Pentamidine uptake and resistance in pathogenic protozoa: past, present and future

Patrick G. Bray[1], Michael P. Barrett[2], Stephen A. Ward[1] and Harry P. de Koning[2]

[1]Molecular and Biochemical Parasitology Group, Liverpool School of Tropical Medicine, Liverpool, L3 5QS, UK
[2]Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, G12 8QQ, UK

Diamidines, and pentamidine in particular, have a long history as valuable chemotherapeutic agents against infectious disease. Their selectivity is due mostly to selective accumulation by the pathogen, rather than the host cell; and acquired resistance is frequently the result of changes in transmembrane transport of the drug. Here, recent progress in elucidating the mechanisms of diamidine transport in three important protozoan pathogens, *Trypanosoma brucei, Leishmania* and *Plasmodium falciparum*, is reviewed, and the implications for drug resistance are discussed.

Diamidines have a long history of involvement in the treatment of human protozoan infections, and pentamidine (PMD) is still the drug of choice against early stage sleeping sickness. The first antiprotozoan diamidine, synthalin (actually a diguanidine, see Fig. 1) was developed as a synthetic analogue of insulin, and tested in experimental trypanosome infections on the basis that induced hypoglycaemia was expected to control or cure trypanosome infections in humans [1]. However, Lourie and Yorke discovered in 1937 that synthalin was as active against trypanosomes *in vitro* as trivalent arsenicals. This led to the synthesis and testing of a large number of related compounds, the most active of which was diamidino-1,11-*n*-undecane [2], and later to the aromatic diamidines, including stilbamidine, propamidine and PMD by A.J. Ewins of the pharmaceutical company May and Baker [3]. These compounds were found to be highly effective against first stage (and early second-stage, see Box 1) human *Trypanosoma brucei gambiense* infections [4] and Indian kala azar [5], although stilbamidine was found to cause serious neurological effects in some patients [6] and was later abandoned. Importantly, PMD and propamidine were proven to be effective against strains resistant to the aromatic arsenicals then in use, such as tryparsamide [7], which had developed as a result of widespread use of these drugs during the preceding 15 years. Resistance to PMD itself has never been a significant problem in the field, despite its widespread use as a prophylactic (Box 2).

In addition to their use as drugs against African trypanosomes, diamidines have been used clinically against various other infectious diseases including *Pneumocystis carinii* pneumonia [8] and leishmaniasis. Various studies, in the decade following the first diamidine synthesis, also found antibacterial, antifungal, antineoplastic, antimalarial and antileishmanial activity (reviewed in Ref. [9]).

PMD uptake in African trypanosomes

PMD uptake in African trypanosomes was first studied by Damper and Patton [10,11], who concluded that high-affinity transporters were involved, and that the parasite continued actively to accumulate drug until a lethal intracellular concentration had been reached. This concentration appeared to be in excess of 1 mM, but it is not clear to what extent this reflects free PMD concentrations in the cell because PMD binds tightly to DNA and might be segregated in an intracellular compartment such as the mitochondrion, as fluorescent diamidines such as stilbamidine have been shown to be associated with kinetoplast DNA (kDNA) and promote linearization of kDNA minicircles [12].

Carter *et al.* [13] provided strong evidence that PMD uptake by *T. b. brucei* is mediated by the P2 aminopurine transporter, which is also implicated in the uptake of melaminophenyl arsenicals [14,15], by showing that PMD inhibited both P2-mediated adenosine transport and prevented melarsen-oxide-induced cell lysis *in vitro*. The apparent inconsistency of a selective aminopurine-only transporter efficiently mediating the uptake of diverse molecules such as melaminophenyl arsenicals and diamidines was overcome with the elucidation of the structural motif recognized by this transporter [16,17]. However, adenosine did not inhibit [^{3}H] PMD transport by *T. b. brucei* to the expected extent [13]. Recently, it has become clear that additional transporters are capable of mediating PMD uptake in bloodstream forms of *T. brucei* (Fig. 2). No fewer than three distinct transporters are capable of mediating PMD uptake, as measured by transport of [^{3}H] PMD [18] and [^{125}I] iodopentamidine [19], with K_m values ranging over three orders of magnitude (Table 1).

Only one of these transporters was sensitive to inhibition by adenosine, and was hence designated adenosine-sensitive pentamidine transporter (ASPT)1. There is strong kinetic evidence that ASPT1 is identical to the P2 adenosine transporter. First, the ASPT1-mediated

Corresponding author: Harry P. de Koning (H.de-Koning@bio.gla.ac.uk).

