Trends in Parasitology

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REVIEWS

Review

Melarsoprol Resistance in African Trypanosomiasis

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Arsenicals were introduced as monotherapies for the treatment of human African trypanosomiasis, or sleeping sickness, over 100 years ago. Toxicity has always been an issue but these drugs have proven to be both effective and quite durable. Unfortunately, melarsoprol-resistant parasites emerged as early as the 1970s and were widespread by the late 1990s. Resistance was due to mutations affecting an aquaglyceroporin (AQP2), a parasite solute and drug transporter. This is the only example of widespread drug resistance in trypanosomiasis patients for which the genetic basis is known. This link between melarsoprol and AQP2 illustrates how a drug transporter can improve drug selectivity but, at the same time, highlights the risk of resistance when the drug uptake mechanism is dispensable for parasite viability and virulence.

> A further advantage of combined therapy is this, that under the influence of two different medicaments the danger of rendering the parasite immune to arsenic, naturally a very great obstacle in connexion with further treatment, is apparently minimized
> Paul Ehrlich, 1913

Highlights

Arsenical monotherapies were previously very successful for treating human African trypanosomiasis (HAT).

Melarsoprol resistance emerged as early as the 1970s and was widespread by the late 1990s.

Melarsoprol resistance represents the only example of widespread drug resistance in HAT patients where the genetic mechanism has been established.

The current goal of elimination of HAT as a public health problem by 2020 may be undermined by the emergence and spread of resistance to current or new drugs.

Insights into potential resistance mechanisms for current and new drugs will facilitate predictions of the likelihood of resistance and will also facilitate rational approaches to minimizing, monitoring, and tackling the future emergence of resistance.

African Trypanosomiasis Drugs and Drug Resistance

The **African trypanosomes** (see Glossary), *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, cause chronic and acute human African trypanosomiasis (HAT), or sleeping sickness (gHAT in west Africa and rHAT in east Africa, respectively). These parasites are transmitted to mammals by the tsetse fly vector, with humans being the main reservoir for gHAT and game animals for rHAT. In the early stages of infection with *T. b. gambiense*, by far the commonest form of the disease, symptoms are less severe but the parasites eventually cross the blood–brain barrier in most patients and, once in the central nervous system (CNS) cause the severe neurological symptoms of the disease, which commonly culminate in coma and death [2]. This key biphasic disease process has important implications in terms of diagnosis and treatment. Since many drugs fail to cross the blood–brain barrier and enter the cerebrospinal fluid (CSF), and those that do often come with associated risks, diagnostics that provide staging information are essential; this typically involves a blood test that, if positive, progresses to a CSF test [3]. Cases are currently relatively low and the focus is on elimination as a public health problem by 2020. However, it should be remembered that there have been major epidemics in the past where historical periods characterised by low numbers of cases have been followed by resurgence [4]. Chemotherapy and vector control strategies have had a major impact on disease control but parasite reservoirs persist in animals and in trypanotolerant individuals [5], presenting a substantial challenge in the absence of a vaccine [6].

Historically, most patients were only diagnosed once symptoms progressed to those associated with CSF involvement, meaning that CSF-penetrant drugs were particularly important. The

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typically less toxic but poorly CSF-penetrant drugs used for the first stage of the disease are **pentamidine** for gHAT and suramin for rHAT [7]. The sufficiently CSF-penetrant drugs used for the second stage of the disease are **melarsoprol**, the only drug available until the 1990s, and NECT, or nifurtimox-eflornithine combination therapy [7,8]. Although it is often said that the drugs mentioned above suffer from problems of drug resistance, melarsoprol resistance is actually the only concrete example of clinically relevant drug-resistant parasites.

