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Trypanocidal drugs: mechanisms, resistance and new targets

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The protozoan parasites *Trypanosoma brucei* and *Trypanosoma cruzi* are the causative agents of African trypanosomiasis and Chagas disease, respectively. These are debilitating infections that exert a considerable health burden on some of the poorest people on the planet. Treatment of trypanosome infections is dependent on a small number of drugs that have limited efficacy and can cause severe side effects. Here, we review the properties of these drugs and describe new findings on their modes of action and the mechanisms by which resistance can arise. We further outline how a greater understanding of parasite biology is being exploited in the search for novel chemotherapeutic agents. This effort is being facilitated by new research networks that involve academic and biotechnology/pharmaceutical organisations, supported by public–private partnerships, and are bringing a new dynamism and purpose to the search for trypanocidal agents.

Trypanosomatids are flagellated protozoan parasites belonging to the order Kinetoplastida. They can infect diverse hosts, ranging from plants through to higher mammals. In humans, *Trypanosoma brucei* and *Trypanosoma cruzi* are responsible for two major tropical diseases: human African trypanosomiasis (HAT) and Chagas disease, respectively. There are 11 million people infected and more than 150 million at risk. These diseases represent a major public health problem in regions of the world least able to deal with the associated economic burden. With no immediate prospect of vaccines, and no satisfactory drug treatments, the requirement for new therapies is a priority. However, drug discovery is high risk and expensive, and the development of agents designed specifically to target trypanosomal diseases is not perceived to be commercially attractive. Until recently, interest from large pharmaceutical corporations has been minimal, with most research and development occurring in academic settings. As a consequence, trypanosome infections are now referred to as 'most neglected diseases'. This review focuses on how existing trypanocidal therapies mediate their activity, the problems associated with their usage and current areas of research that offer hope of new treatments.

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Human African trypanosomiasis

The problem

Insect-transmitted parasites of the *T. brucei* species complex are the causative agents of HAT (also known as African sleeping sickness) and nagana, a disease common in domesticated animals in Africa. The parasite was first identified in 1895 by David Bruce while studying cattle (Ref. 1), and later from human blood by Forde and Dutton (Ref. 1). HAT is considered endemic in 36 countries of sub-Saharan Africa, with over 50 million people at risk (Refs 2, 3). Localised epidemics can arise readily following political and socioeconomic disruption, killing tens of thousands of people. As a result of coordinated surveillance and treatment programmes, the number of people dying from HAT has recently fallen to ~70 000 annually (Ref. 4). However, in regions of Angola, the Democratic Republic of Congo and southern Sudan, the mortality rate exceeds that of malaria and HIV/AIDS (human immunodeficiency virus and acquired immune deficiency syndrome).

HAT pathology has two distinct phases: the early (or haemolymphatic) stage and the late (or encephalitic) stage (Refs 2, 3). In the early stage, parasites are found in both the blood and the lymph systems. Symptoms begin with irregular fevers, headaches and joint pains, but can develop to generate other complications including enlarged lymph glands and spleen, local oedema and cardiac abnormalities. When parasites cross the blood–brain barrier, the disease then enters the late stage. Here, a series of neurologically related symptoms are observed, including severe headaches, alterations to the sleeping pattern, personality change, mental function impairment and weight loss. Without treatment, patients eventually fall into a coma and die.

HAT is caused by two distinct subspecies of *T. brucei*, each having separate geographical foci and differing pathologies (Ref. 2). In West and Central Africa, *T. b. gambiense* is prevalent, while elsewhere in sub-Saharan Africa, *T. b. rhodesiense* predominates. These subspecies are responsible for the West and East African forms of the disease, respectively. The main difference between the two infections is the rate of progression from the blood/lymphatic stage to the cerebral stage. In West African trypanosomiasis, this takes months and hence the infection is termed chronic. By contrast, with East African trypanosomiasis the infection is more acute, with progression occurring in as little as 1–3 weeks. In both cases, the disease is zoonotic, although with *T. b. gambiense*, humans are the main reservoir. Wild and domestic animals, especially cattle, are the major reservoirs with *T. b. rhodesiense*. A third closely related subspecies, *T. b. brucei*, is nonpathogenic to humans, but is responsible for many cases of nagana in cattle. This infection is of huge veterinary and economic importance and has had a profound effect on agricultural development in Africa.

T. brucei is an extracellular pathogen, with a surface coat composed almost entirely of a single antigen, the variant surface glycoprotein (VSG). The parasite avoids immune destruction by a process of antigenic variation, which involves the periodic switching of VSG expression to another of the antigenically distinct surface glycoproteins encoded by the large repertoire of *VSG* genes. As a result of this constant switching, vaccine development against this parasite is not thought to be a realistic prospect.

Treatment of HAT

Treatment of African sleeping sickness is dependent on the subspecies and the disease stage. When parasites are restricted to the blood/lymphatic system, pentamidine is used against *T. b. gambiense*, and suramin against *T. b. rhodesiense* (Table 1). Neither compound can cross the blood–brain barrier effectively, and these drugs are therefore of little use against cerebral disease. The treatments available for this lethal stage are restricted to melarsoprol and eflornithine. Melarsoprol is the only drug active against both *T. brucei* subspecies once the central nervous system (CNS) has been accessed (Table 1). However, it is a highly toxic arsenical, and drug resistance is a major issue (Ref. 5). Eflornithine, in contrast, is relatively safe, but this compound is effective only against West African trypanosomiasis and the cost of treatment is problematic in underdeveloped countries (Table 1). Currently, Phase III clinical trials are evaluating the efficacy of a combination therapy consisting of eflornithine with nifurtimox against the cerebral stages of West African trypanosomiasis (Refs 6, 7, 8). This has proved very effective and has recently been recommended by the World Health Organization (WHO) as a front-line treatment for infections with *T. b. gambiense*.