 1471-4922/03/$ - see front matter doi:10.1016/S1471-4922(03)00069-2

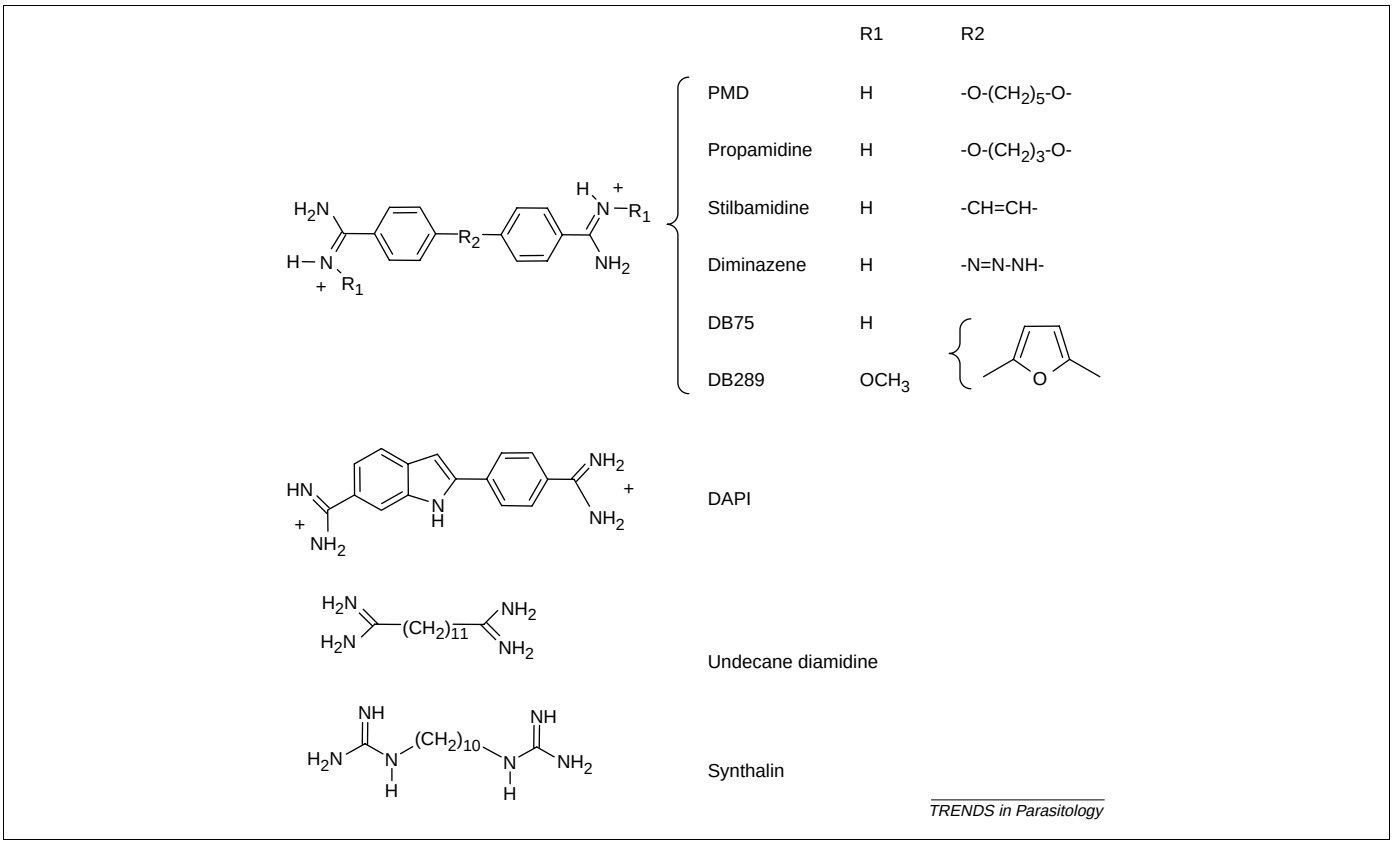


Fig. 1. Structure of various diamidines and related compounds. Structures of the classical aromatic diamidines PMD, propamidine, stilbamidine and diminazene aceturate, of the first orally available diamidine, DB289 and its active metabolite, DB75, of DAPI and of the early diamidine undecane diamidine and the diguanidinium compound synthalin. Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; PMD, pentamidine; R_1, functional group substituted on amidine group; R_2, chain or ring forming the central part of the diamidine.

transport of both [^{3}H] PMD and [^{125}I] iodopentamidine is inhibited by adenine and adenosine (but not by other purines, pyrimidines, pterines and amino acids), with K_i values that are virtually identical to the corresponding K_i values for P2-mediated adenosine transport [18,19]. Likewise, the P2 transporter is inhibited by PMD and iodopentamidine, with K_i values almost identical to the K_m values for ASPT1-mediated PMD transport (Table 1). K_i values for other diamidines (diminazene aceturate) and melaminophenyl arsenicals are also identical for P2 and ASPT1 [17,18]. However, the kinetic similarities alone can offer no formal proof that the adenosine-sensitive PMD transport activity is mediated by the same transporter

Box 1. Diamidines in late-stage sleeping sickness

The main limitation to diamidine use against *Trypanosoma brucei rhodesiense* infections seems to be the far more rapid invasion of the cerebrospinal fluid (CSF) by this parasite. Pentamidine (PMD) is not prescribed against late-stage sleeping sickness, presumably because it does not cross the blood–brain barrier efficiently enough to attain the necessary drug levels in the central nervous system (CNS), although early trials do report considerable efficacy of PMD and propamidine against sleeping sickness with a moderate degree of change to the CSF [63,64] and PMD was found to be equally effective as tryparsamide in these cases [64]. These findings, now largely forgotten, show that diamidines are capable of crossing the blood–brain barrier. The high potential for diamidine use against late-stage sleeping sickness was demonstrated in a much more recent study by Doua *et al.* [65]. These authors showed that early late-stage *Trypanosoma brucei gambiense* human African trypanosomiasis (HAT) could be treated with ten intramuscular injections of 4 mg kg^{-1}, with a cure rate of 94%. It is of great importance to develop novel diamidines with improved CNS permeation, possibly administered as prodrugs with the amidine groups masked. A good example of this approach is the orally available experimental drug DB289, now being clinically tested against early stage HAT, and its derivatives are currently in development [66].

Box 2. Pentamidine as a prophylactic

Field trials for the prophylactic use of pentamidine (PMD) were initiated in 1942 by Van Hoof and co-workers. It appeared that a single injection of 4 mg kg^{-1} protected against *Trypanosoma gambiense* trypanosomiasis for at least six months [67], and a massive programme of PMD prophylaxis was started in the then Belgian and French African colonies and Nigeria, running until the late 1960s [68]. While this program was spectacularly successful in reducing the incidence of sleeping sickness, virtually eradicating the disease from Afrique Occidentale Française and Afrique Equatoriale Française [69], the initial high hopes of eradicating the disease were never realized (see Ref. [70] for review). Despite massive PMD use, regularly treating millions of people during the 1950s and 1960s, there were virtually no reports of induced PMD resistance. The 1976 WHO report on African trypanosomiasis mentions resistance to arsenicals and to diminazene aceturate (berenil) in cattle, but not to PMD [71]. PMD resistance was not detected in the comprehensive survey by the Organization de Coordination pour la Lutte contre les Endemies en Afrique Centrale in 1967, in countries where intense 'pentamidinization' had been practiced for over a decade [68].