The Development of Arsenic-Based Therapies

Inorganic arsenical compounds have been extensively used for nearly 2500 years as both poisons and therapeutic agents. The first recorded use of arsenic in the treatment of trypanosomiasis was in 1858 [9]. Although the cause of **nagana**, or animal trypanosomiasis, was not known at the time, David Livingstone treated a 'fly-struck' mare with 'two grains of arsenic in a little barley daily for about a week' or Fowler's solution (a popular tonic containing 1% potassium arsenite; Figure 1A, Key Figure). Although the mare showed an initial improvement in condition, it ultimately succumbed to the disease. It was not until 1902 that laboratory confirmation of the trypanocidal activity of arsenite in infected rodents was demonstrated by Laveran and Mesnil at L'Institut Pasteur [10]. Unfortunately, all animals either died from infection if treatment was discontinued or from arsenic toxicity if not.

In 1905, a more selective organic arsenical, optimistically named 'Atoxyl' (arsanilic acid; Figure 1A), was demonstrated to show therapeutic efficacy in mice by Thomas and Breinl at Liverpool, and in 1907 Robert Koch stated that 'no doubt can any longer exist as to the specific action of [Atoxyl]' [11]. This pentavalent compound had previously been dismissed by Ehrlich and Hata in Frankfurt, however, as it lacked trypanocidal activity *in vitro*. It was the pivotal discovery of Thomas and Breinl that resurrected Ehrlich and Hata's interest in organic arsenicals, leading to the discovery in 1910 of arsphenamine (compound '606', Salvarsan) for the treatment of syphilis. Meanwhile, the extensive use of Atoxyl for the treatment of HAT patients had revealed serious toxicity issues, particularly blindness due to optic nerve damage. This stimulated further research, leading to the discovery of tryparsamide (Figure 1A) by Jacobs and Heidelberger at the Rockefeller Institute in 1919 [12].

By 1925 clinical trials in the Belgian Congo established that tryparsamide was highly effective, especially in treating late-stage disease. However, by the late 1940s, **treatment failure** with tryparsamide had become widespread (>80%) in the Belgian Congo and French Cameroun. Meanwhile, Ernst Friedheim was developing a novel series of melaminophenyl arsenical compounds. An entertaining autobiographical account of his observation that feeding Swiss cheese to HAT patients ameliorated the neurotoxicity of melarsen oxide reveals how he came to combine the heavy metal chelator **British anti-Lewisite** (BAL, dimercaprol) with melarsen oxide to produce melarsoprol (MelB, Arsobal; Figure 1A) [13]. By 1950, melarsoprol was found to be highly effective (97%) against tryparsamide treatment failures; however, by the turn of the century, its therapeutic value against gHAT had been eroded by extensive treatment failures, particularly in the Democratic Republic of the Congo (Figure 1B). Although NECT has largely supplanted melarsoprol in West Africa for the treatment of gHAT, melarsoprol is still in use today in East Africa as the only effective treatment for advanced stage rHAT. The reader is referred to reviews for a more detailed history of arsenic in medicine [14] and in HAT [15]. Although melarsoprol has been unfairly disparaged as 'arsenic in antifreeze' it has saved countless lives since its introduction for HAT treatment in 1949. Also noteworthy, arsenic as a therapeutic has also undergone resurgence with arsenic trioxide used for the treatment of acute promyelocytic leukemia [16].

Glossary

African trypanosomes: a group of closely related parasites typically (but not always) restricted to Africa due to transmission by tsetse flies, with a geographically limited range. *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* infect humans and animals. *Trypanosoma brucei brucei*, *Trypanosoma congolense*, and *Trypanosoma vivax* infect livestock and wild animals. *Trypanosoma evansi* and *Trypanosoma equiperdum* infect horses, camels, or water buffalo and cause surra and dourine, also in Asia and Latin America. Other related trypanosomatids, *Trypanosoma cruzi* and *Leishmania* spp., are transmitted by distinct vectors displaying different global distribution and cause distinct diseases.

British anti-Lewisite: a heavy metal chelator developed during World War II as an antidote to Lewisite, an arsenic-based chemical warfare agent.

Flagellar pocket: a flagellum-associated invagination of the parasite plasma membrane and the exclusive site for endocytosis where several receptors are concentrated [97].

Melarsoprol: a 398 Da arsenical compound first introduced for HAT therapy in the 1940s. A combination of melarsen oxide and British anti-Lewisite.

