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Commentary

Uptake and mode of action of drugs used against sleeping sickness

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Abstract

Sleeping sickness is resurgent in Africa. Adverse side-effects and drug-resistance are undermining the few drugs currently licensed for use against this disease, which is caused by parasitic protozoa of the *Trypanosoma brucei* group. Pentamidine and suramin are used before parasites become manifest in the central nervous system, after which the organic arsenical melarsoprol is used. Eflornithine is also useful in late-stage disease. A mode of action has been elucidated only for the ornithine decarboxylase inhibitor eflornithine. Both uptake and potential intracellular targets need to be considered when contemplating modes of action. The melaminophenyl arsenicals are accumulated via an unusual amino-purine transporter termed P2, which also seems to have a role in the uptake of the diamidine class of drugs to which pentamidine belongs. Since loss of this transporter leads to drug-resistance, other uptake mechanisms also need to be considered in generating novel trypanocides. Some nitroheterocyclic drugs have prolific activity against trypanosomes, although the fact that they are mutagenic in Ames' tests is acting as a barrier to further development. New drugs are urgently needed and the advent of genome sequencing and target validation using genetic modification will hopefully accelerate this process.

Keywords: Sleeping sickness; Trypanosomiasis; Chemotherapy; Drug-resistance; Megazol; Nitroheterocycles

1. Sleeping sickness

Sleeping sickness (human African trypanosomiasis) is caused by subspecies of *Trypanosoma brucei,* parasitic protozoa transmitted in Africa by tsetse flies. Two forms of the disease are recognised: a chronic form caused by the subspecies *T. b. gambiense* and an acute form caused by *T. b. rhodesiense* [1].

Unfortunately, the gradual breakdown of counter-measures introduced in the early part of the twentieth century has led to a re-emergence of sleeping sickness as a problem in sub-Saharan Africa with prevalence of infected individuals estimated to be approaching 500,000 as we enter a new millenium [2].

The causative parasites dwell free in the lymphatic fluid and bloodstream of their mammalian hosts and then invade the CNS and other organs. Symptoms relating to neurological disorders culminating in coma and then death become manifest in this CNS-involved stage. Progression to the late stage takes weeks in the form of the disease caused by *T. b. rhodesiense,* whereas several years may elapse prior to neurological decline in the disease caused by *T. b. gambiense.*

The surface of the parasite is shrouded by a glycoprotein coat. With up to a thousand different genes encoding antigenically distinct versions of this coat, the parasites have the capacity to engage in an immuno-protective process of antigenic variation [3]. Antigenic variation has rendered the prospects of a vaccine against the parasites poor. Drugs do exist for use against African trypanosomes; however, serious side-effects are a problem with all of them, and resistance is increasing. Since victims of the disease are among the world's poorest people, the incentive for investment in the development of new drugs has been sadly lacking from the pharmaceutical industry. A better understanding of the mode of action of the currently used drugs may underpin the development of new, improved therapy.

2. Drugs in use against sleeping sickness

Due to pharmacological difficulties in breaching the blood–brain barrier, different drugs are used in treating the disease depending on CNS involvement [4]. Before para-

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Abbreviations: DFMO, D,L-α-difluoromethylornithine; and ODC, ornithine decarboxylase.

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sites become manifest in the CNS, suramin and pentamidine are the drugs of choice against *rhodesiense* and *gambiense* forms of the disease, respectively. Late-stage disease is treated with the melaminophenyl arsenical drug melarsoprol. In the case of the disease caused by *T. b. gambiense*, eflornithine (DFMO) is also used.

2.1. Suramin

Suramin, a colourless polysulphonated symmetrical naphthalene derivative, was first used against sleeping sickness in 1922 [5]. Other naphthalene dyes including trypan red and trypan blue were initially developed for their marked trypanocidal activity.

Suramin binds with high avidity to serum proteins, including low-density lipoprotein for which trypanosomes have a receptor [6] that is used to accumulate this complex via receptor-mediated endocytosis. Suramin accumulates in trypanosomes relatively slowly, and it has been speculated that uptake of this drug occurs via endocytosis bound to low-density lipoprotein [6].

In spite of many years of imaginative speculation, its mode of action against trypanosomes remains obscure. At physiological pH, the drug bears six negative charges, and it inhibits many enzymes by electrostatic interactions. The drug has been used recently in clinical trials against hormone-refractory prostate cancer [7].

