in key areas such as pharmacokinetics and safety pharmacology. Thirdly, there has been significant progress in understanding disease biology and developing assays, both cellular and animal, that are likely to be predictive of human disease. All of the compounds in the development pipeline were discovered through phenotypic screening processes. Importantly, methods are being developed to facilitate the identification of the molecular targets that these compounds inhibit (Box 1).

**HAT.** Fexinidazole, a compound re-discovered by DNDi, is an orally active drug against both the peripheral and Central Nervous System stages of HAT. It has now been registered for use against HAT caused by *T. b. gambiense*, and is in advanced clinical trials for HAT caused by *T. b. rhodesiense*. Fexinidazole is activated by a parasite nitro-reductase to a reactive intermediate, which then likely acts against multiple targets within the parasite. Acoziborole is an orally active oxaborole, active against both peripheral and Central Nervous System stages of HAT and now in phase IIb/III clinical studies. This compound has been shown to inhibit *T. brucei* Cleavage and Polyadenylation Specificity Factor 3 (CPSF3), an endonuclease involved in mRNA maturation.

**Leishmaniasis.** The vast majority of drug discovery in this area has been focused against VL, with relatively little against CL, mucocutaneous leishmaniasis or PKDL. Several new treatments are in clinical development, including combinations of existing drugs, to shorten treatment duration, improve safety and reduce the risk of drug resistance (for example miltefosine/ paramomycin: NCT03129646). In a notable development, there are a number of NCEs in clinical development (Figure 2). In most cases, their mode of action has been determined, although in all cases the compounds were initially identified through phenotypic screening and target deconvolution carried out later. These NCEs include GSK3186899/DDD853651, which has been shown to inhibit cyclin-related cyclin dependent kinase (CRK12) as well as two compounds which have been shown to be proteasome inhibitors, one from the Dundee/GSK collaboration (GSK3494245/DDD01305143) and one from Novartis (LXE408). These compounds inhibit the “chymotrypsin”-like active site on the proteasome β5 subunit. Their binding site includes a selectivity pocket that is found in kinetoplastid enzymes, but not in the corresponding human orthologue. Other compounds in clinical development are the oxaborole DNDI-6148, which, like acoziborole, acts through CPSF3
and the nitroimidazole DNDi-0690. The mechanism of action of the latter compound is yet to be determined. Having five compounds in clinical development for VL is a substantial step towards the delivery of new and better treatments for VL and is testament to the concerted efforts of multiple groups in this field. Although very little direct drug discovery has been carried out for CL, it appears that many compounds active against VL have the potential to be re-purposed for CL. However, given the different tissue distribution of CL and MCL compared to VL, compounds which might kill the parasites causing CL, may not have appropriate PK for treating CL. This needs further investigation, to develop a robust Target Candidate Profile for CL.

**Chagas’ disease.** The situation with CD has proved far more challenging. Various new benznidazole regimens are being investigated. There is a high rate of treatment discontinuation with the current treatments due to side effects. The recent small-scale BENDITA study (NCT03378661) looked at both different lengths of treatment and dosage of benznidazole. This suggested that it may be possible to reduce the length of treatment, which would be a significant boost for treatment of CD. Similar responses were seen in patients after 6 months who received a shortened (2 week) regimen of benznidazole compared to the standard treatment for 8 weeks. The Multibenz study (NCT03191162) is looking at this in more detail. Fexinidazole is also undergoing clinical trials for CD (NCT03587766). The oxaborole DNDi-6148 has also been nominated as a clinical candidate for CD and is in phase 1 clinical trials. Its mode of action in *T. cruzi* has yet to be reported. Other than this, there is no other compound reported in clinical trials or GLP toxicology studies. As described elsewhere in this article, there have been real challenges in finding molecules which can achieve radical cure.

A major advance in recent years has been to establish the molecular targets of the compounds in clinical development (Box 1). This has given rise to some highly validated drug targets. The question arises as to whether the limited drug discovery effort should be more focused on developing new chemotypes (backup series) for these highly validated targets or to focus on new targets. As attrition in clinical development is high, the backup strategy with highly validated targets clearly has merits, however, these targets are not fully validated until we obtain clinical evidence in humans. For this reason, and to develop future combination treatments, it remains vital to develop new candidates with differentiated modes of action.

### 3.2 Structural information

Recent developments in protein structure prediction and determination are having an impact in this area. DeepMind have now used artificial intelligence in the form of AlphaFold (https://alphafold.ebi.ac.uk) to predict structures for thousands of *T. cruzi* and *Leishmania* proteins. Emerging protein structural information, combined with more robust validation of drug targets has the potential to refocus drug discovery efforts more towards target-based approaches, as opposed to just phenotypic approaches.

One of the most striking examples of the use of structural information is cryoEM of the proteasome inhibitors for *Leishmania*. Both the Dundee/GSK and Novartis groups have high resolution cryoEM images of their proteasome inhibitors bound in the *L. tarentolae* structure. Analysis of these structures has revealed the binding sites of the inhibitors, which is in the β5 subunit, which forms the chymotrypsin binding site. The inhibitors bind close to the key threonine residue in the active site. The inhibitors also bind into a small hydrophobic pocket, that is not found in the human orthologues, explaining the selectivity for the parasite proteasome. These cryoEM structures have the potential to be used for design purposes. Use of structural information has the potential to greatly facilitate medicinal chemistry optimisation of compounds and to provide new insights; see for example the structure of the *Leishmania* ribosome with paromomycin.

### 4. Key challenges in drug discovery and approaches to tackle them

There are several key challenges in drug discovery for kinetoplastids, which we discuss below.

There remains a severe lack of robustly validated molecular targets that can be exploited for anti-kinetoplastid drug discovery. This situation is slowly improving, due to significant improvements in determining the mechanism of action of phenotypic hits (Box 1). However, it has been shown that significant numbers of phenotypically-active compounds actually interact with the same, small number of molecular targets, such as CYP51, cytochrome b and the proteasome. Furthermore, some of these targets are not progressible to clinical development, as they are very unlikely to give effective treatment as defined by current target product profiles. Of particular note is...
CYP51 in CD, where clinical trials with posaconazole and fosravuconazole had very high levels of relapse.\cite{67,68} This has resulted in very limited drug discovery portfolios and severely limits the scope for development of combination therapies, as there are too few drugs and candidates. The lack of novel drug targets and the repeated “re-discovery” of known targets is probably due to a combination of the limited chemical space that is being screened, the permissive nature of some active sites\cite{66,69} and the setup of the assays used for screening which may be particularly sensitised to inhibition of these targets. In order to address the issue of compounds acting at known targets, it is important to evaluate novel compound series against these targets at an early stage. This is particularly important for un-desirable targets such as CYP51, for which we developed an assay that is early in our screening cascade.\cite{65} Persister parasites pose an additional challenge, as discovering the likely limited number of suitable targets for these forms will be challenging, due to the difficulties in isolating persisters, the lack of understanding of the biology of them and the likelihood that many cellular processes are either very low turnover or suspended. Evaluation of compound series in washout assays is most likely a way of identifying compounds that are active against persisters, which will need to be followed by mechanism of action studies.\cite{23}

There are some common issues for all drug discovery programmes, including toxicology, physiologically-relevant assays, animal models predictive of human disease and understanding pharmacokinetic/pharmacodynamic (PK/PD) issues.\cite{3} It is important to understand these challenges in the context of kinetoplastid drug discovery. It is essential to develop compounds which have a very high safety profile. Most of the people who have kinetoplastid diseases live in either rural or economically deprived areas, where there is a lack of sophisticated health care facilities and trained staff. In particular for CD, where patients may not be experiencing any symptoms, and may never become ill, new drugs need to be highly selective and have no or minimal adverse effects. This is very important both from an ethical viewpoint and for patient compliance.

There are several ways to mitigate the risk of potential toxicity. One of the most common risks is inhibition of a human orthologue of the parasite target. Where the target has been identified, it is often possible to obtain highly selective inhibitors for a pathogen target, even where there is close similarity between the pathogen protein and human orthologue, for example with the proteasome\cite{54,56}, CPSF3\cite{52} or N-myristoyltransferases.\cite{70,71} Thus, potential drug targets in pathogens causing infectious diseases should not be disregarded simply because they have a human orthologue. However, this selectivity must be monitored from a very early stage in a drug discovery programme. Use of protein structural information can greatly facilitate development of selective inhibitors, even where there is high similarity between the parasite protein and its human orthologue.