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Table 1. Problems associated with current drugs for human African trypanosomiasis

Drug	Use	Problem
Suramin	Effective against early stage of *T. b. rhodesiense*	Ineffective against early stage of *T. b. gambiense* and late stage of both HATs
Pentamidine	Effective against early stage of *T. b. gambiense*	Ineffective against early stage of *T. b. rhodesiense* and late stage of both HATs
Melarsoprol	Effective against late stage of both HATs	Toxic (kills up to 5% of patients) Resistance observed in field
Eflornithine	Effective against late stage of *T. b. gambiense*	Ineffective against late stage of *T. b. rhodesiense* Difficult dosing scheme, and hospitalisation costs

Abbreviations: HAT, human African trypanosomiasis; *T. b., Trypanosoma brucei.*

Suramin

Suramin was first used against HAT in 1922 following observations that trypan dyes had trypanocidal activity (Refs 9, 10). This symmetrical polysulphonated naphthalene derivative of urea (Fig. 1) is negatively charged at physiological pH. The drug is typically administered to patients as an intravenous injection with a low dose on day 1 followed by higher amounts on days 3, 5, 11, 23 and 30 (Ref. 11) (Table 2). Its anionic charge prevents it from freely crossing biological membranes, hinders transport across the blood–brain barrier and promotes binding to a variety of other molecules. Suramin is extremely stable in humans, shows no transformation in the liver, and is only slowly excreted in urine. These properties contribute to the relatively long half-life (Ref. 12) (Table 2).

Based on size and charge, it had been assumed that transport of suramin into trypanosomes occurs through bulk-flow uptake. However, in the presence of blood serum proteins, the drug transport rate exceeds the level attainable by this passive process alone (Ref. 13). As suramin can readily complex with blood serum proteins, it has been suggested that the drug enters *T. brucei* through receptor-mediated endocytosis, possibly bound to low-density lipoprotein (LDL) (Refs 14, 15). However, analysis of endocytosis components indicates that suramin and LDL uptake are not directly linked, with the drug entering the parasite via an unidentified mechanism (Ref. 16).

Little is known about how suramin is active against trypanosomes. Suramin-resistant strains of *T. brucei* and *Trypanosoma evansi*, a blood-borne animal parasite, have been selected in vitro, but the mechanisms underlying this phenotype have not been established (Refs 17, 18). By virtue of its negative charge, suramin can hinder uptake of serum proteins, or inhibit endocytosis and key enzymes in metabolic pathways such as glycolysis (Refs 13, 19, 20). Since suramin affects multiple targets this, coupled with its low toxicity, may explain why it has been used to treat early-stage East African trypanosomiasis for nearly 90 years.

Pentamidine

Pentamidine belongs to the aromatic diamidine class of antiprotozoal agents and was first used against *T. b. gambiense* in 1937, following observations that the related compound synthalin had potent antitrypanosomal activity (Refs 9, 10). Treatment involves daily intramuscular injections for 7–10 days (Ref. 11) (Table 2). It is readily metabolised by the mammalian cytochrome P450 system (but not by the trypanosomal system) and excreted in urine, and therefore has a relatively short half-life (Refs 21, 22, 23) (Table 2).

Pentamidine is positively charged. As a result, it binds easily to blood serum proteins, does not readily diffuse across biological membranes, and passes into the cerebrospinal fluid very slowly (Ref. 24). Because the drug must accumulate to high intracellular levels before having a trypanocidal effect (>1 mM), it is probable that active transport mechanisms are necessary for pentamidine activity. Uptake of diamidines by

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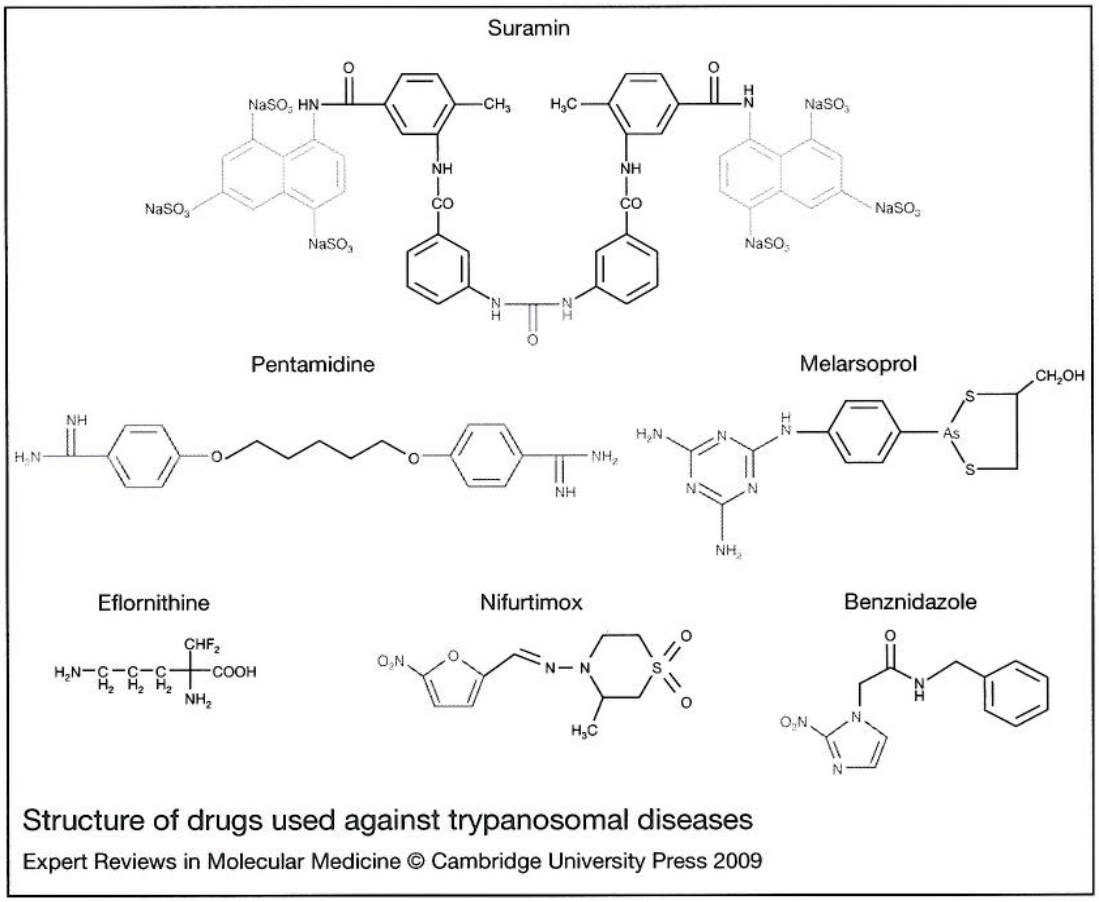