Nagana: animal African trypanosomiasis; a disease of cattle and other livestock characterized by fever and lethargy and typically caused by *T. vivax* or *T. congolense*.

Pentamidine: a 346 Da amidine compound first introduced for HAT therapy in the 1940s and used to treat gHAT prior to CNS involvement.

Safety index: the ratio of the lowest dose that elicits no serious toxicity (no-observed-adverse-effect level, NOAEL) to that which achieves efficacy to the disease. Sometimes expressed as LD_{10}/ED_{90} in animal studies.

Therapeutic index: the ratio of the 'lethal' dose causing 50% death (LD_{50}) in animals to the 'effective' dose required to cure 50% (ED_{50}). For ethical reasons this has been replaced by the ratio of ED_{50}/TD_{50} – the 'toxic' dose that causes serious unwanted side effects in animals.

Clinical Pharmacology and Toxicology of Melarsoprol

The treatment schedule for melarsoprol in the 1950s was derived empirically. The drug is almost insoluble in water and is administered intravenously as a 3.6% solution in propylene glycol. This solvent is highly irritant to tissues and has to be given in glass syringes as it slowly dissolves plastic syringes. Until recently, treatment used to start with incremental doses (to anticipate potential drug toxicity) with 'rest intervals' between treatment series (presumably to avoid cumulative drug toxicity) [7]. However, a concise 10-day treatment schedule of 2.2 mg/kg daily has now replaced previous regimens, as it is equally efficacious but considerably more cost-effective for gHAT [17,18] and rHAT [19]. Melarsoprol is rapidly metabolised *in vivo* to other trypanocidal metabolites (possibly melarsen oxide) [20,21], and melarsoprol is not detectable in the CSF. The active metabolites in CSF achieve only about 3% of the plasma trough level [20]. Elimination is via the urine [20] and bile [22]. The **safety index** for melarsoprol or melarsen oxide

Treatment failure (treatment relapse): this can be due to multiple reasons: the drug, the patient, the clinician, or the infecting organism. Examples: variations in drug metabolism and pharmacokinetic behaviour in different patient populations, poor quality or substandard drugs, poor patient compliance, clinical errors in drug administration, different tissue tropism of parasite strains, parasite dormancy, reinfection, or drug resistance.