Another cause for adverse effects is off-target effects. Most drugs interact with multiple proteins, which it is difficult to predict. Several mitigation approaches are available. These include: counter-screens against mammalian cell lines, human profiling panels of enzymes and receptors and in vivo toxicity studies. There are particular assays for common toxicities, such as screening against particular ion channels (eg hERG) for cardiotoxicity, the Ames assay\cite{72,73} for potential genotoxicity and assays for mitochondrial toxicity. Some chemical functional groups are known to be reactive or potentially toxic. Either these can be removed from lead compound series, or a de-risking strategy put in place from an early stage. For example, some, but not all aromatic nitro compounds and anilines are known to be genotoxic. Therefore, if a compound series contains one of these functionalities, it is important to add the Ames assay into a screening cascade from an early stage or try to remove this functionality altogether. Other approaches to predict off-target activity being developed include artificial intelligence, which may be used to highlight risks at earlier stages during the drug discovery process.

A key challenge is to develop assays which are predictive of human disease, where often the diseases are not fully understood. Many of the more high-throughput primary assays lack the detailed physiological relevance.\cite{2,3} Various approaches to improve relevance are being explored: a) replacement of tissue-culture cell lines with more relevant complex cell systems, e.g. primary human host cells,\cite{74,75} stem cell derived host cells\cite{76} and explant models,\cite{74,75} b) improved assay set-up: many viability assays cannot distinguish between cytostatic and cytocidal compounds as starting pathogen density is below the detection limit and methods that overcome this have been reported,\cite{77,78} c) alternative assay platforms to detect activity against key rare populations such as persisters, e.g. washout outgrowth assays.\cite{29} Often the more relevant models are challenged by limited throughput and/or long duration. It is therefore necessary to develop appropriate screening cascades that
combine high-throughput primary assays with more physiologically relevant secondary assays so that compounds of interest are identified in the most efficient manner (Figure 3).

Animal models of disease do not always replicate the course of infection and disease pathology found in humans. It is important to understand these differences, allowing models to be tailored be more predictive of efficacy of new compounds in humans. A key case in point are animal models for CD (Box 2 and our previous article). It is important the learnings from clinical trials of New Chemical Entities are fed back into the development of both cellular and animal models of disease, to ensure that they are relevant to disease. The interpretation of animal models is also important in the context of vaccine development (see below), where there are differences between animal (typically mouse is the PD model) the human immune systems and also possibly the distribution and physiological context of the parasites.

Related to the development of predictive animal models of infection is understanding the pharmacokinetic/ pharmacodynamic (PK/PD) relationships. Determining what the efficacy drivers are in terms of pharmacokinetics (specifically the blood and tissue compound exposure profile in relation to its in vitro antiparasitic activity) has not proved trivial for VL and CD. Some PK/PD work has been undertaken in recent years for VL, but for CD in particular, there remains poor understanding of the distribution of parasites in the host, which makes developing compounds with appropriate distribution properties challenging. For CL, variable PK profiles are needed for drug efficacy with local accumulation within a single simple CL lesion versus high systemic exposure and distribution to all skin sites for diffuse CL and PKDL. With the need to permeate biological barriers and reach infected dermal macrophages crucial for therapeutic efficacy, skin PK research in animal models of CL needs better exploration. With no impact on infectivity and thus PD readout, integrated blood sampling in efficacy studies is actively encouraged to aid PK/PD understanding.

Resistance to drugs is a major issue with antimicrobials, as is seen with HIV, malaria and TB. There are problems of resistance with drugs used to treat kinetoplastids. Resistance has been found in T. b. gambiense to melarsoprol, previously used to treat HAT through mutation or loss of transporters. In the case of VL, there is resistance to antimonials, through mutations of aqualyceroporins which are thought to be involved in drug uptake. In Bihar state, India, resistance to antimonials has been linked to high levels of arsenicals found in the drinking waters. There is also resistance to miltefosine used to treat VL, likely through changes to uptake and efflux. Whilst there is no room for complacency, and the situation must continue to be monitored, resistance to drugs used to treat kinetoplastids is not as severe as with some other infectious diseases. There are several reasons for this: the causative organisms are diploid, the rates of transmission from people being treated to other people are lower (in particular for the zoonotic diseases) and parasite burdens can be relatively low, particularly with chronic infection. The use of combination therapies should further mitigate against the emergence of drug resistant parasites. Knowledge of the molecular target of drugs (Box 1) is very important in monitoring for resistance; one of the common mechanisms of resistance is single nucleotide polymorphisms. Monitoring for these, where they occur, during or post clinical trials is important in understanding resistance risks. It must be noted that many of the current used drugs for kinetoplastids are reactive. Many of the newer generation compounds under development have specific enzymatic targets, so it will be important to monitor for clinical development of resistance.

Many of the current treatments suffer from poor efficacy, long treatment duration and adverse effects. These issues can potentially be overcome through combination treatment, either of current treatments or with NCEs. However, developing drug combinations is complex. In vitro combination effects can be assessed at multiple levels (potency, efficacy and rate-of-kill interactions) and it is not always clear if in vitro experiments translate well to the in vivo situation. In vivo it is thought to be important to match the partners’ pharmacokinetics (PK), to maintain equivalent drug exposure and avoid treatment failure. This is difficult to achieve due to the multi-factorial nature of each compound’s PK and challenging to model, owing to differences in PK across species. Furthermore, the limited number of validated drug targets and the small number of compounds going into clinical development limits the choice of potential combination partners. Nevertheless, combination therapies have been successfully developed in multiple infectious disease areas, such as HAT, malaria and TB.

These parasitic diseases cause asymptomatic cases, which has multiple implications: long-term health implications for patients; disease elimination due to reservoirs of infection amongst humans; diagnostics, how asymptomatic cases are detected or diagnosed; and treatment, with what
and how. Parasites have a range of mechanisms by which they evade the human immune system, allowing them to establish chronic infections, some of which are asymptomatic.90

**HAT:** The gambiense form of the disease can have long periods of latency before symptoms develop. However, more recently it has been established that there is a sub-population of seropositive patients with no detectable parasitaemia; at least some of this population appear not to develop clinical symptoms over an extended period of time, or to clear infection.91,92 It has been hypothesised that parasites in these patients reside in the skin.97,98 The impact of asymptomatic cases as an infection reservoir is not fully understood, but they are thought to contribute to transmission.90,91 Detecting and treating this asymptomatic population is likely to be important in elimination of disease. The recent development of safer medicines for treatment of HAT mean that this may now be feasible, although a practical means to identify these asymptomatic patients is required.

**Leishmaniasis:** There is evidence of a large number of asymptomatic patients with VL. Some of these go on to develop clinical disease. In general, most people infected with *L. donovani* or *L. infantum* (which cause VL) are asymptomatic. Whether a patient remains asymptomatic or develops active VL depends on multiple factors. Diagnosis of asymptomatic VL is not always straightforward, as patients can be seronegative, and PCR results can be problematic due to very low levels of parasitaemia. However, there is a cellular stimulation assay, followed by detection of chemokines and cytokines, that appears to be sensitive in these cases. The importance of asymptomatic patients as a reservoir of infection and the levels of transmission from asymptomatic patients is poorly understood.90

**CD:** CD is characterised by prolonged periods of asymptomatic infection. The initial acute phase either has relatively mild, non-specific symptoms or is asymptomatic and is followed by an often long asymptomatic chronic phase (the indeterminate phase), with very low levels of parasites circulating in the bloodstream. Only a subset of indeterminate patients will develop symptomatic disease, at which point it may be too late to treat with antiparasitic agents.93 Key issues associated with asymptomatic CD are: patients are often not identified until disease has advanced too far, treatment adherence can be poor as patients do not feel ill, in particular when treatments are long and have side effects, and demonstrating cure in clinical trials is difficult as inability to detect parasites in the bloodstream is not evidence of cure.

Animal reservoirs of infection are not fully understood, but probably have an under-estimated impact on these diseases. Where the main or a significant reservoir of infection is in animal populations, eradication as a strategy to tackle disease will not be possible. HAT caused by *T. b. rhodesiense* is a zoonosis, with the main reservoir of infection being both wild and domesticated animals and livestock. It is unclear what the role of this is as a reservoir of infection.93,94 A recent review of the literature has indicated that multiple mammalian species can be infected with *Leishmania* spp and act as a significant reservoir of infection.95 VL caused by *L. infantum* is thought to be primarily a zoonotic disease with the main reservoir being dogs.96 *L. donovani* has been found in a variety of agricultural animals and dogs on the Indian subcontinent.97 CL is caused by a variety of different species and sub-species of *Leishmania*. These have a variety of different reservoirs, including rodents, sloths and armadillos.98 In the case of *T. cruzi*, infection is widespread amongst domestic and wild animals.98,99 *T. cruzi*, as well as a number of other trypanosomes, has also been found in bats.99,100 Infection in animals is important in transmission to humans.