Figure 1. Structure of drugs used against trypanosomal diseases. The highlighted regions correspond to motifs described in the text. For suramin, urea (blue), napthalenes (green) and sulphonates (red) are shown. In pentamidine, the aromatic diamidine (blue) is outlined. For melarsoprol, the arsenic (red) and melamine ring (blue) are highlighted. The blue regions in nifurtimox and benznidazole correspond to the 5-nitrofuran or the 2-nitroimidazole group, respectively.

bloodstream *T. brucei* involves the P2 aminopurine permease (*Tb*AT1), a transporter that also mediates melarsoprol uptake (Refs 25, 26, 27). However, *T. brucei TbAT1*-null mutants were found to retain sensitivity to pentamidine, whereas they were resistant to other diamidines such as berenil (Ref. 28). These results can now be explained following the characterisation of other uptake activities, including a high-affinity pentamidine transporter (HAPT) and low-affinity pentamidine transporters (LAPTs) (Refs 26, 29). As pentamidine uptake by *T. brucei* occurs by several routes, it has been speculated that resistance by reduced drug uptake is unlikely to arise in a clinical context. However, parasites deficient in two of the pentamidine transporters (*Tb*AT1 and HAPT) have been generated in the laboratory, demonstrating that such events can occur (Ref. 30). In addition, recombination between resistant strains in the field is a possibility.

The mode of action for pentamidine is not fully understood. It can interact with anionic molecules and readily bind to DNA (Ref. 31). Analogues of pentamidine accumulate in the nucleus and kinetoplast, a disc-shaped structure containing the mitochondrial genome (Ref. 32). It has been proposed that pentamidine inhibits mitochondrial topoisomerase II activity, leading to the linearisation of the kinetoplast DNA and the generation of dyskinetoplastic trypanosomes (Ref. 33). Exposure of parasites to

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Table 2. Pharmacokinetic properties of drugs against human African trypanosomiasis

Drug	Absorption	Distribution	Metabolism	Excretion	Refs
Suramin	5 mg/kg intravenous injection on day 1, then 20 mg/kg at days 3, 5, 11, 23 and 30	99.7% binds to proteins; unable to pass through blood–brain barrier	Not metabolised; half-life 35–60 days	Slowly in urine	11, 12
Pentamidine	4 mg/kg intramuscular injection given once daily or on alternate days for 7–10 days	69% binds to protein; passes poorly through blood–brain barrier	Metabolised in liver by cytochrome P450 system; half-life 6.4–9.4 h	Urine (<10%)	11, 24, 251
Melarsoprol	1.2 mg/kg intravenous injection on day 1, 2.4 mg/kg on day 2 and 3.6 mg/kg on days 3 and 4; repeat three or four times with 7–10 day interval 2.2 mg/kg intravenous injection on days 1–10	Crosses the blood–brain barrier	Metabolised to melarsen oxide; half-life 35 h	Rapidly in urine	11, 42
Eflornithine	400 mg/kg in four daily infusions for 7 or 14 days	Limited passage through blood–brain barrier	Not metabolised; half-life 3.3 h	Urine	11, 252
NECT	Nifurtimox at 15 mg/kg per day orally administered over 10 days, plus eflornithine at 400 mg/kg per day in infusions of 12 h for 7 days	See above and Table 3	See above and Table 3	See above and Table 3	8

Abbreviations: HAT, human African trypanosomiasis; NECT, nifurtimox–eflornithine combination therapy.

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pentamidine analogues appears to confirm this observation, as prolonged treatment leads to breakdown of the kinetoplast and its eventual disappearance (Refs 31, 32). However, it has been argued that formation of dyskinetoplastic parasites may not be sufficient to cure an infected mammalian host, and the mechanism of pentamidine transport into the mitochondrion has yet to be determined (Ref. 34). Thus, diamidines may exert their trypanocidal activities through other mechanisms, possibly via inhibition of *T. brucei* *S*-adenosylmethionine decarboxylase or Ca^{2+}-ATPase activities, by affecting mitochondrial membrane potential, or by acting to uncouple oxidative phosphorylation (Refs 35, 36, 37, 38). Intriguingly, pentamidine analogues accumulate in organelles lacking DNA, possibly acidocalcisomes, and this possible mode of action warrants further investigation (Ref. 32).