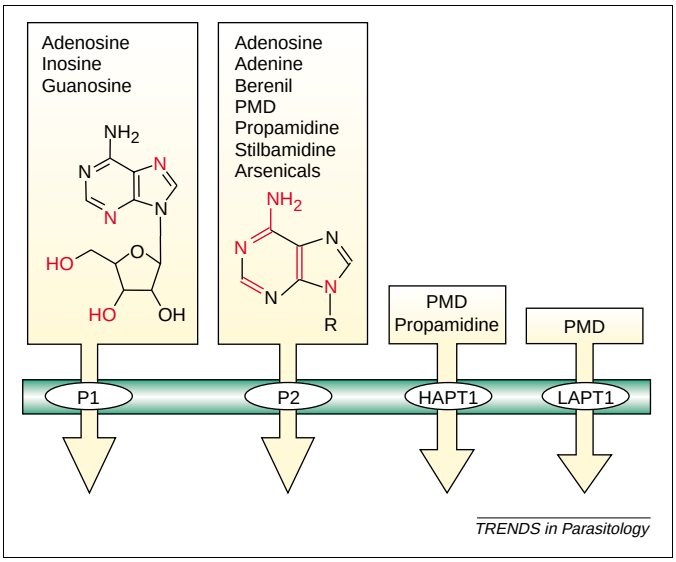


Fig. 2. Nucleoside and diamidine transporters in *Trypanosoma brucei brucei*. The substrate recognition motifs of the P1 and P2 transporters are indicated in red. P1 interacts with adenosine through hydrogen bonds to nitrogen residues 3 and 7 of the purine ring, and the 5′ and 3′ hydroxyl groups of ribose. P2 binds the amidine group comprising N1 and the position 6 amine. Additional interactions are formed by pi-stacking of the purine ring with an aromatic residue in the transporter-binding pocket, and N9 also contributes to binding, although not through a hydrogen bond [17]. Abbreviations: HAPT1, high-affinity PMD transporter 1; LAPT1, low-affinity PMD transporter 1; PMD, pentamidine; R, hydrogen (adenine) or ribose (adenosine).

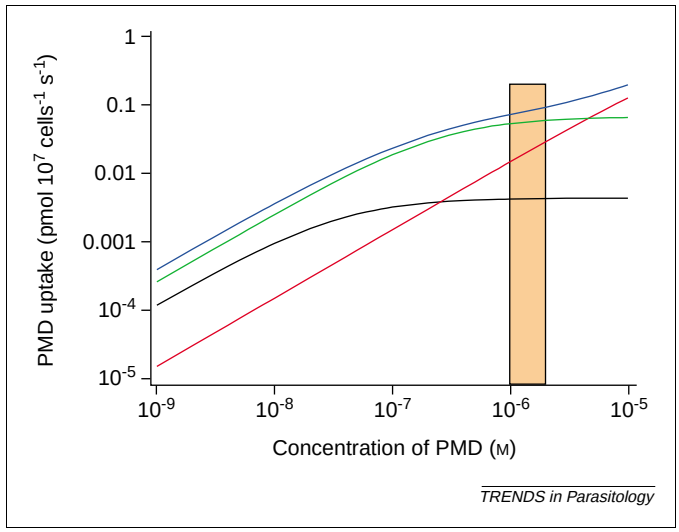


Fig. 3. PMD uptake rates by various transporters in *Trypanosoma brucei brucei* bloodstream forms. Uptake rates were plotted as a function of the extracellular PMD concentration from $V = (V_{max} \times L)/(L + K_m)$ in which L is the ligand concentrations, and the K_m and V_{max} values for individual transporters were used from Table 1. Plasma concentrations in patients treated with the standard 4 mg kg^{-1} dosing have been determined at 0.5–2.5 μM [20]. Over this range, the contribution of LAPT1 increased from 13% to 35% of total PMD uptake, due to its very high capacity for PMD transport. Key: black line, HAPT1; blue line, combined uptake by HAPT1, LAPT1 and P2; green line, P2; orange bar, maximum plasma concentration of PMD in patients; red line, LAPT1. Abbreviations: HAPT1, high-affinity PMD transporter 1; LAPT1, low-affinity PMD transporter 1; PMD, pentamidine.

as the PMD-sensitive adenosine transport activity. The construction of null mutants of *TbAT1* (the gene believed to encode P2 [15]) and our transport studies with [^{3}H] PMD and [^{3}H] adenosine in these cells are consistent with this gene encoding both P2 and ASPT1 activities (E. Matovu *et al.*, unpublished).

The P2 transporter consistently mediated 50–70% of the PMD uptake [18,19]. Two additional PMD transport activities could be distinguished: a high-affinity pentamidine transporter (HAPT1; $K_m = 36 \pm 6$ nM) and a low-affinity pentamidine transporter (LAPT1; $K_m = 56 \pm 8$ μM). HAPT1 was competitively inhibited by propamidine, but neither transporter was sensitive to inhibition by other trypanocides, including stilbamidine and melarsen oxide, or by various amino acids, vitamins, purines and pyrimidines.

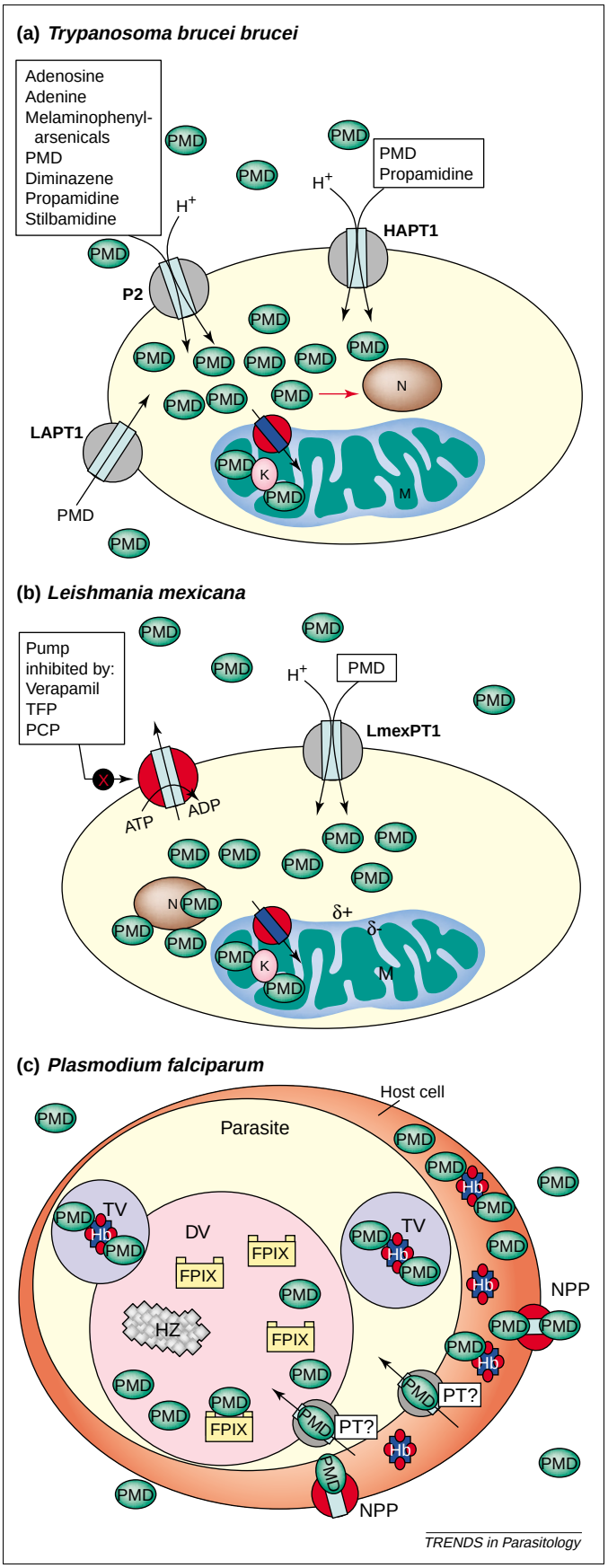
Because of the different kinetic parameters of the three PMD transporters, the relative contribution of each to the total flux is a function of the extracellular PMD concentration (Fig. 3), which reaches up to 2.5 μM in plasma of sleeping sickness patients treated with the standard dose of 4 mg kg^{-1} [20]. The combination of high affinity and relatively high V_{max} of ASPT1 ensures that, at any concentration, at least 50% of the flux is mediated by this carrier. HAPT1, as a result of its very high affinity, contributes relatively little because it is saturated at low concentrations.