2.2. Pentamidine

Pentamidine is an aromatic diamidine [8]. It was developed after the observation that a related compound that induces hypoglycaemia in mammals, synthalin, had prolific anti-trypanosomal activity. Diamidines actually work directly against the parasites, independently of their physiological action on the host. It is active against early stages of the *gambiense* form of sleeping sickness. It is also used against antimony refractory leishmaniasis and *Pneumocystis carinii* pneumonia [8].

The drug is concentrated to high levels by the parasites. It seems that pentamidine can enter *T. brucei* via the same P2 amino-purine transporter that accumulates melaminophenyl arsenicals (see later) [9]. Loss of this transporter can render parasites cross-resistant to both diamidines and arsenicals. However, some parasites without P2 remain sensitive to pentamidine. This may be because the parasite has other routes of internalisation of this drug.[1]

The mode of action has not been established. As a polycation, the molecule interacts electrostatically with cellular polyanions. Its interaction is particularly tight with the unique intercatenated network of circular DNA molecules, which make up the mitochondrial genome of all kinetoplastid flagellates. The order Kinetoplastida takes its name from this network, which is termed the kinetoplast [10]. In the case of African trypanosomes, an interaction with the kinetoplast may appear to be of limited interest as these organisms do not have a classical mitochondrial metabolism; moreover, parasites can retain viability when the kinetoplast has been removed (dyskinetoplastidy). On the other hand, dyskinetoplastid parasites have been shown to be less sensitive than wild-type cells to the related diamidine drug, used in the treatment of animal trypanosomiasis, berenil [11]. Numerous other potential targets have been proposed, although none have been verified. Given that the drug reaches millimolar concentrations within the cells [12], it could be that its toxic effect arises from inhibition of multiple cellular targets.

2.3. Melarsoprol

Melarsoprol is a melaminophenyl based organic arsenical that was introduced as an anti-trypanosomiasis reagent in 1949 [13]. It was Paul Ehrlich who introduced arsenicals as drugs for use against sleeping sickness at the turn of the century. His compound, salvarsan or ‘606,’ is often considered as the prototypic chemotherapeutic reagent [14].

Melarsoprol is accumulated by an unusual amino-purine transporter, and loss of this transporter leads to drug resistance. *T. brucei* contains several purine nucleoside transporter activities [9]. One of these, called P2, carries adenosine and also its nucleobase adenine, while the second, P1, appears to be a general purine nucleoside transporter. P2 also interacts with melaminophenyl arsenicals and diamidines (as judged by the ability of these reagents to inhibit adenosine uptake) [9, 12, 15, 16]. Trypanosomes selected for resistance to sodium melarsen had lost the P2 transporter [9], and a *T. equiperdum* line selected for resistance to berenil, with some cross-resistance to arsenicals, had a P2 transporter with markedly reduced affinity for substrate [15]. These combined data indicate that in wild-type *T. brucei* group organisms the P2 transporter is responsible for the uptake of arsenicals and diamidines including berenil (the situation for pentamidine is more complicated, as described above).

A motif recognised by the P2 transporter is shared by amino-purines, melamine-based arsenicals, and diamidines [16, 17]. Grafting this motif to compounds that would otherwise not enter *T. brucei* offers a means of delivering new drugs to these cells [18].

Trypanosomes exposed to arsenicals lyse very rapidly. A mode of action has yet to be established. Loss of ATP due to inhibition of glycolysis could underlie lysis caused by the drug. However, it seems that the cells lyse before ATP supplies are seriously depleted [19], leading several workers to question whether glycolysis is a target for arsenical action. A recent experiment designed to isolate potential targets of

[1]De Konig HP, personal communication. Cited with permission.

arsenicals revealed that glycerol-3-phosphate dehydrogenase specifically fixes to column-bound Cymelarsan (an analogue of melarsoprol used in veterinary trypanosomiasis), and it is inhibited by the drug [20]. An action of arsenicals related to energy metabolism should not be ruled out yet.

Trypanothione ($N^{1,}N^{8}$-bis-glutathionylspermidine) is an unusual low molecular weight thiol, comprising two glutathione molecules conjugated with spermidine. In trypanosomatids, it performs most of the roles carried out by glutathione in mammalian cells. Since arsenic is known to interact very stably with thiols, trypanothione has also been proposed as the definitive target for these compounds. However, since arsenicals actually interact more tightly with other thiols including lipoic acid [21] and at the point of arsenical-induced lysis only a small fraction of trypanothione is conjugated with the drug, it is not certain that trypanothione or the enzymes involved in its metabolism are the *in situ* targets of these drugs.