Melarsoprol

Melarsoprol (MelB) was introduced in 1949 and is currently the only treatment used against the late stage of both forms of HAT (Ref. 39). This trivalent melaminophenyl arsenical (Fig. 1) functions as a prodrug, with activation leading to the formation of melarsen oxide (MelOx). MelB is highly toxic and causes reactive encephalopathy in approximately a fifth of patients, and has a mortality rate of 5% (Ref. 40). Additionally, it is responsible for other side effects including convulsions, fever, loss of consciousness, rashes, bloody stools, nausea and vomiting. The drug is administered as an intravenous injection with a number of different schedules (Ref. 41) (Table 2). For *T. b. rhodesiense* infections, a regime consists of one injection daily over 3–4 days, followed by a rest period of 7–10 days; this is then repeated twice more. Based on pharmacokinetic properties, an alternative scheme is now routinely used for *T. b. gambiense* infections, consisting of a daily injection for 10 days (Refs 11, 42). Although this regime can lead to adverse skin reactions, it is as effective as the previous schedules and significantly cheaper: less drug is used and hospitalisation is shorter. Additional problems are also encountered because MelB has poor solubility in water, alcohol or ether, and the drug is normally dissolved in propylene glycol. As a result, MelB can only be given using glass syringes and the injections are very painful, with patients frequently reporting a burning sensation during administration.

Accumulation of melaminophenyl arsenical drugs by trypanosomes occurs through the P2 aminopurine permease *Tb*AT1, and loss of this activity has been implicated in drug resistance in both laboratory and field isolates (Refs 5, 28, 43, 44). The transporter was identified using competition studies, which demonstrated that uptake of purine nucleosides occurs by two distinct activities (Ref. 43). One transport system (designated P1) functions as a general purine nucleoside transporter, and a second (*Tb*AT1) carries adenosine and its nucleobase adenine. MelB and MelOx hinder adenine uptake through the latter mechanism. Comparison of *Tb*AT1 substrates has identified a common amidinium-like motif (Refs 45, 46, 47, 48). This pattern has now been incorporated into several novel chemical structures, some of which display considerable trypanocidal activity (Refs 46, 49, 50, 51, 52). Intriguingly, *TbAT1*-null mutants are only threefold more resistant to MelB and MelOx than controls, significantly lower than the levels of resistance reported in laboratory-selected cells adapted to grow in the presence of arsenical drugs (Refs 5, 53). This has led to the proposal of alternative uptake routes, with HAPT being a likely candidate (Refs 28, 30, 54). The involvement of this pentamidine transporter in arsenical uptake stems from observations that sequential loss of *Tb*AT1 followed by HAPT generates parasites with a greater resistance to MelOx than the *TbAT1*-null mutant (Ref. 30).

Arsenical drugs lyse *T. brucei* rapidly, but their modes of action are not fully understood. MelB inhibits a range of glycolytic and pentose phosphate pathway enzymes (Refs 55, 56), and can readily form stable adducts with trypanothione, the glutathione–spermidine conjugate that is the major thiol in trypanosomes (Refs 57, 58). These adducts can block activity of trypanothione reductase (TR), the parasite-specific enzyme that maintains trypanothione in its reduced form (Refs 58, 59). However, in all cases the rapidity with which the drug kills parasites cannot be explained by the systems so far implicated in its mode of action. For example, in the time taken for MelB-treated cells to lyse, ATP levels have not been sufficiently depleted to kill the parasite (Ref. 58). In addition, only a small fraction of

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trypanothione is actually conjugated to the arsenical, and *T. brucei* with reduced TR levels are as sensitive to MelOx as controls (Ref. 60).

In some regions, the relapse rate to MelB is up to 30% (Ref. 11). The emergence of resistance is of concern, given that the drug is the only cheap treatment available against the late stage of both forms of HAT. In a significant number of cases, MelB-nonresponsive parasites retrieved from HAT patients were found to contain mutations in *TbAT1*, suggesting that resistance correlates with reduced drug uptake (Ref. 5). However, *TbAT1*-null mutants do not display a high level of resistance (Ref. 28), although it has not yet been established whether other transporters such as HAPT contribute to this phenotype. In other organisms, multidrug resistance is often mediated through active efflux mechanisms involving ABC transporters. Analysis of the *T. brucei* genome data suggests that the parasite possesses 22 potential members of this protein class (Ref. 61). Overexpression of one of these, *Tb*MRPA, results in tenfold resistance to MelB (Ref. 62). This protein is a putative thiol-conjugate transporter and it has been postulated that *Tb*MPRA functions by removing arsenical/trypanothione moieties from the cell. However, its role in mediating resistance in a clinical context is open to debate (Ref. 63).

Eflornithine

Originally developed as an anticancer therapy, eflornithine (DL-α-difluoromethylornithine) was first used to treat late-stage West African trypanosomiasis in the early 1980s (Ref. 64) (Fig. 1). It quickly gained the nickname 'resurrection drug' for its ability to cure and revive comatose patients. However, despite being relatively safe, usage is restricted as a result of the difficult dosing scheme (Ref. 65): eflornithine is administered under medical supervision by intravenous infusion in four large doses daily for 7–14 days (Ref. 11) (Table 2). One explanation for the high concentrations of drug needed to cure the disease is poor transport across the blood–brain barrier (Ref. 66). Despite these problems, eflornithine is now recommended as the front-line treatment for late-stage West African trypanosomiasis (Refs 65, 67, 68, 69).