It is not known whether the P2 or ASPT1 transporter is energy-dependent, but the related P1 nucleoside transporter of *T. b. brucei* procyclics is a proton symporter [21], as are its purine and pyrimidine nucleobase transporters [22].

Table 1. Kinetic parameters and inhibition constants of the trypanosomal pentamidine transporters[a].

	Trypanosoma brucei brucei[b]			*Leishmania mexicana*[c]
	P2	**HAPT1**	**LAPT1**	**PT1**
Kinetic parameters				
*K*m (μM)	0.26 ± 0.03	0.036 ± 0.006	56 ± 8	7.4 ± 1.8
*V*max	0.068 ± 0.007[d]	0.0044 ± 0.0004[c]	0.85 ± 0.15[d]	452 ± 42[e]
K_i values of inhibitors (μM)				
Stilbamidine	2.4 ± 0.3	56 ± 3	>250	21 ± 5
Propamidine	1.9 ± 0.8	4.6 ± 0.7	>250	12 ± 1
Diminazene	2.5 ± 0.8	63 ± 3	>250	27 ± 3
Melarsen oxide	0.63 ± 0.20	>100	NE, 100	ND

[a]Abbreviations: HAPT1: high-affinity pentamidine transporter 1; LAPT1: low-affinity pentamidine transporter 1; ND, not determined; NE, no effect at indicated concentration; P2, purine transporter 2; PT1, pentamidine transporter 1.
[b]From Ref. [18]
[c]From Ref. [39]
[d]In pmol (10^7 cells)$^{-1}$
[e]In fmol s^{-1} μg protein^{-1}



HAPT1-mediated PMD uptake was inhibited by the proton-ionophore carbonyl cyanide chlorophenylhydrazone (CCCP) (>90% at 10 μM CCCP [18]), indicating that HAPT1 might also be proton-dependent. This was more thoroughly investigated in the equivalent procyclic pentamidine transporter (PPT1), which is kinetically indistinguishable from HAPT1 and could well be the same gene product [18]. PPT1-mediated PMD uptake was inhibited by CCCP ($IC_{50} = 4.2 \pm 0.3$ μM), as well as by nigericin, gramicidin, N, N′-dicyclohexylcarbodiimide (DCCD) and N-ethylmaleimide (NEM), reagents that have been shown to reduce the proton-motive force over the procyclic plasma membrane [21]. These observations do not constitute proof that HAPT1 is a proton symporter but, even if some of these agents act also on the mitochondrion, or on the transporter itself, it is likely that HAPT1 accumulates PMD in an energy-dependent way.

Drug resistance

It has been noted that, in laboratory strains, cross-resistance between melaminophenyl arsenicals and diamidines is not uncommon and this has been widely attributed to the loss of the P2 transporter, which takes up both classes of drugs (see Refs [16,23] for reviews). However, the situation is by no means straightforward, and crossresistance between any arsenical and any diamidine is not a foregone conclusion. Examples are the lack of crossresistance between PMD and melarsen oxide in *T. b. brucei* cRU15 [24], the low resistance to cymelarsan in *Trypanosoma equiperdum* and *Trypanosoma evansi* strains adapted to high diminazene aceturate concentrations [25], or the slight reduction in melarsoprol sensitivity in *T. b. rhodesiense* isolates with high levels of diminazene resistance [26]. The overview in Fig. 4a explains that, whereas resistance to diminazene is relatively easily introduced by loss of P2 function, PMD resistance requires the loss of three different transporters, and propamidine resistance requires the loss of two. The inconsistent level of crossresistance between diminazene and melarsoprol appears to suggest that melaminophenyl arsenicals, similar to some diamidines, have a secondary route of entry into the trypanosome.

PMD resistance that is unconnected to drug accumulation has been reported in a *T. b. brucei* clone adapted

Fig. 4. PMD uptake in parasitic protozoa. (a) *Trypanosoma brucei brucei* bloodstream trypomastigote. PMD is accumulated through the P2, HAPT1 and LAPT1 carriers [18], and associates mostly with the N or is taken up into the M where it is tightly bound to K. (b) *Leishmania mexicana* promastigote. PMD is taken up by a high-affinity pentamidine transporter, LmexPT1, probably a proton symporter [39]. From the cytosol, it is further concentrated in the M, by an unknown carrier or channel, dependent on the MMP. It is removed from the cell by an energy-dependent pump that is inhibited by verapamil, PCP and TFP, presumably a P-glycoprotein or multidrug-resistance protein. (c) *Plasmodium falciparum* trophozoite. PMD enters the infected erythrocyte through the NPP and binds to Hb. It might then enter the parasite through phagocytosis [61] and/or a PT. The PMD then binds to FPIX in the food vacuole, preventing HZ crystallization and leading to cell death. PMD might enter the DV either through phagocytosis and/or a PT. Abbreviations: DV, digestive vacuole; FPIX, ferriprotoporphyrin IX; HAPT1, high-affinity PMD transporter 1; Hb, haemoglobin; HZ, haemozoin; K, kinetoplast; LAPT1, low-affinity PMD transporter 1; LmexPT1, *Leishmania mexicana* pentamidine transporter 1; M, mitochondrion; MMP, mitochondrial membrane potential; N, nucleus; NPP, new permeability pathway; PCP, prochlorperazine; PMD, pentamidine; PT, putative transporter; TFP, trifluoperazine; TV, transport vesicle.

to high levels of PMD *in vitro* [27]. There was no crossresistance to other diamidines and the resistance mechanism is still unknown. However, it is unlikely that this resistance will arise or persist 'in the field' because the resistant clone was much less virulent than the parent strain [27].