Another critical challenge is the clinical development of both New Chemical Entities or new combinations or formulations of existing drugs. There are challenges over the identification of sites suitable for clinical trials and then setting them up with appropriate facilities and personnel, as many of the patients live in remote rural areas. Some of these areas are also in politically unstable places or where there is conflict. There is also the question of funding these trials, as there are no strong economic drivers to develop new medicines. The clinical trials are costly and require sustained funding. All of these factors mean that there is a limit to the number of compounds that can be progressed through the clinical development process, even when promising molecules are identified. Furthermore, as in all disease areas, there will be a high attrition rate during clinical trials, leading to disappointment in many cases. It is estimated that for anti-infectives only 19-25% of compounds that are advanced into clinical trials make it through to registration.101,102 Therefore a much larger number of compounds needs to be advanced into clinical trials than are required, particularly considering the aim of developing combination therapies. Fortunately, there are some mechanisms in place to support the clinical development process; in particular DNDi is very active in coordinating and
supporting clinical development of compounds and there are mechanisms such as the European and Developing Countries Clinical Trials Partnership and the Global Health Innovative Technology Fund. However, it is imperative that clinical trial sites and funding are in place to enable clinical development of much needed new therapies for these diseases.

5. Other Approaches

5.1 Vaccines
Development of vaccines for kinetoplastid infections has proven very challenging. *T. brucei* spp have a variable surface glycoprotein which constantly changes, meaning the human immune system is constantly “catching up”; whilst *T. cruzi* and *Leishmania* spp are predominantly intracellular parasites, remaining significantly hidden from the human immune system.

Recently, the Wright lab has identified a potential vaccine antigen that induces protective immunity against the animal pathogen *T. vivax*. They selected potential candidate proteins, predicted to be exposed at the cell surface or secreted, using a genetic approach. Following expression of 39 potential antigens which were used to inoculate mice prior to challenge with *T. vivax*, they identified an antigen, IFX, which is found at the flagellum. This vaccine, based on IFX, was shown to provide protective immunity in a mouse model of *T. vivax* infection.\(^\text{103}\) Unfortunately, when this vaccine was evaluated in a goat infected with *T. vivax* (a natural host of *T. vivax*), there was no protection against infection, despite production of high levels of antibody to IFX.\(^\text{104}\) Therefore, there appear to be other factors which are important in the immune response to *T. vivax* in goats. This illustrates the difficulties in extrapolating data from one animal species to another, particularly when developing vaccines, where subtle differences between immune systems can have significant consequences.

Although there are gaps in understanding that must be addressed before effective *Leishmania* vaccines are delivered for humans or for companion animals, there have been notable examples of recent progress in this area.\(^\text{105}\) Prophylactic vaccines, therapeutic vaccines, and vaccines for use in combination with host-directed immuno-chemotherapy, are all under active development. For example, the ChAd63-KH (KMP-11 / HASPB) adenovirus-based vaccine, for use against VL and PKDL, proved to be safe, and to elicit a robust CD8+ T cell response in healthy volunteers in a Phase I trial.\(^\text{106}\) An effective, yet historically less safe approach, involves ‘leishmanization’, vaccination with live *L. major*. A safer alternative to leishmanization could be achieved using live, but genetically attenuated *Leishmania*. Indeed, a recent study using a CRISPR Cas9 edited *L. major* vaccine shows promise in experimental models,\(^\text{107}\) and suggests that these attenuated, marker-free vaccines can now progress to human trials. There are of course additional challenges associated with delivering live attenuated vaccine in resource-poor settings. More sophisticated vaccine delivery systems are also under development, using dissolvable microneedle skin patches, for example, which, combined with the LiHyp1 vaccine, induced protective immunity in BALB/c mice.\(^\text{108}\) The LetiFend® vaccine, based on recombinant Protein Q, and for use against canine leishmaniasis caused by *L. infantum*, was also shown to be safe and effective, reducing confirmed cases by 72%.\(^\text{109}\)

There has been a rapid recent development of vaccine technology, such as mRNA vaccines; it is unknown if any of this may have applicability to the kinetoplastids. Controlled human infection models have been very powerful in accelerating clinical trials, notably in malaria. Recently, work has been published towards development of a human challenge model of cutaneous leishmaniasis, using a strain of *L. major*.\(^\text{110}\) If this is successfully translated from mice into humans, it has the potential to be useful for vaccine development and possibly drug development. However, such models are not likely to be viable in the other kinetoplastid diseases, due to the uncertainty in rescue treatment for these lethal diseases.

5.2 Host-directed therapies
Complex host-parasite interactions occur, as the kinetoplastid parasites evade the human immune system, while also obtaining sufficient nutrients for growth and replication, and also for onward transmission to other hosts. There is growing interest in developing agents that target host-pathogen interactions in infectious diseases, for treating these diseases.\(^\text{111-114}\) Tackling these host-pathogen interactions is still in its infancy, since the biology remains poorly understood. There is also the risk that targeting the host, for example modulating the immune or inflammatory systems, could have unintended consequences, such as increasing the vulnerability of the host to infection by the parasite in question or other pathogens. However, there is potential in this area, likely in combination with...
compounds acting directly on the parasite. Furthermore, it is thought that compounds that target host-parasite interactions are less likely to be subject to resistance.\textsuperscript{111,114}

Various approaches have been investigated for identifying host-targets for treating infectious diseases generally, including transcriptomic and proteomic analysis of host cells and gain / loss of function studies using cDNA or siRNA respectively.\textsuperscript{112} Approaches adopted include modulation of the immune response through interfering with signalling processes (e.g. kinase inhibitors, GPCRs) affecting processes such as NF-kB activation (a transcription factor which stimulates the immune response) or cytokine signalling (interleukins, interferons, TNF\(_{\alpha}\)). Many of the approaches for disrupting host-pathogen interactions are very expensive (e.g. antibodies and recombinant proteins) and outside the Target Product Profiles for kinetoplastid diseases, although some approaches may be more feasible, such as nutritional products or repurposing of commonly used small molecule drugs for non-communicable diseases. One particularly promising immune-stimulator is CpG oligonucleotide D35, which increases the efficacy of short-course, low-dose antimony treatment in a macaque model of CL\textsuperscript{115} and may also serve as an anti-leishmanial vaccine adjuvant.\textsuperscript{108} CpG-D35 progressed to a Phase 1 clinical trial in 2021.\textsuperscript{116} Immunomodulators may also be combined with chemotherapy to tackle CL. For example, although the difference failed to achieve statistical significance, an antimony treatment was associated with improved cure rate when combined with a TLR7 agonist in a clinical trial in Peru.\textsuperscript{117}

\textit{Leishmania} spp and \textit{T. cruzi} are predominantly intracellular parasites. It may be possible to activate the immune system against these parasites. In the case of CD, the damage to the heart and colon is thought to result from an inflammatory reaction of the host to the parasite, so approaches to dampen this response may have a role in treatment during chronic infection. Similarly, over-activation of the immune system (through over-production of TNF\(_{\alpha}\)) is thought to be responsible for the extensive tissue damage seen in mucocutaneous leishmaniasis.\textsuperscript{111} \textit{T. brucei} spp. are extracellular pathogens but have a variable surface glycoprotein which constantly changes. At initial stages of infection, stimulation of the immune system may be suitable. In later stages, where the parasites have infected the Central Nervous System, inflammation causes damage, hence anti-inflammatory treatment may be investigated.\textsuperscript{111}

6. Summary

Over the last few years significant progress has been made in understanding kinetoplastid disease biology. This knowledge has enabled assays (both in vitro and in vivo) to be developed that can identify compounds with realistic potential to treat kinetoplastid diseases in humans. However, there is still much to learn about these complex parasites and the diseases that they cause, especially in regard to \textit{Trypanosoma cruzi}, the causative agent of Chagas’ disease (CD). Parasite persistence and tissue distribution perhaps represent the biggest challenges in dealing with CD. In the past, these factors were not considered. Thus, there has been little or no progress in filling the pipeline for CD. It remains to be seen how our increasing understanding of parasite and disease biology will impact the discovery for CD, but the knowledge gained to date is certainly impacting upon the approaches being employed.