In mammals, eflornithine enters cells by passive diffusion, a mechanism that may operate in bloodstream *T. brucei* (Refs 70, 71, 72). However, as uptake by trypanosomes is temperature sensitive and follows Michaelis–Menten-type kinetics, it could be carrier-mediated, possibly by amino acid transporters (Ref. 73; M. Barrett, pers. commun.). Once in the parasite, the drug functions as an irreversible inhibitor of ornithine decarboxylase (ODC), the rate-controlling enzyme in polyamine biosynthesis (Ref. 74). The interaction between *T. brucei* ODC and eflornithine has been extensively studied and the mechanism of drug selectivity has been determined (Refs 75, 76). It stems from the differential stability of the parasite ODC (half-life ~18 h) and mammalian ODC (half-life ~20 min) (Refs 75, 77, 78). When eflornithine irreversibly binds to the mammalian enzyme, the resultant complex is rapidly degraded and replaced with newly synthesised ODC. By contrast, the *T. brucei* eflornithine–ODC molecule is relatively stable and the enzyme slowly replaced. Thus, the level of active ODC is effectively decreased, leading to a cessation of putrescine formation. Eventually, parasites stop growing and the nondividing cells are cleared by the immune system.

Chagas disease

The problem

Chagas Disease (or American trypanosomiasis) is caused by *T. cruzi*. This is a zoonotic infection spread by blood-feeding triatomid (or kissing) bugs. The parasite can infect a wide range of mammals including dogs, cats, monkeys, rodents, ground squirrels, opossums and armadillos. It is found throughout Latin America, where as many as 8–11 million people are infected, resulting in over 15 000 deaths per year (Refs 2, 3, 79). Normally, the disease occurs in rural areas where triatomid bugs transmit parasites from animals to humans. Recently, as a result of human migration, the disease has also been found in urban areas. This, in combination with secondary routes of infection, such as through blood and organ transplantation, has resulted in Chagas disease becoming a problem in the USA, which has an estimated 100 000 infected people (Ref. 80).

Chagas disease has three distinct phases: acute, indeterminate and chronic (Refs 2, 3). The acute stage commonly affects children, with up to 5% of diagnosed cases resulting in death. During

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this phase, parasites multiply as intracellular amastigotes in macrophages and tissue cells at the site of the insect bite. Although generally asymptomatic, the acute stage can present clinical signs in the form of fever, skin lesions (chagoma), oedema, enlarged lymph nodes and conjunctivitis (Romana's sign). These can appear 1–6 weeks after infection. In the indeterminate stage, patients are asymptomatic and parasites largely disappear from the bloodstream, although they can be detected in cardiac and smooth muscle. In this phase, patients remain a reservoir of infection. In most cases, the disease does not advance further. However, 30% of individuals eventually progress to the chronic stage, often 10–20 years after the initial infection. The long-lasting, symptomatic chronic phase frequently presents with extensive cardiac and digestive tract pathologies and in these instances prognosis is poor. Reactivation of latent Chagas disease in HIV/AIDS patients has also been observed, sometimes with unusual clinical manifestations, including CNS involvement (Ref. 81).

In Latin America, the economic impact of Chagas disease outweighs the combined effects of other parasitic diseases, including malaria and leishmaniasis. Although there is no effective cure for the chronic disease, it is possible to halt transmission through the elimination of the insect vector. In 1991, governments of the six Southern Cone countries launched an initiative to control Chagas disease, which has had remarkable success, with transmission halted in previously endemic regions (Ref. 82). New programmes, aimed at repeating this accomplishment in other areas of Latin America, are now under way (Ref. 79). However, even if all transmission could be blocked, Chagas disease would remain a public health problem for many years.

Chagas disease chemotherapy

Treatment of Chagas disease is controversial because the two available drugs, nifurtimox and benznidazole, are toxic, may be carcinogenic, and have poor efficacy against the chronic stage (Refs 83, 84, 85). Additionally, the proposition that antiparasitic drugs are appropriate for treating the chronic stage has been questioned, given the belief that there is a large autoimmune component associated with this phase of the disease (Ref. 86). Recently, a series of reports using murine models has shown that parasite persistence is both necessary and essential for development of Chagasic heart disease, that drugs prevent development of chronic cardiomyopathy, and that drug-induced pathogen clearance results in a stable protective T cell memory (Refs 87, 88, 89, 90, 91). These studies demonstrate that chemotherapeutic intervention against any stage of Chagas disease is an appropriate course of action.

Nifurtimox and benznidazole have been used to treat the acute phase of Chagas disease for more than 40 years. Both compounds are orally administered, and because they are metabolised by the cytochrome P450 system (Refs 92, 93, 94), multiple doses have to be given daily (Refs 2, 95) (Table 3). A course of treatment lasts 1–4 months for nifurtimox and 1–2 months for

Table 3. Pharmacokinetic properties of drugs against Chagas disease

Drug	Absorption	Distribution	Metabolism	Excretion	Refs
Nifurtimox	8–10 mg/kg/day in three oral doses for 30–120 days	Rapidly absorbed from the GI tract	Metabolised in liver; half-life 3 h	No significant elimination	2, 95, 253
Benznidazole	5–10 mg/kg/day in two oral doses for 30–60 days	Rapidly absorbed from the GI tract; 44% binds to protein; good tissue penetration	Metabolised in liver; half-life 12 h	Urine and faeces	2, 95, 254

Abbreviation: GI, gastrointestinal.