It is recommended that diamidine analogues considered for future use against human African trypanosomiasis (HAT) are tested for uptake by all three PMD transporters to delay the onset of resistance. The recent suggestion that diminazene aceturate could be used against HAT [28] does not seem sensible in the light of this information because it would quickly induce high levels of resistance, as well as a low-level resistance to melarsoprol. Because *T. b. gambiense* isolates from melarsoprol-treated relapse patients display only a low level of melarsoprol resistance [29], this could rapidly escalate the current crises of melarsoprol refractoriness [30].

PMD uptake and resistance in *Leishmania mexicana*

Pentavalent antimonial drugs are the standard first-line treatment for leishmaniasis [31], although resistance is a growing problem [32]. PMD is used clinically as an alternative to pentavalent antimony in leishmaniasis treatment [33–35]. A definitive mode of action has yet to be elucidated for the drug, although studies on PMD uptake and distribution in drug-sensitive and -resistant parasites have recently given some insight into events.

PMD was found to be a competitive inhibitor of arginine transport [36] in *Leishmania donovani* and a noncompetitive inhibitor of putrescine and spermidine transport in *Leishmania infantum* [37], *L. donovani* and *L. mexicana* [38]. The availability of radiolabelled PMD helped to determine whether the drug shared a transporter with arginine, putrescine or spermidine [39]. These metabolites did not inhibit [^{3}H] PMD uptake; therefore, the observed inhibition by PMD must relate to the drug's ability to inhibit permeases through some mechanism other than it being a competitive substrate. The drug enters both promastigote and amastigote forms of *L. mexicana* via a carrier-mediated process that recognizes the drug with measured K_m values of 7.4 μM in promastigotes and 13.9 μM in amastigotes [39]. The maximum velocity of uptake is substantially higher in amastigotes than in promastigotes (8540 fmol s^{-1} μg $protein^{-1}$ in amastigotes and 452 fmol s^{-1} μg $protein^{-1}$ for promastigotes) [39]. A wide variety of common metabolites also failed to inhibit PMD uptake. Three inhibitors of P-glycoproteins (trifluoperazine, prochlorperazine and verapamil) were competitive inhibitors of uptake, but the physiological role of the *Leishmania* transporter that carries PMD into the cell is unknown. Some divalent inorganic cations also showed inhibitory activity and it is tempting to speculate that a divalent cation channel or transporter could operate as the transporter for PMD, although with a mechanism markedly different from that normally used for the natural substrates.

Resistance to PMD has been observed in *Leishmania* [40–46]. The mechanism(s) of resistance are poorly understood, but events at the mitochondrion appear to be associated with the drug's action. Electron microscopy revealed that treatment of *Leishmania* with PMD leads to disintegration of the network of intercatenated circular DNA molecules that comprise the mitochondrial genome, termed the kinetoplast, and also the mitochondrion [47,48], and a collapse in the mitochondrial membrane potential (MMP) [49] is one of the first manifestations of this drug treatment. In yeast, a mitochondrial localization was identified for the product of the *PNT1* gene that confers resistance to PMD when overexpressed [50,51]. In mammalian cells, PMD affects MMP as well [52].

The affinity for PMD shown by its transporter in *Leishmania* is unaltered in resistant parasites (7.4 ± 1.8 μM and 5.9 ± 2.1 μM, in wild-type and resistant strains, respectively). However, PMD accumulates to a lower level, and the apparent V_{max} for uptake is diminished in resistant parasites. A similar situation has been noted in resistance to isometamidium in *Trypanosoma congolense* [53]. Resistance in this case was concluded not to associate with alterations at the level of a plasma membrane transporter, but with changes in the MMP. Isometamidium rapidly enters the *T. congolense* mitochondrion where it binds tightly to kDNA. The apparent decrease in V_{max} in resistant cells correlated with diminished capacity to accumulate drug intracellularly. Similar to isometamidium, diamidines are organic cations, which also bind to the kinetoplast. 4,6-diamidino-2-phenylindole (DAPI), a fluorescent analogue of PMD (Fig. 1), enters cells and accumulates rapidly in the mitochondrion, binding to the kinetoplast in wild-type, but not in high-level PMD-resistant, *Leishmania* [39]. The fluorescent diamidine binds equally well to wild-type and resistant kinetoplasts, so access to the kinetoplast, rather than its ability to bind to the DNA, differentiates wild-type and resistant cells. A diminution of MMP in PMD-resistant *Leishmania* [39], as measured by accumulation of rhodamine-123, indicates that MMP is diminished in the drug-resistant line. Because rhodamine-123 is a substrate for P-glycoproteins, a diminished accumulation of this substance could also indicate enhanced P-glycoprotein efflux activity at the leishmanial plasma membrane. However, while an efflux mechanism for PMD was identified in *Leishmania* [39], the activity was the same in wild-type and resistant parasites [39].

The data on a resistance mechanism are consistent with a model, outlined in Fig. 4b, whereby PMD enters both resistant and wild-type cells via the same plasma membrane carrier that is unaltered in the resistant lines [39]. However, in wild-type but not in resistant parasites, PMD is rapidly accumulated into the mitochondrion with uptake being driven by the greater MMP of wild-type cells. Inhibitors of the MMP (e.g. potassium cyanide, sodium azide, 2,4-dinitrophenol and oligomycin) decrease PMD uptake into wild-type cells. By contrast, in resistant parasites, the drug does not accumulate rapidly into the mitochondrion, but remains free in the cytosol. Efflux pumps appear to be operative in removing cytosolic or membrane-associated PMD from the cell (as judged by the ability of inhibitors of pumps shown to be operative in *Leishmania*, e.g. verapamil, trifluoperazine and prochloperazine, to allow accumulation of drug in resistant cells). It is likely that this PMD efflux system can remove the

majority of PMD from the non-mitochondrial compartment of both cell types but, in the wild-type cells, most of the PMD becomes bound to the kinetoplast within the mitochondrion, and so is not available for efflux. This is not the case in resistant parasites. A balance involving rate of uptake into the cell, efflux from the cell at the plasma membrane, and accumulation into the mitochondrion would then underlie the events reported here. Agents that inhibit efflux might lead to reversal of resistance by allowing PMD to accumulate in the cytosol to levels that drive accumulation into the mitochondrion, through an unknown transporter or channel.