2.4. Eflornithine

Eflornithine (DFMO) is an analogue of ornithine, which acts as a specific suicide inhibitor of the enzyme ODC [22]. It was developed as an anti-cancer reagent; however, it remains at the trial stage against neoplastic disease [7]. The drug also has prolific activity against sleeping sickness caused by *T. b. gambiense,* even in the late CNS-involved stage [4].

It has been reported that uptake of DFMO in *T. brucei* occurs via passive diffusion across the plasma membrane [23], although a separate report did note a saturable process typical of transport-associated uptake [24]. Reduced drug accumulation has been noted in parasites resistant to eflornithine; however, whether this was due to decreased uptake or increased efflux was not determined [24]. A separate study failed to identify a decreased accumulation associated with resistance [25].

DFMO inhibits ODC. It has similar affinity for the mammalian and trypanosomal enzymes. Its specificity against the parasite arises because this organism has an ODC that is degraded within the cell and replenished at a rate several orders of magnitude slower than its mammalian counterpart [26]. Thus, a pulse of DFMO can deprive trypanosomes of ODC and polyamine synthesis for a prolonged period compared with mammalian cells, leading to a cessation of growth. A functional immune system is required to kill the growth-arrested trypanosomes.

To be effective against sleeping sickness, the drug needs to be given in large doses. An additional drawback is its lack of activity against *rhodesiense* sleeping sickness. *T. b. rhodesiense* may be innately less susceptible to the drug than *T. b. gambiense,* since it has a higher overall ODC activity and the enzyme has a shorter half-life than the *gambiense* counterpart [27].

3. Nitroheterocyclic experimental trypanocides

3.1. Nifurtimox

The nitrofuran drug nifurtimox is used against Chagas' disease, caused by another trypanosome, *T. cruzi,* in Latin America [28]. The drug contains a nitro group that is central to activity. It also has been used in trials, with limited success, against arsenical refractory *T. brucei* in West Africa [29].

Uptake of nifurtimox into *T. cruzi* has been reported to occur via passive diffusion across the plasma membrane [30]. Studies have not been extended yet to *T. brucei,* but it is also likely to enter these cells via passive diffusion.

The one-electron reduction of the nitro group generates a potent free radical, which, in turn, generates reduced oxygen metabolites (such as superoxide, hydroxyl free radical, and hydrogen peroxide) believed to cause the death of the parasite [31]. The specificity towards the parasite (which is not great, the compounds being quite toxic to mammals) is thought to relate to the compounds being more readily reduced by the parasite than the host cells and also because mammalian cells have better protection against oxidative damage.

The pathways that might lead to the preferential reduction of these compounds in trypanosomes have been studied. One intriguing hypothesis was that trypanothione reductase might be responsible for the reduction [32]. Certainly a number of nitro-containing compounds can act as "subversive-substrates" for the enzyme; however, nifurtimox was one of the less successful substrates, and it is unlikely that trypanothione reductase-mediated nitro-reduction underlies the activity of the clinically used nitroheterocyclic compounds [32].

3.2. Megazol

Megazol is a 5-nitroimidazole that has good efficacy against both *T. cruzi* [33] and *T. brucei* [34]. The drug possesses the motif recognised by the P2 amino-purine transporter responsible for the uptake of several anti-trypanosomal drugs [9, 15–17]. Should this drug share the P2 transporter as a portal of entry, it would be of limited use against arsenical-resistant parasites. However, strains of parasite lacking the P2 transporter, resistant to other drugs that use this portal of entry into trypanosomes, were not cross-resistant to megazol [35]. Uptake of radiolabelled megazol revealed that this drug, although capable of an interaction with the P2 transporter, enters cells predominantly via passive diffusion [35].