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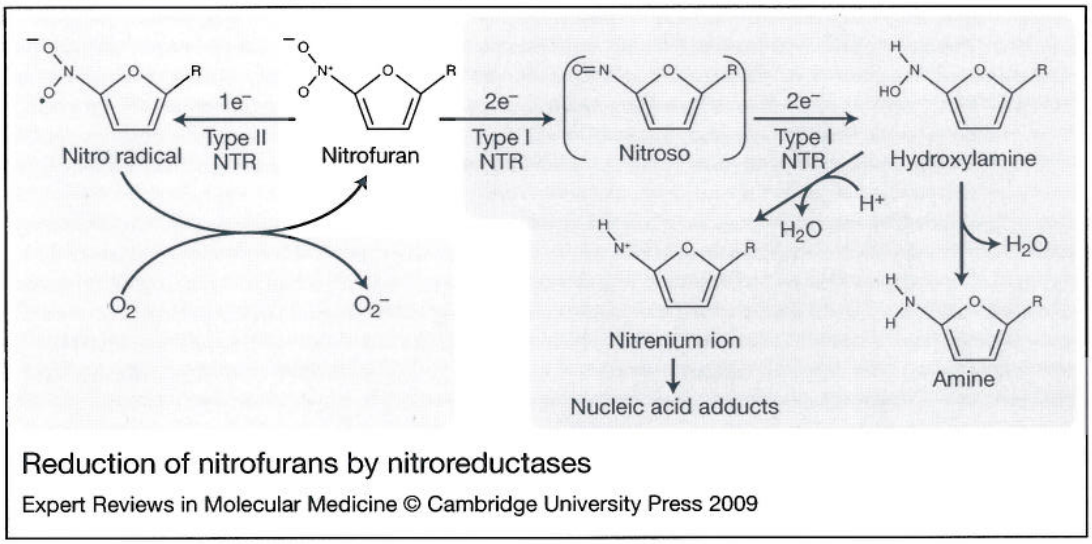

Figure 2. Reduction of nitrofurans by nitroreductases. Type I nitroreductases (NTRs) (blue background) mediate a two-electron reduction of the conserved nitro group on the nitrofuran drug to generate the unstable nitroso derivative. This then undergoes further reduction to the amine form via a hydroxylamine intermediate. The hydroxylamine can also react to form a nitrenium ion, which then promotes DNA damage. Type II NTRs (lilac background) mediate a one-electron reduction of the conserved nitro group, leading to the formation of an unstable nitro radical. In the presence of oxygen, this can react to form superoxide anions (O_2^-). This process, known as futile cycling, also results in the regeneration of the drug.

benznidazole. In many cases the recommended drug schedules are not completed, often as a result of side effects, resulting in considerable scope for the development of resistance.

Both nifurtimox and benznidazole are nitroheterocyclic compounds. They contain a nitro group linked to a furan or imidazole ring, respectively (Fig. 1). These agents function as prodrugs and must undergo enzyme-mediated activation to have cytotoxic effects, reactions catalysed by nitroreductases (NTRs). The precise mode of action of nitroheterocyclic drugs in trypanosomes was originally unclear, with two hypotheses proposed.

The first stemmed from observations that activation of either drug can lead to the formation of reactive oxygen species (ROS) (Refs 96, 97, 98) (Fig. 2), a process that involves a one-electron reduction of the drug by a type II NTR activity. In the presence of oxygen, this promotes superoxide anion production and drug regeneration, a process known as futile cycling (Fig. 2). To date, several trypanosomal flavin adenine dinucleotide (FAD)-containing enzymes, including TR, lipoamide dehydrogenase and cytochrome P450 reductase have been implicated in nifurtimox reduction (Refs 98, 99, 100). This mode of action was considered attractive because trypanosomes were thought to lack many of the 'classical' eukaryotic enzymes responsible for ROS detoxification (Refs 101, 102). However, far from being deficient in such activities, trypanosomatids actually possess a series of novel oxidative defence pathways (Refs 103, 104, 105, 106). The only direct link between drug-induced ROS formation and trypanocidal activity stems from gene deletion experiments on *T. brucei SODB1*, which encodes a superoxide dismutase. Parasites lacking SODB1 are hypersensitive to nifurtimox and benznidazole (Ref. 107). Functional analysis of other oxidative defence pathways has failed to find a link with the trypanocidal activity of nitroheterocyclic drugs (Refs 104, 108, 109, 110, 111, 112, 113).

The second hypothetical mechanism was based on the demonstrated antimicrobial activity of nitrofurans (Refs 114, 115). Here, flavin mononucleotide (FMN)-containing, oxygen-insensitive type I NTRs mediate a series of two-electron reductions of the conserved nitro

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group, through nitroso, to a hydroxylamine derivative, using reduced nicotinamide adenine dinucleotide (phosphate) [NAD(P)H] as the source of reducing equivalents. The hydroxylamine can react to generate nitrenium cations that promote DNA breakage (Refs 116, 117) (Fig. 2). In addition to this, the highly electrophilic intermediaries may affect other molecules in the cell. The decrease in free thiol content observed in nifurtimox- and benznidazole-treated *T. cruzi* could be due to conjugation of the thiol with the nitroso form of the drugs, thus affecting the redox status of the cell (Ref. 118). Two trypanosomal enzymes with this type I activity have been reported. The first is a prostaglandin F2α synthase (PGFS), although this can only mediate two-electron reduction of nifurtimox under anaerobic conditions (Ref. 119). The second, for which there is now strong experimental evidence, is a type I NTR (Ref. 120). This class of enzyme had been regarded as specific to bacteria, and absent from eukaryotes; trypanosomes are now a major exception.

T. cruzi resistance to nifurtimox and benznidazole is encountered throughout Latin America and can be readily mimicked through continuous culturing of the parasite in the presence of drug (Refs 120, 121, 122). Until recently though, the molecular basis for resistance had not been elucidated and appeared complex. Parasites selected for nitroheterocyclic drug resistance often displayed significant karyotypic alterations (Refs 120, 121), with the resulting effects on global gene expression masking the precise mechanism(s) (Refs 123, 124, 125, 126, 127, 128, 129). A link between the activity of a trypanosome type I NTR and cross-resistance to nifurtimox and benznidazole has now been established in *T. cruzi* and *T. brucei*. In both cases, deletion of the corresponding gene generates parasites resistant to nitroaromatic compounds, while overexpression confers hypersensitivity (Ref. 120). Furthermore, laboratory selection of nifurtimox-resistant *T. cruzi* gives rise to parasites that lack one of the chromosomes containing the *NTR* gene. This phenotype can be complemented by reintroduction of the gene. These experiments clearly implicate NTR as a key player in the activation of both drugs and demonstrate the potential for resistance by a simple mechanism (Ref. 25). The extent to which this occurs in the field remains to be established. Another possible resistance mechanism involving drug detoxification has also been proposed (Ref. 120). Overexpression of a trypanosomal cytochrome P450 reductase (CPR) confers resistance to benznidazole. However, given that nitroheterocyclic drugs are readily metabolised by hepatic CPRs to generate toxic products, it is unclear how this mechanism would mediate resistance within the parasite. This warrants further attention to dissect the precise details underlying the pathway.