PMD against Plasmodium falciparum

Diamidines were tested against experimental malaria almost immediately after their synthesis [54]. Monkeys infected with *Plasmodium knowlesi* at parasitaemias that would, without intervention, be fatal could be saved with a daily dose of 2.5 mg kg^{-1} of undecane diamidine [54] and, in 1940, PMD and stilbamidine were shown to have similar curative effects in monkeys [55]. Sen Gupta also observed that, even in highly endemic areas, patients treated for kala azar with stilbamidine were rarely suffering from malaria [56]. However, other drugs, such as quinine and atebrin were effective, and diamidines were not pursued as much as antimalarials until recently. In 1990, Tidwell and co-workers synthesized new classes of diamidines, and demonstrated that these have considerable antimalarial activity [57]. Our recent studies confirm the potential of diamidines against *P. falciparum*, which showed a lack of crossresistance with standard antimalarials [58].

PMD is selectively accumulated into the *Plasmodium*-infected erythrocyte, reaching concentrations 500-fold greater than the surrounding medium. By contrast, no significant accumulation into uninfected erythrocytes is observed. Hence, it is probable that this specific uptake process underlies the antimalarial activity of PMD and other diamidines [58].

Kinetic studies reveal an initial rapid phase of PMD uptake (half life = 90 s) followed by a slower phase that is linear over several hours. The initial phase is nonsaturable in the micromolar range, but the slower phase shows two saturable processes with dissociation constants (K_d) of ~2 μM and 200 μM.

At first, we focused on the rapid initial phase of uptake because this most likely represents the passage of PMD across the host erythrocyte membrane. As well as being nonsaturable, the initial uptake phase is insensitive to known inhibitors of PMD transport into other protozoan parasites. Instead, initial uptake is effectively blocked by inhibitors of the new permeability pathway (NPP) [58]. The NPP is induced by the malaria parasite on the host cell membrane and has properties of an inwardly rectified anion channel rather than a transporter [59]. It is nonsaturable and anion-selective, but it exhibits significant permeability to cations [60,61]. Changing the counter ion (major permeant ion) in the bathing medium markedly influences passage of cations through the NPP [60]. We find similar effects of changing the counter ion on the initial uptake of PMD [58].

Taken together, all of these data suggest that PMD is not transported as in other protozoan parasite species, but penetrates the *Plasmodium*-infected erythrocyte membrane via the induced NPP channel (Fig. 4c). Once through the host cell membrane, PMD binds to haemoglobin but with low affinity. Here, the drug will have direct access to the external membranes of the intracellular parasite. From here, the drug must be able to access the interior of the intracellular parasite to exert its antimalarial effect, but the means by which this is achieved is not known. It is possible that the drug is taken up by the intracellular parasite in the cytostomal feeding process (f, [61]). Alternatively, a putative transporter could carry the drug across the parasite plasma membrane (Fig. 4c). Regardless of the way PMD is taken up by the intracellular parasite, it is likely that a significant amount of the drug eventually ends up in the food vacuole: PMD binds avidly to ferriprotoporphyrin IX (FPIX) *in vitro* with an affinity of ~2 μM. It also binds to aggregated FPIX with lower affinity. FPIX is generated inside the food vacuole from the digestion of haemoglobin and it is likely that PMD binds to FPIX in this location. During the slower phase of PMD accumulation, a saturable process with an affinity of 2 μM is revealed by Scatchard analysis [58]. We believe that this saturable process reflects the binding of PMD to FPIX in the food vacuole. This argument is based on the K_d being identical to that obtained for PMD–FPIX binding *in vitro* and is supported by the data obtained using plasmepsin inhibitors [58]. Plasmepsins are the food vacuole enzymes thought to be responsible for the first steps in the degradation of haemoglobin [62]. Blocking these enzymes stops haemoglobin digestion in the parasite and effectively stops the generation of FPIX [61]. We were able to demonstrate that this action effectively blocks the high-affinity binding of PMD to infected cells and antagonizes the antimalarial activity of all of the diamidines [58].

All of the diamidines tested so far are efficient blockers of haemozoin crystallization *in vitro*. As such, their ultimate mode of action is probably similar to that of chloroquine (CQ). Interestingly, however, there is no crossresistance with CQ or any other standard antimalarials [58], indicating that the diamidine motif could be an interesting lead structure for the development of new antimalarials.

Concluding remarks

Diamidines have a long and distinguished history in the fight against infectious disease and in particular protozoan infections. While much remains unclear about their precise mechanism of action in kinetoplastids, longstanding issues such as selective uptake and mechanisms of (cross)resistance have now largely been resolved. New classes of orally available diamidines with fewer side-effects or improved central nervous system penetration are being developed, and we are now in a better position to predict or prevent the emergence of resistance to the new drugs. The fact that this class of drugs has action against a variety of organisms including opportunistic fungal infections, such as *Pneumocystis carinii* and *Aspergillus*, will make development more commercially attractive. Although prophylaxis on the massive scale of the colonial era is now impracticable, it could be considered to bring epidemics in

defined foci under control, especially in areas with high incidence of melarsoprol treatment failure.

Acknowledgements
P.G.B, M.P.B., S.A.W. and H.P.dK. are supported by the Wellcome Trust.