Despite concerted efforts to determine the mechanism of action of phenotypically-active compounds, there are still relatively few robustly validated molecular targets in kinetoplastid parasites, with a small subset of known targets identified repeatedly through cell-based screening. Thus, there is a pressing need for novel, exploitable drug targets to be identified. Great progress has been made in developing drugs and identifying new drug candidates for human African trypanosomiasis (HAT). In the case of visceral leishmaniasis (VL), it is encouraging to see a number of new chemical entities entering phase 1 studies. However, there is no room for complacency, bearing in mind the high attrition rate of compounds in clinical development due to issues including efficacy and toxicity. In the case of cutaneous leishmaniasis, mucocutaneous leishmaniasis, post-kala-azar dermal leishmaniasis and CD, drug discovery pipelines are sparse and there remains an urgent need for new drugs. Alternative approaches, such as interfering with host-pathogen interactions and vaccines are also being explored. Outstanding issues that will need to be addressed to ensure the long-term control and ultimate eradication of kinetoplastid diseases include identification and treatment of asymptomatic patients. Therefore, much work remains to fully understand kinetoplastid diseases and to develop effective drugs to treat these infections.
Box 1: New genetic and proteomics methods: Mechanism of action determination

Determining the mechanism of action and molecular targets of compounds identified via phenotypic screening can be vital. The information that such studies provide can facilitate the medicinal chemistry optimisation of compounds, facilitate improved compound selectivity, aid selection of appropriate compounds for combination studies and allow the emergence of target-driven resistance in the clinic to be monitored. Determining the molecular targets of active compounds can be challenging. However, through implementation of a variety of novel approaches to drug target identification great strides are now being made. The ability to advance the development of drugs through knowledge of their mechanisms of action is proving transformative and can ultimately lead to a more diverse drug discovery portfolio. Focused mechanism of action studies have provided information allowing prioritisation of series acting on the same molecular targets and removal of series shown to act with un-desirable modes of action.

Genetic tools and technologies, applied to the trypanosomatids, have had substantial impacts on efforts to characterise protein function and to identify drug resistance mechanisms and drug targets in recent years. To increase throughput, ‘loss-of-function’, ‘gain-of-function’ and tagging approaches have all been scaled-up, to a genomic scale in several cases. Indeed, high-throughput approaches have been parallelised, whereby millions of parasites, each with a specific single protein depleted or over-expressed, can be screened in one experiment. These latter approaches have been applied to drug mode-of-action and resistance studies.

RNA interference loss-of-function screening has been used for these mechanism of action studies for several years and, has more recently facilitated identification of the proteasome as the target of a new preclinical candidate in T. brucei and Leishmania and, for a separate series, facilitated identification of divalent metal chelation as the mechanism underpinning toxicity; the latter compounds were deprioritised as anti-leishmanial candidates as a result. This approach also revealed a novel African trypanosome-specific pro-drug metabolism and potential resistance mechanism for a candidate veterinary benzoxaborole.

Over-expression gain-of-function screening has emerged more recently and is more likely to yield direct drug-target identification. For example, a T. brucei cleavage and polyadenylation specificity factor (CPSF3) was identified as the target of both clinical and veterinary trypanocidal benzoxaboroles; including aciziborole, currently in Phase II/III clinical trials against sleeping sickness. In addition, the approach was used to validate Leishmania N-myristoyltransferase as a target. A kinase over-expression screen was also used to identify the kinetoplastid kinetochore protein KKT10/CLK1 as a promising target of a potent amidobenzimidazole with potential to treat all three human trypanosomal diseases, while the Cos-Seq approach continues to reveal genes linked to drug resistance in Leishmania.

Genome editing is also increasingly impacting drug discovery efforts against the trypanosomatids. CRISPR Cas9-based approaches have revolutionized biotechnology by allowing RNA-programmed targeting of specific chromosomal loci. Precision editing of drug targets, for example, facilitates the assembly of drug resistance strains and provides insight into structure-activity relationships. Quantitative measures of drug resistance can also be important in determining whether particular mutations are likely to have a detrimental impact in a clinical setting. Cas9-based editing has been applied to T. brucei CPSF3, providing insights into selective anti-trypanosomal action; and to the T. cruzi proteasome, revealing cross-resistance between arylsulfonamides and distinct anti-leishmanials. Oligo-targeting, which is Cas9-independent, has also now been used to edit priority drug targets in the trypanosomatids. In recent years, compounds that inhibit the promising chemical, genetically and often pharmacologically validated targets detailed above have progressed into and through clinical development. New target-based screening programs have also been initiated in parallel. Further insights relating to these drug targets should facilitate surveillance for drug resistance, understanding of toxicity, rational combination design and rational back-up planning.
Box 2: Animal models
Animal models of infection are important in the selection of molecules to progress into human clinical trials. It is important to develop animal models that are predictive of efficacy in humans. An example for the importance of this has been the failure of the CYP51 inhibitors posaconazole and ravuconazole (the E1224 prodrug) to clear parasites as effectively as benznidazole in clinical trials for CD.67,68,128 This has driven the establishment of mouse models of disease (both acute and chronic) that can distinguish between posaconazole-type compounds, where there is a very high level of relapse in humans, and benznidazole, which has clinical efficacy.129

In HAT, it is important to be able to treat stage 2 infection, when the parasite has invaded the central nervous system. The classical model uses GVR35 mice assessed over 180-days.130,131 The length of this experiment has a very detrimental effect on timelines for drug discovery. Recent bioluminescent imaging procedures have reduced this to a 90-day experiment and have also reduced the number of animals required.132

In VL, both mice and hamsters are used as the animal model of disease. The Syrian golden hamster is highly susceptible to infection with visceralizing Leishmania species (L. donovani, L. infantum) and is considered the best experimental model to study VL because it reproduces the clinicopathological features of human disease.133 However, because it is challenging to sample blood from hamsters for integrated PKPD, mouse models are generally preferred as the front line disease model in a drug discovery setting. In mice, during the first weeks of infection, the parasites multiply rapidly in the liver; however, four weeks later, the mice develop an effective immune response, clear the liver parasites and become resistant to re-infection. While pathology in the liver is limited, the parasites persist in the spleen and the infection progresses for a longer period of time. Drug treatment in mice therefore only usually assesses the ability of the compound to clear liver parasites, but not those in spleen or bone marrow as this takes several weeks to develop to a robustly detectable level. Depending on compound distributional properties there is therefore a risk of poor translational PKPD from mouse to human. The development of a bioluminescent mouse model of VL for drug screening has overcome this potential weakness in drug efficacy assessment in mice, allowing investigation of drug efficacy on both liver, spleen and bone marrow parasites within two weeks of infection.134 A bioluminescent mouse model for systematic screening of vaccines for VL is also now available.135

In vivo models of CL for the evaluation of drug efficacy are mainly mouse based with the L. major Balb/c mouse model the most commonly used.136 Skin lesions develop rapidly (within 2-4 weeks), an advantage for drug screening. Because humans often self-cure CL and Balb/c mice are incapable of self-curing, lack of clinical relevance is the main limitation of the model. Alternative rodent models, such as C57/bl6 mice or golden hamsters do self-cure, but disease onset is much slower. Drug efficacy is determined by reduction in lesion size compared to vehicle dosed control. With inflammation an important factor in lesion size and a confounding factor for drug efficacy, quantification of parasite load is also considered for therapeutic effect.

In CD, a number of different animal models have been developed. The review by Chatelain and Konar provides a comprehensive overview of these.137 The mouse models developed by the Kelly laboratory are widely used,138 which use bioluminescence to monitor the infection (Figure 4). The advantage of bioluminescence is that it can be used to monitor where the infection occurs longitudinally and also only identifies live parasites. Following treatment, the mice are left for some time to see if relapse occurs and then by several rounds of immunosuppression to allow for replication of small numbers of any remaining parasites. This could potentially include “persister” cells, although this needs experimental confirmation. It is also possible to carry out ex vivo imaging on individual tissues, which is effective in pin-pointing reservoirs of infection. The Kelly team developed both an acute and a chronic model. In the acute stage of infection, there is a very high level of infection, with parasites found throughout the body, reaching a maximum at around 14 days. The innate immune system then reduces the level of infection and in the chronic phase (reached at around 50-60 days), parasites are mainly found in colon, skin and stomach, although some parasites are also found in other tissues and this is also somewhat dependent on the species of mouse used. In general, lower doses of clinical compounds such as benznidazole are required to achieve efficacy in the chronic model compared to the acute mode, which could be a reflection of the lower parasite burden. These models are effective in distinguishing between compounds known to be clinically active, such as benznidazole and those for which relapse in the clinic is an issue, such as posaconazole.
One of the arguments against using mice as a Chagas’ disease model in drug discovery is the differing immune response in mice versus human toward *T. cruzi* infection. Recently a 6 α-1,3-galactosyltransferase (α-GalT-KO) knockout mouse has been developed as a model of Chagas disease.139 Mice naturally express the α-Gal epitope and therefore do not produce anti-α-Gal antibodies, such that the anti-α-Gal immune response, a critical factor for protection against *T. cruzi* infection in humans, is absent. Infection in these mice with *T. cruzi* led to an increased immune response in the heart tissue and thus offers an interesting possibility for testing novel effective therapeutics at different stages of infection to explore the optimal window for when to commence treatment to reduce or prevent cardiac damage.