Future perspectives in chemotherapy

The research landscape

As outlined, new treatments for HAT and Chagas disease are urgently needed in the respective regions. This will require a major international effort since important specifications must be fulfilled by any new drugs targeted at these diseases. In the case of HAT, the priority is a safe, oral, short-course therapy for late-stage *T. b. gambiense* CNS infection. The requirement for active compound to reach parasites within the brain is a major issue in drug design, since the blood–brain barrier impedes the passage of most small molecules. For Chagas disease, a drug able to arrest or alleviate the symptoms of the chronic stage of the infection is the challenging goal. Progress in both areas has been limited, in part, by the lack of good animal models that accurately mirror the course of disease in humans. However, the outlook for research is improving with schemes such as the Drugs for Neglected Diseases Initiative (DNDi) and the Consortium for Parasitic Drug Development (CPDD), as well as the engagement of organisations such as the Bill & Melinda Gates Foundation and the Wellcome Trust. In addition, academic and biotechnology/pharmaceutical organisations, facilitated by public–private partnerships, are building portfolios of projects based on validated targets and high-throughput screening of chemical libraries. These approaches build on significant advances that have been made in our understanding of trypanosome biology and genetics, including the completion of the *T. brucei* and *T. cruzi* genome projects in 2005 (Refs 131, 132). The development of sophisticated and flexible genetic manipulation systems, particularly for *T. brucei* (Refs 133, 134), has also been an important factor, in that it facilitates rapid analysis of gene function and target validation.

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New targets for chemotherapy

There are now a large number of trypanosomatid enzymes and/or biochemical pathways that have been identified as targets for drug development. Below are listed some examples that are currently under investigation in this context.

Sterol biosynthesis

Analysis of *T. cruzi* sterol composition revealed that the parasite contains ergosterol, a molecule previously identified as a component of fungal cell membranes (Ref. 135). Candidate genes encoding enzymes in the ergosterol biosynthetic pathway have been identified in *T. cruzi*, with many of these having now been characterised and chemically validated as drug targets (Refs 136, 137, 138, 139, 140, 141, 142). The pathway is present in *T. brucei*, but this parasite can also scavenge cholesterol from the host bloodstream (Ref. 143). Several inhibitors of the fungal sterol biosynthetic pathway have been found to have activity against *T. cruzi*. These include compounds that block sterol 14α-demethylase (CYP51), such as triazoles (Refs 144, 145, 146), imidazoles (Refs 136, 140, 147, 148, 149), indomethacin amides (Ref. 142) and a novel structure (C155-0123) based on the moiety *N*-[4-pyridyl]-formamide (Refs 141, 150). A number of these are reported to display curative activity against acute/chronic Chagas disease, with some able to eradicate nifurtimox- and benznidazole-resistant *T. cruzi* from mice, even in immunosuppressed hosts (Refs 146, 151, 152).

The allylamine derivative terbinafin, and heteroallyl 5-nitrofuranes, which are both inhibitors of squalene epoxidase, are also active against *T. cruzi* (Refs 153, 154). Terbinafin is of particular interest, as it potentiates the activity of the triazole CYP51 inhibitors (Refs 153, 155). Inhibitors of lanosterol synthase (oxidosqualene cyclase) have been reported to have good in vitro activity against *T. cruzi*, but in vivo effects have yet to be identified (Refs 138, 156). Some azasterols have been shown to target sterol methyltransferase activity, whereas others do not (Refs 157, 158, 159, 160, 161, 162). These latter molecules do however still display trypanocidal activity, even against *T. b. rhodesiense*, an organism able to scavenge sterols from its surrounding environment, indicating that these compounds may target other essential parasitic enzymes (Refs 160, 161, 162, 163).

Cysteine proteases

Several trypanosomal cysteine proteases (CPs) have been identified and biochemically characterised, including the cathepsin L-like enzymes *Tc*CATL (cruzipain) and *Tb*CATL (rhodesain or brucipain) (Refs 164, 165, 166, 167), and the cathepsin B-like CPs *Tc*CATB and *Tb*CATB (Refs 168, 169, 170, 171) (nomenclature in accordance with Ref. 172). Validation of these enzymes as drug targets is under way. Chemical validation of both cathepsin L-like CPs and *Tb*CATB has resulted in the identification of inhibitors that block enzymatic function, with several compounds displaying trypanocidal activity in vivo and parasitological cure in animal models (Refs 150, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183). For *Tc*CATL and *Tb*CATL, structural data have informed the molecular mechanisms underlying some of these enzyme–inhibitor interactions (Refs 184, 185, 186, 187, 188). Genetic validation of the *T. cruzi* CPs has not been reported, presumably because of their essential nature, although overexpression of *Tc*CATL does enhance metacyclogenesis (Ref. 189). Intriguingly, RNA interference (RNAi) experiments in *T. brucei* have demonstrated that *Tb*CATB, but not *Tb*CATL, is essential for parasite viability (Refs 171, 190). However, in murine models, trypanosomes with reduced *Tb*CATL are less efficient at crossing the blood–brain barrier and parasitaemia is suppressed, leading to the suggestion that anti-*Tb*CATL inhibitors may function by preventing the onset of late-stage HAT (Ref. 190).