Key Figure

Arsenicals and Melarsoprol-Resistant Trypanosomiasis

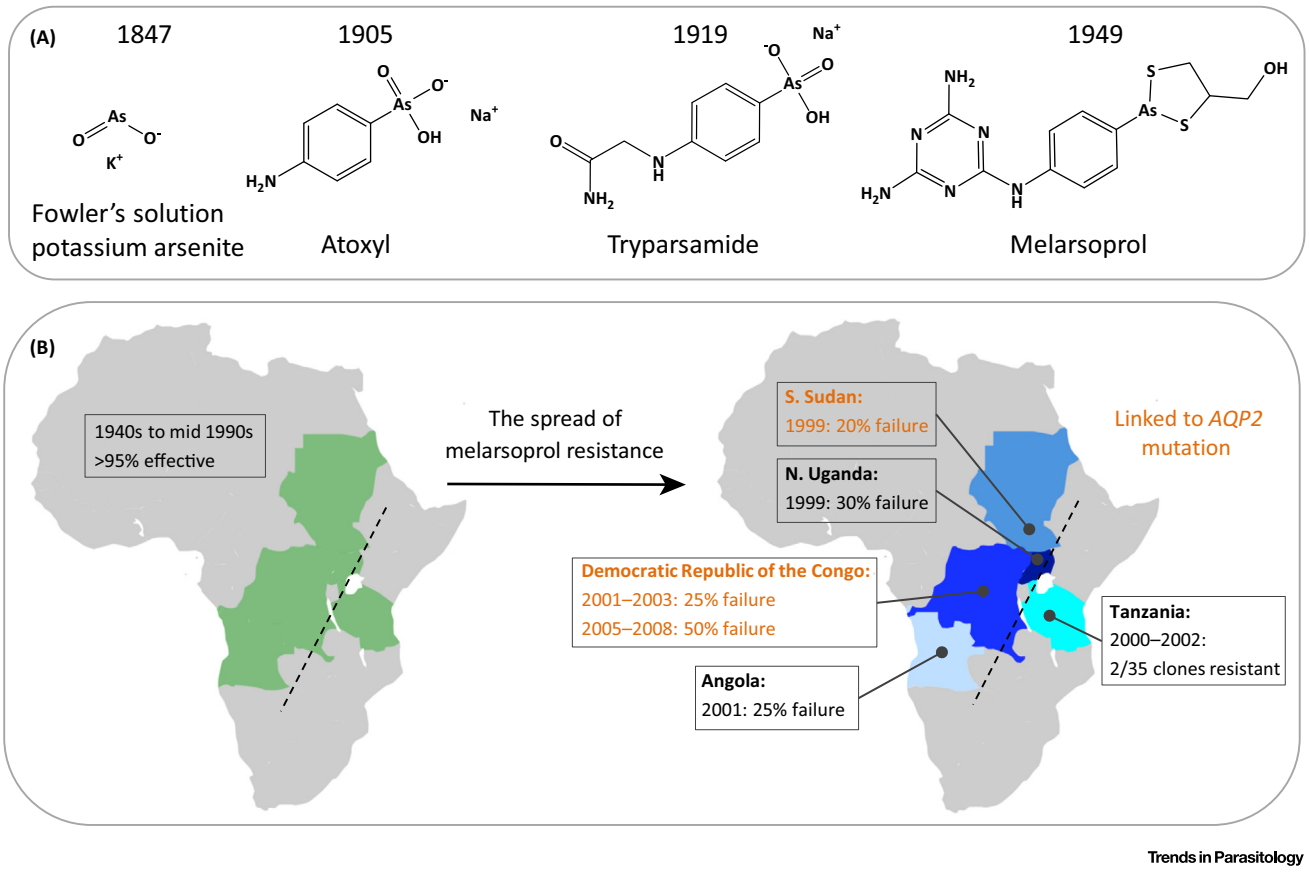

Figure 1. (A) Arsenical drugs and the years they were introduced for the treatment of African trypanosomiasis. (B) Melarsoprol was highly effective, but resistance was widespread by the late 1990s and was linked to *AQP2* mutation more recently. The broken line indicates the range of *Trypanosoma brucei gambiense* (West) and *Trypanosoma brucei rhodesiense* (East).

is ~5 in mice infected with *T. b. rhodesiense* [23], and the **therapeutic index** is 50–100 for melarsoprol in *T. equiperdum* infections in mice [13].

The most important serious side effect of melarsoprol treatment is post-treatment reactive encephalopathy (PTRE) that typically occurs 1–10 days after the start of treatment. PTRE is characterised by increased mental deterioration followed by coma and, in some cases, convulsions and death. PTRE occurs in 5–10% of melarsoprol-treated patients, with an overall fatality rate of about 50%. The pathogenesis of PTRE is not fully understood [24], but is proposed to involve an immune reaction resulting from rapid lysis of trypanosomes in the CNS, rather than drug toxicity, as survivors of PTRE are usually able to resume treatment with no further serious adverse events [25]. However, PTRE can occur in stage I HAT patients; and arsenical encephalopathies have been reported with melarsoprol for leukaemia [26], with a phenylarsenoxide derivative for solid tumours [27], with arsenic trioxide for metastatic carcinoma [28], and with various organic arsenicals in the treatment of syphilis [29]. The integrity of the blood–brain barrier that prevents entry of most drugs into the CNS is compromised in stage II HAT [30], and restoration of a functional blood–brain barrier following partial treatment might also account for the lack of toxicity once melarsoprol therapy is resumed.

Treatment with prednisolone has been reported to reduce the incidence and mortality from PTRE in gHAT [31–33], although the situation is less clear for rHAT [7,34,35]. Unfortunately, the optimised 10-day melarsoprol therapy failed to reduce the incidence of PTRE in either gHAT [17] or rHAT [19].