Whilst animal models of infectious disease prove very valuable in drug discovery, there are key differences between animal models and human infection. This includes differences in disease pathology and immune response to pathogens between species and differences in pharmacokinetics of compounds in different species. However genetically modified mice are becoming important tools in understanding and addressing these differences and to aid selection of compounds most likely to have clinical efficacy in humans. For progressing compounds to any of these mouse models of infectious disease, a level of drug optimisation for a mouse is required, so that the PK characteristics in mice will deliver the desired efficacious outcome following dosing, be that oral or parenterally for proof of concept. To overcome this and also potentially providing better translational PKPD, there is a strong argument for better exploring the use of recently developed mouse models, humanised for key drug metabolising enzymes, as disease models for any of the kinetoplastid diseases. Provided the humanised mouse model infection characteristics is consistent with that in current wild type mouse models, humanised models where the mouse CYP450s and promotors have been removed and replaced with human CYP450s and promotors offer potential significant advantages over the current models, especially for those compounds demonstrating mouse specific metabolism issues.140 Various knockout mice are also being used to understand the effect of various components of the immune system on disease pathology and progression.141,142
Figure 1. Persisters and potential outcomes of drug treatment on intracellular *T. cruzi*. Multiple outcomes after drug treatment have been observed. Some compounds induce reversible growth arrest (e.g. GNF7686\(^{15}\)), and parasites start dividing rapidly upon removal of drug. Other compounds kill the majority of intracellular parasites, with only a very small number surviving treatment (persisters). The surviving parasites have been shown to be in a state of spontaneous and reversible growth arrest\(^{12}\) which is likely a key factor in their ability to survive drug treatment. The ideal outcome of drug treatment is that all parasites are killed, this can be achieved by treating parasites for extended durations and/or with high concentrations of drugs\(^{23,27}\) or potentially with compounds that target mechanisms essential for the survival of persisters.
Figure 2: New compounds and targets
Figure 3. Representative screening cascades for VL and CD.

Critical path: Key assays for a phenotypic hit discovery programme. For visceral leishmaniasis the main purpose is to identify quickly compounds that are active in intracellular models, as such compounds have a high chance demonstrating proof-of-concept in animal models (provided appropriate PK). High-throughput screening for VL is typically carried out using axenically grown...
parasites, in particular axenic amastigotes. Initially screening can also be carried out in intracellular models, if high-throughput assays are available, or for smaller compound libraries. Following axenic assays, compounds that are non-selective over human cells are removed and compounds of interest are progressed to intracellular assays. At this point, any compounds with suitable activity should be validated through structure/purity determination and/or resynthesis. Confirmed actives are next subjected to analysis of known modes of action, in particular to identify compounds that have a mode of action that is already being tested in the clinic. For CD the cascade needs to quickly remove compounds with undesirable modes of action and identify compounds that can achieve complete cure. Hits are usually identified in high-throughput intracellular systems, as there are no suitable axenic models. Typically, a large fraction of hits act through undesirable modes of action such as CYP51, and screening to remove these is carried out early in the cascade. Remaining compounds of interest should at this stage be validated for structure and purity. For CD it is thought that compounds that can kill all parasites have the highest chance success in the clinic. To assess ability to achieve sterile cure, compound washout / parasite outgrowth assays are applied.

For both diseases validated hits are next progressed to hit to lead chemistry, with as main aim to achieve proof of concept efficacy in a suitable animal model of disease.

Full in vitro biological profile: For key compounds in each series a full biological profile should be determined. This includes determination of potency against multiple relevant strains and host cells, determination of the rate-of-kill of compounds and profiling against different life stages. In addition, to understand potential for future combination treatments, key series can be profiled in combination experiments.
Figure 4: Efficacy Studies

Efficacy of Benznidazole® at 100 mg·kg⁻¹ p.o. u.i.d. for 20 days (A) or Posaconazole® at 20mg·kg⁻¹ p.o. u.i.d. for 20 days (B) as a treatment for chronic stage of *Trypanosoma cruzi* infection. Whole body (dorsal [D] and ventral [V]) and ex vivo imaging. Heat-maps are on log10 scales and indicate intensity of bioluminescence from low (blue) to high (red). p.i.: post-infection. Mice dosed from day 113 to 132 p.i. and immunosuppressed on days 163, 167 and 171 p.i. using cyclophosphamide (200 mg/kg i.p.).
| Causative Pathogen (s) | Major symptoms | Vector | Endemic regions | Prevalence | DALYs | Years of Life Lost | Years Lived with Disability | Deaths per year | Treatments |
|------------------------|----------------|--------|----------------|------------|-------|--------------------|---------------------------|---------------|------------|
| Trypanosoma brucei gambiense | 1st stage: Fever, headache, enlarged lymph nodes, joint pain, itching. 2nd stage: Confusion, sensory, coordination and sleep cycle disturbance. | Tsetse fly | Sub-Saharan Africa | 3,800 | 83,000 | 82,000 | 1,000 | 1,400 | pentamidine (stage 1, *T. b. gambiense*) suramin (stage 1, *T. b. rhodiense*) , NECTc fexinidazole (acoziborole in late-stage clinical trials) |
| T. b. rhodesiense | Acute phase: asymptomatic or mild. Fever, occasionally swelling at site of inoculation. Chronic phase: Cardiac and digestive disorders, heart failure. | Reduviid bug | South America Now spread through migration to large parts of the world | 6,500,000 | 280,000 | 217,000 | 58,000 | 9,500 | benznidazole nifurtimox |
| T. cruzi | | | | 8,600 | 400,000 | 403,000 | 600 | 5,700 | amphotericin B miltefosine antimonials paromomycin |
| | | Sandflies | Key foci: India, East Africa, Brazil | | | | | | antimonials miltefosine amphotericin B |
| Leishmania donovani, L. infantum | Fever, weight loss, enlargement of the spleen and liver, anaemia. | | | | | | | | |
| L. tropica, L. aethiopica, L. major, L. infantum, L. mexicana, L. amazonensis, L. braziliensis, L. guyanensis | CL: Skin lesions, mainly ulcers. MCL: Destruction of mucous membranes of nose, mouth and throat. | | | | | | | | |
| Melarsoprol (stage 2, *T. b. rhodesiense*) |

Data from the Global Burden of Disease study 2019 (http://ghdx.healthdata.org/gbd-results-tool). Data is rounded.

- PKDL, Post Kala-Azar Dermal Leishmaniasis, a complication following cure from VL is not included in this table.
- CL and MCL are combined in this table. The data for these are combined in the Global Burden of Disease. Some species cause both diseases. MCL is found in South America, while CL is found across large parts of the tropical and sub-tropical world.

NECT, Nifurtimox Eflornithine Combination Therapy
Acknowledgements
The authors wish to acknowledge the support of Wellcome for their work in kinetoplastid biology and drug discovery projects over many years (100476, 105021, 092340, 203134, 204672, 218448).