The mode of action of the drug is not clear. Its redox potential of −438 mV is far lower than that of nifurtimox (−260 mV). 5-Nitroimidazoles are not normally reduced by aerobic cells; however, megazol is susceptible to nitro-reduction in the presence of several enzymatic systems including some found in *T. cruzi* extracts [36]. Fumarate

reductase activity from *T. cruzi* is inhibited by megazol [37] and may possess megazol reductase activity; however, other enzymes are also likely to possess this capability. Interestingly, parasites resistant to megazol are cross-resistant to other nitroheterocycles including nifurtimox but not to other drugs thought to induce oxidative stress, or typical P-glycoprotein substrates (Barrett MP, unpublished data). This may indicate that the same nitroreductase activity may be responsible for activation of all of these drugs in trypanosomes, and that this activity may be altered in resistant lines.

4. Need for flexibility in licensing anti-sleeping sickness drugs

With sleeping sickness resurgent across much of Africa and arsenical resistance increasing, the need for anti-trypanosomiasis drugs has never been greater. However, pharmaceutical companies are reluctant to develop new agents because they perceive the market and associated profits to be insignificant. It therefore falls to academic scientists to develop new reagents. Given the effectively incurable nature of arsenical-resistant late-stage disease, relaxation of licensing criteria is needed to accelerate the introduction of novel trypanocides. Some of the current leads, for example megazol, have been disregarded due to the fact that they are positive in Ames' tests [38]. The drug, however, cures simians of trypanosomes in a single dose[2] with no sign of toxicity. While a case for human trials cannot be justified prior to successful completion of a full toxicological study, Ames' test positivity is an inadequate reason to abandon all further development of a drug that may be useful against an otherwise incurable disease. A full study on the toxic effects of megazol is required.

5. Rational and nouvelle-empirical approaches to the development of new trypanocidal drugs

Megazol, and other leads, may prove to be unsuitable after toxicological evaluation. New drugs and legitimate targets need to be unravelled. Traditionally, the route to drug target identification has been through a process of comparative biochemistry. Enzymes, metabolites, or proteins identified in parasites and known to be absent from, or strikingly different in, the mammalian host were considered ideal targets. Thus, glycolysis of trypanosomatids has been perceived as an excellent target owing to the unusual compartmentation of the glycolytic enzymes in peroxisome-like organelles called glycosomes [39]. Similarly, the discovery that trypanothione rather than glutathione itself is the major redox reactive metabolite in trypanosomatids has elevated this molecule and the enzymes involved in its metabolism to paradigms of anti-parasite drug targets [40].

Systematic sequencing of pathogen genomes is now possible, and a natural progression from comparative biochemistry has been to proceed to a large-scale sequencing project to identify new drug targets (http://parsun1.path.cam.ac.uk/).

A simple host:parasite difference, however, is insufficient to give a molecule legitimate "target" status. Target validation is the cornerstone of the rational approach to chemotherapy. In recent years, it has become possible to use the "gene-knockout" approach to provide evidence on whether a trypanosome gene (and hence its encoded product) is essential, or not, and thus a credible target for inhibitory drugs [41]. Failure to knockout a gene is often taken to infer that the gene is essential for the parasite, although it is an over-simplification to imply that an essential gene encodes a validated drug target.

For example, removal of genes for key glycolytic enzymes would certainly be impossible. However, mathematical modelling of glycolytic flux has revealed how difficult it would be to design pharmacological inhibitors of glycolytic enzymes that would have sufficient impact on flux to kill the parasite [42]. Nevertheless, irreversible inhibitors or competitive inhibitors with K_i values substantially below K_m values for substrate (such as transition state analogues) would be sufficiently potent to kill the cells [41]. Caution is required in interpreting gene-knockout data.

The relative ease with which recombinant proteins may be produced also allows a nouvelle-empirical approach to drug identification to be pursued. Validated targets can be produced in bulk and the inhibitory activity of a wide range of compounds, existing in chemical libraries, tested for inhibitory activity.

Conclusions

Resurgent trypanosomiasis is a major cause of death in sub-Saharan Africa. As the disease was, until recently, scarce and drugs have been available for many years, there has been a complacency associated with the development of novel trypanocides. Since resistance to currently used drugs is on the increase, the need to develop novel reagents has never been greater. In spite of the reluctance of the pharmaceutical industry to invest in development of new drugs against a disease exclusive to Africa, genome sequencing and recombinant DNA technology offer routes to the accelerated discovery and validation of new drug targets. Flexibility in taking drugs forward for trials and ultimately in licensing is also required if new drugs arising from these studies are to be developed for clinical use.

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[2]Enanga B, personal communication. Cited with permission.

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