References

1 Von Janscó, N. and Von Janscó, H. (1935) Chemotherapeutische Wirkung und Kohlehydratstoffwechsel: die Heilwirking von Guanidinderivaten auf die Trypanosomeninfektion. *Z. Immunitätsforsch. Exper. Ther.* 86, 1–30
2 King, H. *et al.* (1937) New trypanocidal substances. *Lancet* 223, 1360–1363
3 Lourie, E.M. and Yorke, W. (1939) Sudies in chemotherapy XXI. The trypanocidal action of certain aromatic diamidines. *Ann. Trop. Med. Parasitol.* 33, 289–304
4 Lourie, E.M. (1942) Treatment of sleeping sickness in Sierra Leone. *Ann. Trop. Med. Parasitol.* 36, 113–131
5 Napier, L.E. *et al.* (1942) The treatment of Kala-Azar by diamidino stilbene: analysis of 101 cases. *Indian Med. Gaz.* 77, 71–74
6 Napier, L.E. and Sen Gupta, P.C. (1942) *A peculiar neurological sequel to administration of 4:4″-diamidino-diphenyl-ethylene (M&B 744). Indian Med. Gaz.* 77, 71–74
7 Van Hoof, L. *et al.* (1944) Pentamidine in the prevention and treatment of trypanosomiasis. *Trans. R. Soc. Trop. Med. Hyg.* 37, 271–280
8 Goa, K.L. and Camploi-Richards, D.M. (1987) Pentamidine isethionate: a review of its antiprotozoal activity, pharmacokinetic properties and therapeutic use in *Pneumocystis carinii* pneumonia. *Drugs* 33, 242–258
9 Schoenbach, E.B. and Greenspan, E.M. (1948) The pharmacology, mode of action and therapeutic potentialities of stilbamidine, pentamidine, propamidine and other aromatic diamidines – a review. *Medicine (Baltimore)* 27, 327–377
10 Damper, D. and Patton, C.L. (1976) Pentamidine transport and sensitivity in brucei-group trypanosomes. *J. Protozool.* 23, 349–356
11 Damper, D. and Patton, C.L. (1976) Pentamidine transport in *Trypanosoma brucei*: kinetics and specificity. *Biochem. Pharmacol.* 25, 271–276
12 Shapiro, T.A. and Englund, P.T. (1990) Selective cleavage of kDNA minicircles promoted by antitrypanosomal drugs. *Proc. Natl. Acad. Sci. U. S. A.* 87, 950–954
13 Carter, N.S. *et al.* (1995) Uptake of diamidine drugs by the P2 nucleoside transporter in melarsen-sensitive and –resistant *Trypanosoma brucei brucei*. *J. Biol. Chem.* 270, 28153–28157
14 Carter, N.S. and Fairlamb, A.H. (1993) Arsenical resistant trypanosomes lack an unusual adenosine transporter. *Nature* 361, 173–175
15 Mäser, P. *et al.* (1999) A nucleoside transporter from *Trypanosoma brucei* involved in drug resistance. *Science* 285, 242–244
16 Barrett, M.P. and Fairlamb, A.H. (1999) The biochemical basis of arsenical-diamidine crossresistance in African trypanosomes. *Parasitol. Today* 15, 136–140
17 De Koning, H.P. and Jarvis, S.M. (1999) Mol. Pharmacol. *Purine transporters in bloodstream forms of T. b. brucei: Substrate recognition motifs and affinity for trypanocidal drugs* 56, 1162–1170
18 De Koning, H.P. (2001) Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by three distinct transporters. Implications for crossresistance with arsenicals. *Mol. Pharmacol.* 59, 586–592
19 De Koning, H.P. and Jarvis, S.M. (2001) Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by the P2 adenosine transporter and at least one novel, unrelated transporter. *Acta Trop.* 80, 245–250
20 Waalkes, T.P. and DeVita, V.T. (1970) The determination of pentamidine (4, 4-diamidinophenoxypentane) in plasma, urine, and tissues. *J. Lab. Clin. Med.* 75, 871–878
21 De Koning, H.P. *et al.* (1998) Characterisation of a nucleoside/proton symporter in procyclic *Trypanosoma brucei brucei*. *J. Biol. Chem.* 273, 9486–9494
22 De Koning, H.P. and Diallinas, G. (2000) Nucleobase transporters (review). *Mol. Membr. Biol.* 75, 75–94
23 De Koning, H.P. (2001) Transporters in African trypanosomes: role in drug action and resistance. *Int. J. Parasitol.* 31, 511–521
24 Fairlamb, A.H. *et al.* (1992) Characterisation of melarsen-resistant *Trypanosoma brucei brucei* with respect to cross-resistance to other drugs and trypanothione metabolism. *Mol. Biochem. Parasitol.* 53, 213–222
25 Zhang, Z.Q. *et al.* (1992) *In vivo* and *in vitro* sensitivity of *Trypanosoma evansi* and *T. equiperdum* to diminazene, suramin, MelCy, quinapyramine and isometamidium. *Acta Trop.* 50, 101–110
26 Matovu, E. *et al.* (2001) Susceptibility of Ugandan *Trypanosoma brucei rhodesiense* isolated from man and animal reservoirs to diminazene, isometamidium and melarsoprol. *Trop. Med. Int. Health* 2, 13–18
27 Berger, B.J. *et al.* (1995) Characterisation of pentamidine-resistant *Trypanosoma brucei brucei*. *Mol. Biochem. Parasitol.* 69, 289–298
28 Gastellu-Etchegorry, M. *et al.* (2001) Availability and affordability of treatment for human African trypanosomiasis. *Trop. Med. Int. Health* 6, 957–959
29 Matovu, E. *et al.* (2001) Melarsoprol refractory *T. b. gambiense* from Omugo, north-western Uganda. *Trop. Med. Int. Health* 6, 407–411
30 Brun, R. *et al.* (2001) The phenomenon of treatment failures in human African trypanosomiasis. *Trop. Med. Int. Health* 6, 906–914
31 Croft, S.L. and Yardley, V. (2000) Chemotherapy of leishmaniasis. *Curr. Pharm. Des.* 8, 319–342
32 Croft, S.L. (2001) Monitoring drug resistance in leishmaniasis. *Trop. Med. Int. Health* 6, 899–905
33 Becker, I. *et al.* (1999) The efficacy of pentamidine combined with allopurinol of patients with diffuse cutaneous leishmaniasis. *Parasitol. Res.* 85, 165–170
34 Sands, M. *et al.* (1985) Pentamidine: a review. *Rev. Infect. Dis.* 7, 625–634
35 Soto-Mancipe, J. *et al.* (1994) Successful treatment of Colombian cutaneous leishmaniasis with four injections of pentamidine. *Am. J. Trop. Med. Hyg.* 50, 107–111
36 Kandpal, M. *et al.* (1995) Kinetics and molecular characteristics of arginine transport by *Leishmania donovani* promastigotes. *Mol. Biochem. Parasitol.* 71, 193–201
37 Reguera, R. *et al.* (1994) Putrescine uptake inhibition by aromatic diamidines in *Leishmania infantum* promastigotes. *Biochem. Pharmacol.* 47, 1859–1866
38 Basselin, M. *et al.* (2000) Putrescine and spermidine transport in *Leishmania*. *Mol. Biochem. Parasitol.* 109, 37–46
39 Basselin, M. *et al.* (2002) Resistance to pentamidine in *Leishmania mexicana* involves exclusion from the mitochondrion. *Antimicrob. Agents Chemother.* 46, 3731–3738
40 Basselin, M. *et al.* (1997) Effects of pentamidine on polyamine level and biosynthesis in wild-type, pentamidine-treated and pentamidine-resistant *Leishmania*. *Exp. Parasitol.* 85, 274–282
41 Basselin, M. *et al.* (1997) Altered transport properties of pentamidine-resistant *Leishmania donovani* and *L. amazonensis* promastigotes. *Parasitol. Res.* 83, 413–418
42 Basselin, M. *et al.* (1998) Modification of kinetoplast DNA minicircle composition in pentamidine-resistant *Leishmania*. *Acta Trop.* 70, 43–61
43 Basselin, M. and Robert-Gero, M. (1998) Alterations in membrane fluidity, lipid metabolism, mitochondrial activity, and lipophosphoglycan expression in pentamidine-resistant *Leishmania*. *Parasitol. Res.* 60, 78–83
44 Giri, O.P. (1994) Treatment of visceral leishmaniasis unresponsive to pentamidine with amphotericin B. *J. Assoc. Physicians India* 42, 688–689
45 Sereno, D. *et al.* (1997) Phenotypic characterization of *Leishmania mexicana* pentamidine-resistant promastigotes. Modulation of the resistance during *in-vitro* developmental life cycle. *C. R. Acad. Sci. III* 320, 981–987
46 Sereno, D. and Lemesre, J.L. (1997) *In vitro* life cycle of pentamidine-resistant amastigotes: stability of the chemoresistant phenotypes is dependent on the level of resistance induced. *Antimicrob. Agents Chemother.* 41, 1898–1903
47 Croft, S.L. and Brazil, R.P. (1982) Effect of pentamidine isethionate on the ultrastructure and morphology of *Leishmania mexicana amazonensis in vitro*. *Ann. Trop. Med. Parasitol.* 76, 37–43
48 Hentzer, B. and Kobayasi, T. (1977) The ultrastructural changes of *Leishmania tropica* after treatment with pentamidine. *Ann. Trop. Med. Parasitol.* 71, 157–166
49 Verseci, A.E. and Docampo, R. (1992) Ca^{2+} transport by digitonin-permeabilized *Leishmania donovani*. Effects of Ca^{2+}, pentamidine and