References
1. Bonney, K. M., Luthringer, D. J., Kim, S. A., Garg, N. J. & Engman, D. M. Pathology and Pathogenesis of Chagas Heart Disease. *Annu Rev Pathol* **14**, 421-447 (2019).
2. Field, M. C. et al. Anti-trypanosomatid drug discovery: an ongoing challenge and a continuing need. *Nat Rev Microbiol* **15**, 217-231 (2017).
3. De Rycker, M., Baragana, B., Duze, S. L. & Gilbert, I. H. Challenges and recent progress in drug discovery for tropical diseases. *Nature* **559**, 498-506 (2018).
4. Ponte-Sucre, A. An Overview of *Trypanosoma brucei* Infections: An Intense Host-Parasite Interaction. *Frontiers in microbiology* **7**, 2126-2126 (2016).
5. Pérez-Molina, J. A. & Molina, I. Chagas disease. *Lancet* **391**, 82-94 (2018).
6. Echeverria, L. E. & Morillo, C. A. American Trypanosomiasis (Chagas Disease). *Infect Dis Clin North Am* **33**, 119-134 (2019).
7. Burza, S., Croft, S. L. & Boelaert, M. Leishmaniasis. *Lancet* **392**, 951-970 (2018).
8. Büscher, P., Cecchi, G., Jamonneau, V. & Priotto, G. Human African trypanosomiasis. *Lancet* **390**, 2397-2409 (2017).
9. Kande Betu Ku Mesu, V. et al. Oral fexinidazole for stage 1 or early stage 2 African *Trypanosoma brucei gambiense* trypanosomiasis: a prospective, multicentre, open-label, cohort study. *Lancet Glob Health* **9**, e999-e1008 (2021).
10. Dickie, E. A. et al. New Drugs for Human African Trypanosomiasis: A Twenty First Century Success Story. *Trop Med Infect Dis* **5**, 29 (2020).
11. WHO. Ending the neglect to attain the Sustainable Development Goals: a road map for neglected tropical diseases 2021–2030. (2020).
12. Sanchez-Valdez, F. J., Padilla, A., Wang, W., Orr, D. & Tarleton, R. L. Spontaneous dormancy protects *Trypanosoma cruzi* during extended drug exposure. *eLife* **7**, e34039 (2018).
13. Barrett, M. P., Kyle, D. E., Sibley, L. D., Radke, J. B. & Tarleton, R. L. Protozoan persister-like cells and drug treatment failure. *Nat Rev Microbiol* **17**, 607-620 (2019).
14. Mandell, M. A. & Beverley, S. M. Continual renewal and replication of persistent *Leishmania major* parasites in concomitantly immune hosts. *Proc Natl Acad Sci U S A* **114**, E801-e810 (2017).
15. Bigger, J. W. Treatment of Staphylococcal infections with penicillin by intermittent sterilisation. *Lancet* **244**, 497-500 (1944).
16. Fisher, R. A., Gollan, B. & Helaine, S. Persistent bacterial infections and persister cells. *Nat Rev Microbiol* **15**, 453-464 (2017).
17. Balaban, N. Q. et al. Definitions and guidelines for research on antibiotic persistence. *Nat Rev Microbiol* **17**, 441-448 (2019).
18. Voorberg-van der Wel, A., Kocken, C. H. M. & Zeeman, A. M. Modeling Relapsing Malaria: Emerging Technologies to Study Parasite-Host Interactions in the Liver. *Front Cell Infect Microbiol* **10**, 606033 (2020).
19. Cerutti, A., Blanchard, N. & Besteiro, S. The Bradyzoite: A Key Developmental Stage for the Persistence and Pathogenesis of Toxoplasmosis. *Pathogens* **9**, 234 (2020).
20. Ward, A. I., Olmo, F., Atherton, R. L., Taylor, M. C. & Kelly, J. M. *Trypanosoma cruzi* amastigotes that persist in the colon during chronic stage murine infections have a reduced replication rate. *Open Biol* **10**, 200261 (2020).
Morillo, C. A. et al. Randomized Trial of Benznidazole for Chronic Chagas’ Cardiomyopathy. *N. Engl. J. Med.* **373**, 1295-1306 (2015).

Díaz-Bello, Z. et al. Ten-year follow-up of the largest oral Chagas disease outbreak. Laboratory biomarkers of infection as indicators of therapeutic failure. *Acta Trop* **222**, 106034 (2021).

MacLean, L. M. et al. Development of *Trypanosoma cruzi* in vitro assays to identify compounds suitable for progression in Chagas’ disease drug discovery. *PLoS Negl Trop Dis* **12**, e0006612 (2018).

Ward, A. I. et al. *In Vivo* Analysis of *Trypanosoma cruzi* Persistence Foci at Single-Cell Resolution. *mBio* **11**, e01242-01220 (2020).

Dumoulin, P. C. & Burleigh, B. A. Stress-Induced Proliferation and Cell Cycle Plasticity of Intracellular *Trypanosoma cruzi* Amastigotes. *mBio* **9**, e00673-00618 (2018).

Álvarez, M. G. et al. New Scheme of Intermittent Benznidazole Administration in Patients Chronically Infected with *Trypanosoma cruzi*: Clinical, Parasitological, and Serological Assessment after Three Years of Follow-Up. *Antimicrob Agents Chemother* **64**, e00439-00420 (2020).

Bustamante, J. M. et al. A modified drug regimen clears active and dormant trypanosomes in mouse models of Chagas disease. *Sci Transl Med* **12**, eaabb7656 (2020).

Mandal, S., Njikan, S., Kumar, A., Early, J. V. & Parish, T. The relevance of persisters in tuberculosis drug discovery. *Microbiology* **165**, 492-499 (2019).

Goyal, V. et al. Long-term incidence of relapse and post-kala-azar dermal leishmaniasis after three different visceral leishmaniasis treatment regimens in Bihar, India. *PLoS Negl Trop Dis* **14**, e0008429 (2020).

Gitari, J. W. et al. Leishmaniasis recidivans by *Leishmania tropica* in Central Rift Valley Region in Kenya. *Int J Infect Dis* **74**, 109-116 (2018).

Kloehn, J., Saunders, E. C., O’Callaghan, S., Dagley, M. J. & McConville, M. J. Characterization of metabolically quiescent *Leishmania* parasites in murine lesions using heavy water labeling. *PLoS Pathog* **11**, e1004683 (2015).

Tegazzini, D. et al. A Replicative In Vitro Assay for Drug Discovery against Leishmania donovani. *Antimicrob Agents Chemother* **60**, 3524-3532 (2016).

Wolbach, S. B. & Binger, C. A. L. A contribution to the parasitology of trypanosomiasis. *J Med Res* **27**, 83-107 (1912).

Peruzzim, R. I. Pathologico-anatomical and serological observations on trypano- somiases. Final report, League of Nations International Committee on Human Trypanosomiasis. *3*, 245-328 (1928).

Ikede, B. O. & Losos, G. J. Pathology of the Disease in Sheep Produced Experimentally by Trypanosoma brucei. *Vet Pathol* **9**, 278-289 (1972).

Trindade, S. et al. *Trypanosoma brucei* Parasites Occupy and Functionally Adapt to the Adipose Tissue in Mice. *Cell Host Microbe* **19**, 837-848 (2016).

Capewell, P. et al. The skin is a significant but overlooked anatomical reservoir for vector-borne African trypanosomes. *eLife* **5**, e17716 (2016).

Camara, M. et al. Extravascular Dermal Trypanosomes in Suspected and Confirmed Cases of gambiense Human African Trypanosomiasis. *Clin Infect Dis* **73**, 12-20 (2021).

Crilly, N. P. & Mugnier, M. R. Thinking outside the blood: Perspectives on tissue-resident *Trypanosoma brucei*. *PLoS Pathog* **17**, e1009866 (2021).

De Niz, M. et al. Organotypic endothelial adhesion molecules are key for *Trypanosoma brucei* tropism and virulence. *Cell Rep* **36**, 109741 (2021).