Thiol metabolism

Trypanothione is a low molecular weight thiol unique to trypanosomes (Refs 57, 102, 191). Therefore, enzymes involved in its metabolism, especially TR and trypanothione synthetase, are good candidates for drug design (Refs 102, 192, 193). Many of these systems have been genetically validated as drug targets (Refs 60, 106, 111, 191, 194, 195, 196, 197), whereas chemical inhibitors studies have focused primarily on TR (Refs 99, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219); several compounds blocking TR activity have been identified, with some displaying trypanocidal activity. A significant aid to such TR inhibitor studies is the availability of a three-dimensional structure

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(Refs 201, 220, 221, 222, 223). This has facilitated high-throughput virtual enzyme–ligand screens that then guide the synthesis of candidate compounds (Refs 224, 225, 226). The structures of several other enzymes involved in trypanothione metabolism have now been elucidated and it is envisaged that this resource should assist in the identification of inhibitors (Refs 227, 228, 229). However, despite considerable efforts, no inhibitor targeting trypanothione metabolism has yet entered clinical trials.

Polyamine biosynthesis

The use of eflornithine against HAT has prompted the characterisation of other components of polyamine biosynthesis (Ref. 64). In *T. brucei*, several of these enzymes have been biochemically characterised (Refs 35, 230, 231, 232, 233) and shown to be essential for parasite viability (Refs 74, 233, 234, 235). Compounds targeting these activities have been identified (Refs 35, 236, 237, 238, 239, 240), some of which cure *T. brucei*-infected mice (Refs 241, 242, 243). Of these, the *S*-adenosylmethionine decarboxylase irreversible inhibitor MDL 73811 is the most potent, although it is rapidly cleared in rodent models (Refs 241, 244). Recently, a stable derivative, Genz-644131, has been developed that overcomes these problems and shows considerable promise in the treatment of East African trypanosomiasis (Refs 245, 246).

Bridging the gap until the development of novel drugs

There are currently no novel classes of chemotherapeutic agents undergoing trials against late-stage HAT or chronic Chagas disease. As noted by Bernard Pécoul, Executive Director of DNDi, 'No new drugs for stage 2 [late] sleeping sickness are expected in the next five years, so there is an urgent need to develop new treatments based on currently available drugs, especially through combinations' (http://www.msfaccess.org/media-room/press-releases). Given this scenario, the evaluation of combinatorial therapies is important, especially in the short term. Evaluation of cotherapies using existing drugs has several other advantages: it could delay emergence of drug resistance to the parental compounds, it may allow a reduction in drug dosage (thereby minimising toxicity while maintaining efficacy), and it could streamline drug administration. Different combinations of three trypanocidal agents (melarsoprol, eflornithine and nifurtimox) have recently been trialled against West African trypanosomiasis, with one cotherapy (nifurtimox–eflornithine) showing particular promise in terms of safety and efficacy (Refs 6, 7, 8, 247). Follow-up studies at different geographical locations have confirmed the potential of this combination, resulting in NECT (nifurtimox–eflornithine combination therapy) being added to the Essential Medicines List of the WHO (Refs 6, 7, 8, 247).

Another well-tried approach to improving chemotherapy is to investigate derivatives related to drugs already in use. An example of this is parfuramide, an orally available prodrug based on furamidine, a diamidine with trypanocidal activity (Refs 248, 249, 250). This compound did reach Phase III clinical trials against early-stage HAT and was shown to have good efficacy. However, a retrospective Phase I trial in South Africa required by the US Food and Drug Administration revealed nephrotoxicity in a small number of the volunteers. Currently, these unexpected findings are being investigated. In other studies, several nitroheterocyclic derivatives have been shown to have considerable trypanocidal activity (Refs 49, 50, 51, 52). Notable among these is fexinidazole, an orally administered 5-nitroimidazole that displays trypanocidal activity against both HAT subspecies, has little toxicity in animal studies and can cross the blood–brain barrier. This has resulted in fexinidazole entering clinical development with the ultimate goal of using it to target both stages of HAT. The precise mode of action of nitroaromatic compounds is currently unknown, but they may undergo activation involving a type I NTR (Ref. 120). Further dissection of this activity in terms of substrate specificity will provide a valuable resource for designing the next generation of NTR-activatable nitroheterocyclic drugs.

Conclusion

Although several drugs are available for use against HAT and Chagas disease, none is satisfactory. Each has limited efficacy, resistance is an increasing problem, dosage regimes can be complex and drug administration requires medical supervision. There has been significant progress in resolving the mechanisms by which these trypanocidal drugs operate and in

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identifying how resistance might arise. In addition, advances in our understanding of basic parasite biochemistry and genetics have led to identification of a large number of potential drug targets. New funding initiatives, prompted by the recognition that research on these debilitating neglected diseases has long been under resourced, now provide an opportunity to exploit these findings and improve the range of treatments.

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Further reading, resources and contacts

The World Health Organization and The Centres for Disease Control and Prevention websites provide up-to-date information relating to trypanosomal diseases:

http://www.who.int/
http://www.cdc.gov/

Trypanosomal genome sequence data can be accessed at:

http://www.sanger.ac.uk/Projects/T_brucei/
http://www.genedb.org/genedb/tcruzi/
http://tritrypdb.org/tritrypdb/

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Further reading, resources and contacts *(continued)*

Not-for-profit organisations involved in helping combat trypanosomal infections include Drugs for Neglected Diseases Initiative, Bill & Melinda Gates Foundation and the Wellcome Trust:

http://www.dndi.org/
http://www.gatesfoundation.org/Pages/home.aspx
http://www.wellcome.ac.uk/

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Figures

Tables

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