WR-6026 on mitochondrial membrane potential *in situ*. *Biochem. J.* 284, 463–467
50 Ludewig, G. and Staben, C. (1994) Characterization of the PNT1 pentamidine resistance gene of *Saccharomyces cerevisiae*. *Antimicrob. Agents Chemother.* 38, 2850–2856
51 He, S. and Fox, T.D. (1999) Mutations affecting a yeast mitochondrial inner membrane protein Pnt1p, block export of a mitochondrially synthesized fusion protein from the matrix. *Mol. Cell. Biol.* 19, 6598–6607
52 Moreno, S.N. (1996) Pentamidine is an uncoupler of oxidative phosphorylation in rat liver mitochondria. *Arch. Biochem. Biophys.* 326, 15–20
53 Wilkes, J.M. *et al.* (1997) Modulation of mitochondrial electrical potential: a candidate mechanism for drug resistance in African trypanosomes. *Biochem. J.* 326, 755–761
54 Christophers, S.R. and Fulton, J.D. (1938) Observations on the course of *Plasmodium knowlesi* infection in monkeys (*Macacus rhesus*), with notes on its treatment by (1) atebrin and (2) 1:11 normal undecane diamidine together with a note on the latter on bird malaria. *Ann. Trop. Med. Parasitol.* 32, 257–278
55 Fulton, J.D. (1940) The course of *Plasmodium relictum* infection in canaries and the treatment of bird and monkey malaria with synthetic bases. *Ann. Trop. Med. Parasitol.* 34, 53–66
56 Sen Gupta, P.C. (1945) 4:4′-Diamidino-diphenyl ether in the treatment of Indian kala azar. *Indian Med. Gaz.* 80, 495–498
57 Bell, C.A. *et al.* (1990) Structure-activity relationships of analogs of pentamidine against *Plasmodium falciparum* and *Leishmania mexicana* amazonensis. *Antimicrob. Agents Chemother.* 34, 1381–1386
58 Stead, A.M.W. *et al.* (2001) Diamidine compounds: Novel antimalarials with two levels of selectivity against *Plasmodium falciparum*. *Mol. Pharmacol.* 59, 1298–1306
59 Desai, S.A. *et al.* (2000) A voltage-dependent channel involved in nutrient uptake by red blood cells infected with the malaria parasite. *Nature* 406, 1001–1005
60 Kirk, K. (2001) Membrane transport in the malaria-infected erythrocyte. *Physiol. Rev.* 81, 495–537
61 Bray, P.G. *et al.* (1999) Cellular uptake of chloroquine is dependent on binding to ferriprotoporphyrin IX and is independent of NHE activity in *Plasmodium falciparum*. *J. Cell Biol.* 145, 363–376
62 Francis, S.E. *et al.* (1997) Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. *Annu. Rev. Microbiol.* 51, 97–123
63 Harding, R.D. (1945) Late results of treatment of sleeping sickness in Sierra Leone by antrypol, tryparsamide, pentamidine, and propamidine singly and in various combinations. *Trans. R. Soc. Trop. Med. Hyg.* 39, 99–124
64 Duggan, A.J. and Hutchinson, M.P. (1951) The efficacy of certain trypanocidal compounds against *Trypanosoma gambiense* infection in man. *Trans. R. Soc. Trop. Med. Hyg.* 44, 535–544
65 Doua, F. *et al.* (1996) The efficacy of pentamidine in the treatment of early-late stage *Trypanosoma brucei gambiense* trypanosomiasis. *Am. J. Trop. Med. Hyg.* 55, 586–588
66 Legros, D. *et al.* (2002) Treatment of human African trypanosomiasis – present situation and needs for research and development. *Lancet Infect. Dis.* 2, 437–440
67 Van-Hoof, A. *et al.* (1946) A field experiment on prophylactic value of pentamidine in sleeping sickness. *Trans. R. Soc. Trop. Med. Hyg.* 39, 327–329
68 Organisation de Coordination pour la Lutte contre les Endemies en Afrique Centrale (OCEAC) (1968) Rapport final de la deuxieme conference technique
69 Labusquière, R. and Dutertre, J. (1966) La lutte contre les derniers foyers de trypanosomiase humaine africaine à *T. gambiense*. *Med. Trop.* 26, 357–362
70 Ollivier, G. and Legros, D. (2001) Trypanosomiase humaine africaine: historique de la thérapeutique et de ses échecs. *Trop. Med. Int. Health* 6, 855–863
71 WHO (1976) The African Trypanosomiases: Report of a joint WHO Expert Committee and FAO Expert Consultation. WHO Technical Report Series 635