Girard, A. et al. Raman spectroscopic analysis of skin as a diagnostic tool for Human African Trypanosomiasis. *PLoS Pathog* **17**, e1010060 (2021).
42 Reithinger, R. et al. Cutaneous leishmaniasis. *Lancet Infect Dis* **7**, 581-596 (2007).
43 Caridha, D. et al. Route map for the discovery and pre-clinical development of new drugs and treatments for cutaneous leishmaniasis. *Int J Parasitol Drugs Drug Resist* **11**, 106-117 (2019).
44 Steverding, D. The history of leishmaniasis. *Parasit Vectors* **10**, 82 (2017).
45 Meleney. The Histopathology of Kala-Azar in the hamster, Monkey and man. *Am J Pathology* **1**, 147-168 (1925).
46 Domínguez-Asenjo, B. et al. Bioluminescent Imaging Identifies Thymus, As Overlooked Colonized Organ, in a Chronic Model of *Leishmania donovani* Mouse Visceral Leishmaniasis. *ACS Infect Dis* **7**, 871-883 (2021).
47 Feilj, H., Muller, L. & Cappa, S. M. G. Direct micromethod for diagnosis of acute and congenital Chagas' disease. *J Clin Microbiol* **18**, 327-330 (1983).
48 Silva, A. E. et al. Acute Chagas' disease in postrenal transplant and treatment with benzonidazole. *Annals of Diagnostic Pathology* **14**, 199-203 (2010).
49 Lewis, M. D. & Kelly, J. M. Putting Infection Dynamics at the Heart of Chagas Disease. *Trends in Parasitology* **32**, 899-911 (2016).
50 Lewis, M. D. et al. Bioluminescence imaging of chronic *Trypanosoma cruzi* infections reveals tissue-specific parasite dynamics and heart disease in the absence of locally persistent infection. *Cell. Microbiol*. **16**, 1285-1300 (2014).
51 Wittlin, S. & Mäser, P. From Magic Bullet to Magic Bomb: Reductive Bioactivation of Antiparasitic Agents. *ACS Infect Dis* **7**, 2777-2786 (2021).
52 Wall, R. J. et al. Clinical and veterinary trypanocidal benzoxaboroles target CPSF3. *Proc Natl Acad Sci U S A* **115**, 9616-9621 (2018).
53 Wyllie, S. et al. Cyclin-dependent kinase 12 is a drug target for visceral leishmaniasis. *Nature* **560**, 192-197 (2018).
54 Wyllie, S. et al. Preclinical candidate for the treatment of visceral leishmaniasis that acts through proteasome inhibition. *Proc Natl Acad Sci U S A* **116**, 9318-9323 (2019).
55 Thomas, M. et al. Scaffold-Hopping Strategy on a Series of Proteasome Inhibitors Led to a Preclinical Candidate for the Treatment of Visceral Leishmaniasis. *J Med Chem* **64**, 5905-5930 (2021).
56 Khare, S. et al. Proteasome inhibition for treatment of leishmaniasis, Chagas disease and sleeping sickness. *Nature* **537**, 229-233 (2016).
57 Mowbray, C. E. et al. DNDI-6148: A Novel Benzoxaborole Preclinical Candidate for the Treatment of Visceral Leishmaniasis. *J Med Chem* **64**, 16159-16176 (2021).
58 Van den Kerkhof, M. et al. In vitro and in vivo pharmacodynamics of three novel antileishmanial lead series. *Int J Parasitol Drugs Drug Resist* **8**, 81-86 (2018).
59 Wijnant, G. J. et al. Pharmacokinetics and Pharmacodynamics of the Nitroimidazole DNDI-0690 in Mouse Models of Cutaneous Leishmaniasis. *Antimicrob Agents Chemother* **63**, e00829-00819 (2019).
60 Torrico, F. et al. New regimens of benznidazole monotherapy and in combination with fosravuconazole for treatment of Chagas disease (BENDITA): a phase 2, double-blind, randomised trial. *Lancet Infect Dis* **21**, 1129-1140 (2021).
61 Molina-Morant, D. et al. Efficacy and safety assessment of different dosage of benznidazol for the treatment of Chagas disease in chronic phase in adults (MULTIBENZ study): study protocol for a multicenter randomized Phase II non-inferiority clinical trial. *Trials* **21**, 328 (2020).
62 Jumper, J. et al. Highly accurate protein structure prediction with AlphaFold. *Nature* **596**, 583-589 (2021).
Nagle, A. et al. Discovery and Characterization of Clinical Candidate LXE408 as a Kinetoplastid-Selective Proteasome Inhibitor for the Treatment of Leishmaniases. J Med Chem 63, 10773-10781 (2020).

Shalev-Benami, M. et al. Atomic resolution snapshot of Leishmania ribosome inhibition by the aminoglycoside paromomycin. Nat Commun 8, 1589 (2017).

Riley, J. et al. Development of a Fluorescence-based Trypanosoma cruzi CYP51 Inhibition Assay for Effective Compound Triaging in Drug Discovery Programmes for Chagas Disease. PLoS Negl. Trop. Dis. 9, e0004014 (2015).

Wall, R. J. et al. The Q(i) Site of Cytochrome b is a Promiscuous Drug Target in Trypanosoma cruzi and Leishmania donovani. ACS Infect Dis 6, 515-528 (2020).

Molina, I. et al. Randomized trial of posaconazole and benznidazole for chronic Chagas' disease. N Engl J Med 370, 1899-1908 (2014).

Torrico, F. et al. Treatment of adult chronic indeterminate Chagas disease with benznidazole and three E1224 dosing regimens: a proof-of-concept, randomised, placebo-controlled trial. Lancet Infect Dis 18, 419-430 (2018).

Gunatilleke, S. S. et al. Diverse inhibitor chemotypes targeting Trypanosoma cruzi CYP51. PLoS Negl Trop Dis 6, e1736 (2012).

Corpas-Lopez, V. et al. Pharmacological Validation of N-Myristoyltransferase as a Drug Target in Leishmania donovani. ACS Infect Dis 5, 111-122 (2019).

Brand, S. et al. Discovery of a novel class of orally active trypanocidal N-myristoyltransferase inhibitors. J. Med. Chem. 55, 140-152 (2012).

Ames, B. N., Lee, F. D. & Durston, W. E. An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc. Natl. Acad. Sci. USA 70, 782-786 (1973).

Ames, B. N., McCann, J. & Yamasaki, E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat Res 31, 347-364 (1975).

Peniche, A. G. et al. Development of an ex vivo lymph node explant model for identification of novel molecules active against Leishmania major. Antimicrob. Agents Chemother. 58, 78-87 (2014).

Domínguez-Asenjo, B. et al. Ex Vivo Phenotypic Screening of Two Small Repurposing Drug Collections Identifies Nifurtamil as a Potential New Treatment against Visceral and Cutaneous Leishmaniasis. ACS Infect Dis 7, 2390-2401 (2021).

da Silva Lara, L. et al. Trypanosoma cruzi infection of human induced pluripotent stem cell-derived cardiomyocytes: an in vitro model for drug screening for Chagas disease. Microbes Infect 20, 312-316 (2018).

De Rycker, M. et al. A static-cidal assay for Trypanosoma brucei to aid hit prioritisation for progression into drug discovery programmes. PLoS Negl. Trop. Dis. 6, e1932 (2012).

Nuhs, A. et al. Development and validation of a novel Leishmania donovani screening cascade for High-Throughput screening using a novel axenic assay with high predictivity of leishmanicidal intracellular activity. PLoS Negl. Trop. Dis. 9, e0004094 (2015).

Jiménez-Antón, M. D. et al. Pharmacokinetics and disposition of miltefosine in healthy mice and hamsters experimentally infected with Leishmania infantum. Eur J Pharm Sci 121, 281-286 (2018).

Voak, A. A., Harris, A., Qaiser, Z., Croft, S. L. & Seifert, K. Pharmacodynamics and Biodistribution of Single-Dose Liposomal Amphotericin B at Different Stages of Experimental Visceral Leishmaniasis. Antimicrob Agents Chemother 61, e00497-00417 (2017).
Van Bocxlaer, K. et al. Topical Treatment for Cutaneous Leishmaniasis: Dermatopharmacokinetic Lead Optimization of Benzoxaboroles. *Antimicrob Agents Chemother* **62**, e02419-02417 (2018).

Van Bocxlaer, K., Yardley, V., Murdan, S. & Croft, S. L. Drug permeation and barrier damage in Leishmania-infected mouse skin. *J Antimicrob Chemother* **71**, 1578-1585 (2016).

Van Bocxlaer, K., Yardley, V., Murdan, S. & Croft, S. L. Topical formulations of miltefosine for cutaneous leishmaniasis in a BALB/c mouse model. *J Pharm Pharmacol* **68**, 862-872 (2016).

Perry, M. R., Wyllie, S., Raab, A., Feldmann, J. & Fairlamb, A. H. Chronic exposure to arsenic in drinking water can lead to resistance to antimonial drugs in a mouse model of visceral leishmaniasis. *Proc Natl Acad Sci U S A* **110**, 19932-19937 (2013).

Gamarro, F., Sánchez-Cañete, M. P. & Castany, S. in *Drug Resistance in Leishmania Parasites: Consequences, Molecular Mechanisms and Possible Treatments* (eds Alicia Ponte-Sucre, Emilia Diaz, & Maritza Padrón-Nieves) 351-379 (Springer Vienna, 2013).

Alvar, J. et al. Implications of asymptomatic infection for the natural history of selected parasitic tropical diseases. *Semin Immunopathol* **42**, 231-246 (2020).

Kushwaha, A. K. et al. Domestic mammals as reservoirs for Leishmania donovani on the Indian subcontinent: Possibility and consequences on elimination. *Transbound Emerg Dis* **10.1111/tbed.14061** (2021).

Rodríguez-Monguí, E., Cantillo-Barraza, O., Prieto-Alvarado, F. E. & Cucunubá, Z. M. Heterogeneity of *Trypanosoma cruzi* infection rates in vectors and animal reservoirs in Colombia: a systematic review and meta-analysis. *Parasit Vectors* **12**, 308 (2019).

Jansen, A. M., Xavier, S. C. d. C. & Roque, A. L. R. *Trypanosoma cruzi* transmission in the wild and its most important reservoir hosts in Brazil. *Parasit Vectors* **11**, 502 (2018).

Austen, J. M. & Barbosa, A. D. Diversity and Epidemiology of Bat Trypanosomes: A One Health Perspective. *Pathogens* **10**, 1148 (2021).

Mullard, A. Parsing clinical success rates. *Nat Rev Drug Discov* **15**, 447 (2016).

Wong, C. H., Siah, K. W. & Lo, A. W. Estimation of clinical trial success rates and related parameters. **20**, 273-286 (2019)
103 Autheman, D. et al. An invariant *Trypanosoma vivax* vaccine antigen induces protective immunity. *Nature* **595**, 96-100 (2021).

104 Romero-Ramirez, A. I. Antigen discovery in *Trypanosoma vivax*. PhD Thesis, University of Liverpool. [https://livrepository.liverpool.ac.uk/3088027/1/201193495_Feb2020.pdf](https://livrepository.liverpool.ac.uk/3088027/1/201193495_Feb2020.pdf) (2020).

105 Kaye, P. M. et al. Overcoming roadblocks in the development of vaccines for leishmaniasis. *Expert Rev Vaccines*, 1-12 (2021).

106 Osman, M. et al. A third generation vaccine for human visceral leishmaniasis and post kala azar dermal leishmaniasis: First-in-human trial of ChAd63-KH. *PLoS Negl Trop Dis* **11**, e0005527 (2017).

107 Zhang, W. W. et al. A second generation leishmanization vaccine with a markerless attenuated *Leishmania major* strain using CRISPR gene editing. *Nat Commun* **11**, 3461 (2020).

108 Lanza, J. S. et al. A TLR9-adjuvanted vaccine formulated into dissolvable microneedle patches or cationic liposomes protects against leishmaniasis after skin or subcutaneous immunization. *Int J Pharm* **586**, 119390 (2020).

109 Fernández Cotrina, J. et al. A large-scale field randomized trial demonstrates safety and efficacy of the vaccine LetiFend® against canine leishmaniosis. *Vaccine* **36**, 1972-1982 (2018).

110 Ashwin, H. et al. Characterization of a new *Leishmania major* strain for use in a controlled human infection model. *Nat Commun* **12**, 215 (2021).

111 Zumla, A. et al. Host-directed therapies for infectious diseases: current status, recent progress, and future prospects. *Lancet Infect Dis* **16**, e47-63 (2016).

112 Varikuti, S. et al. Host-Directed Drug Therapies for Neglected Tropical Diseases Caused by Protozoan Parasites. *Frontiers in microbiology* **9**, 2655 (2018).

113 Rao, S. P. S. et al. Drug Discovery for Kinetoplastid Diseases: Future Directions. *ACS Infect Dis* **5**, 152-157 (2019).

114 Lamotte, S., Späth, G. F., Rachidi, N. & Prina, E. The enemy within: Targeting host-parasite interaction for antileishmanial drug discovery. *PLoS Negl Trop Dis* **11**, e0005480 (2017).

115 Thacker, S. G. et al. CpG ODN D35 improves the response to abbreviated low-dose pentavalent antimonial treatment in non-human primate model of cutaneous leishmaniasis. *PLoS Negl Trop Dis* **14**, e0008050 (2020).

116 [https://dndi.org/research-development/portfolio/cpg-d35/](https://dndi.org/research-development/portfolio/cpg-d35/).

117 Miranda-Verastegui, C. et al. First-line therapy for human cutaneous leishmaniasis in Peru using the TLR7 agonist imiquimod in combination with pentavalent antimony. *PLoS Negl Trop Dis* **3**, e491 (2009).

118 Paradela, L. S. et al. Multiple unbiased approaches identify oxidosqualene cyclase as the molecular target of a promising anti-leishmanial. *Cell Chem Biol* **28**, 711-721.e718 (2021).

119 Wyllie, S. et al. Activation of bicyclic nitro-drugs by a novel nitroreductase (NTR2) in *Leishmania*. *PLoS Pathog.* **12**, e1005971 (2016).

120 Saldívar, M. et al. Targeting the trypanosome kinetochore with CLK1 protein kinase inhibitors. *Nat Microbiol* **5**, 1207-1216 (2020).

121 Wall, R. J. et al. Antitrypanosomal 8-Hydroxy-Naphthyridines Are Chelators of Divalent Transition Metals. *Antimicrob Agents Chemother* **62**, e00235-00218 (2018).

122 Horn, D. Genome-scale RNAi screens in African trypanosomes. *Trends Parasitol*, in press (2021).

123 Giordani, F. et al. Veterinary trypanocidal benzoxyzaboroles are peptidase-activated prodrugs. *PLoS Pathog* **16**, e1008932 (2020).

124 Fernandez-Prada, C. et al. High-throughput Cos-Seq screen with intracellular *Leishmania infantum* for the discovery of novel drug-resistance mechanisms. *Int J Parasitol Drugs Drug Resist* **8**, 165-173 (2018).
125 Jones, N. G., Catta-Preta, C. M. C., Lima, A. & Mottram, J. C. Genetically Validated Drug Targets in *Leishmania*: Current Knowledge and Future Prospects. *ACS Infect Dis* **4**, 467-477 (2018).

126 Lima, M. *et al.* Identification of a proteasome-targeting arylsulfonamide with potential for the treatment of Chagas' disease. *Antimicrob Agents Chemother*, Aac0153521 (2021).

127 Altmann, S. *et al.* Oligo targeting for profiling drug resistance mutations in the parasitic trypanosomatids. *Nucleic Acids Res* (2022).

128 Morillo, C. A. *et al.* Benznidazole and Posaconazole in Eliminating Parasites in Asymptomatic *T. cruzi* Carriers: The STOP-CHAGAS Trial. *J Am Coll Cardiol* **69**, 939-947 (2017).

129 Chatelain, E. & Scandale, I. Animal models of Chagas disease and their translational value to drug development. *Expert Opinion on Drug Discovery* **15**, 1381-1402 (2020).

130 Kennedy, P. G. *et al.* A substance P antagonist, RP-67,580, ameliorates a mouse meningoencephalitic response to *Trypanosoma brucei brucei*. **94**, 4167-4170 (1997).

131 Jennings, F. W. *et al.* Human African trypanosomiasis: potential therapeutic benefits of an alternative suramin and melarsoprol regimen. *Parasitol Int* **51**, 381-388 (2002).

132 Burrell-Saward, H., Rodgers, J., Bradley, B., Croft, S. L. & Ward, T. H. A sensitive and reproducible *in vivo* imaging mouse model for evaluation of drugs against late-stage human African trypanosomiasis. *J. Antimicrob. Chemother.* **70**, 510-517 (2015).

133 Loria-Cervera, E. N. & Andrade-Narváez, F. J. Animal models for the study of leishmaniasis immunology. *Rev Inst Med Trop Sao Paulo* **56**, 1-11 (2014).

134 Mendes Costa, D., Cecílio, P., Santarém, N., Cordeiro-da-Silva, A. & Tavares, J. Murine infection with bioluminescent *Leishmania infantum* axenic amastigotes applied to drug discovery. *Sci Rep* **9**, 18989 (2019).

135 Ong, H. B., Clare, S., Roberts, A. J., Wilson, M. E. & Wright, G. J. Establishment, optimisation and quantitation of a bioluminescent murine infection model of visceral leishmaniasis for systematic vaccine screening. *Sci Rep* **10**, 4689 (2020).

136 Mears, E. R., Modabber, F., Don, R. & Johnson, G. E. A Review: The Current In Vivo Models for the Discovery and Utility of New Anti-leishmanial Drugs Targeting Cutaneous Leishmaniasis. *PLoS Negl Trop Dis* **9**, e0003889 (2015).

137 Chatelain, E. & Konar, N. Translational challenges of animal models in Chagas disease drug development: a review. *Drug Des Devel Ther* **9**, 4807-4823 (2015).

138 Francisco, A. F. *et al.* Challenges in Chagas Disease Drug Development. *Molecules* **25** (2020).

139 Ayala, E. V. *et al.* C57BL/6 α-1,3-Galactosyltransferase Knockout Mouse as an Animal Model for Experimental Chagas Disease. *ACS Infect Dis* **6**, 1807-1815 (2020).

140 Henderson, C. J. *et al.* An Extensively Humanized Mouse Model to Predict Pathways of Drug Disposition and Drug/Drug Interactions, and to Facilitate Design of Clinical Trials. *Drug Metab Dispos* **47**, 601-615 (2019).

141 Magez, S. & Caljon, G. Mouse models for pathogenic African trypanosomes: unravelling the immunology of host-parasite-vector interactions. *Parasite Immunol* **33**, 423-429 (2011).

142 Antoine-Moussiaux, N., Magez, S. & Desmecht, D. Contributions of experimental mouse models to the understanding of African trypanosomiasis. *Trends Parasitol* **24**, 411-418 (2008).

143 Mitra, A. K. & Mawson, A. R. Neglected Tropical Diseases: Epidemiology and Global Burden. *Trop Med Infect Dis* **2**, 36 (2017).