Mode of Action of Melarsoprol

The mechanism of action of arsenical drugs is not clear. Organic arsenicals can exist as either valence +3 or +5. Pentavalent arsenicals are inactive against African trypanosomes *in vitro* and have to be converted *in vivo* by reduction to the biologically active trivalent forms [36]. Trivalent arsenical compounds have an affinity for sulfhydryl groups, particularly vicinal (neighbouring) thiols on proteins, but this is a rather nonspecific effect since arsenite and aminophenylarsene oxide lack any cellular selectivity between host and parasite. Indeed, as described below, selective toxicity is primarily conferred via selective uptake of melaminophenyl arsenicals by the parasite-specific P2 adenosine transporter (*AT1* gene) and aquaglyceroporin 2 (AQP2).

Once within the cell, the primary event appears to be reaction with the dithiol form of trypanothione to form a 1:1 complex known as Mel T [37]. Mel T is a moderately potent competitive inhibitor of the pivotal antioxidant enzyme, trypanothione reductase, but definitive evidence that this is the major mechanism leading to cell death is lacking [38]. Knockdown of trypanothione synthetase and trypanothione reductase supports the idea that MelT is toxic to the cell [39], but the precise mechanism remains elusive. Bloodstream forms of *T. brucei* rapidly lose motility when exposed to trivalent phenyl arsenoxides such as melarsen oxide, suggesting that inhibition of energy metabolism via glycolysis is involved in cell death. However, a careful study concluded that inhibition of glycolysis is not the cause, but rather the consequence, of cell lysis [40]. Thus, the putative target responsible for rapid cell lysis remains to be identified, and the possibility that arsenicals interact with a critical membrane component has not been explored.

Studies on Laboratory-Acquired Melarsoprol Resistance

Melarsoprol Transport and Cross-Resistance with Amidines

Paul Ehrlich 'the father of chemotherapy' and colleagues first described drug resistance over 100 years ago and this work was based on laboratory studies using trypanosomes [1]. Efforts to understand arsenical resistance have since included contributions from Frank Hawking [41]

(father of the late theoretical astrophysicist Stephen Hawking), and have been linked to efforts to understand amidine resistance, since cross-resistance between these two structurally unrelated classes of drugs was reported in 1951 [42]. Subsequent studies have confirmed that selection for resistance to melarsoprol typically yields cross-resistance to pentamidine and vice versa [43,44]. Resistance was found to be due to reduced drug uptake for both melarsen oxide [45] and pentamidine [46,47]. Arsenical efflux is another possible mechanism of resistance, and overexpression of the ABC transporter multidrug resistance protein A (MRPA) does indeed result in melarsoprol resistance [48]. This was not the case in a mouse model, however, and no MRPA overexpression was detected in melarsoprol-resistant isolates from HAT patients [48].

The first trypanosome transporter linked to arsenical and amidine cross-resistance was the P2 adenosine transporter in *T. b. brucei* [46,49]. Functional cloning in yeast was subsequently used to identify the *AT1* gene that encodes this transporter, which also rendered the yeast susceptible to melarsen oxide [50]. Gene knockout was used to generate cells lacking *AT1*, and these cells were approximately twofold resistant to both melarsoprol and pentamidine [51]. It may have seemed at that time then that the genetic basis of melarsoprol–pentamidine cross-resistance was clear. There was more to the story, however. The correlation between mutant *AT1* alleles and melarsoprol resistance remained incomplete after extensive study; see [52–54] for example. In addition, it was possible to select cells lacking *AT1* for higher levels of resistance and this involved the loss of a 'high-affinity pentamidine transporter' (HAPT) function in both *T. b. brucei* and *T. b. gambiense* [44].

AQP2 Is a Melarsoprol–Pentamidine Transporter

As described above, a powerful approach for investigating mechanisms of drug resistance is to select resistant parasites in the laboratory and then to identify the underlying changes, both pharmacological and genetic. Indeed, it was a variation on this theme that revealed a candidate for the gene encoding the HAPT. Genome-scale screens were used to perturb each gene individually and to ask which genes affected melarsoprol or pentamidine resistance [39]. This RNA interference target-sequencing (RIT-seq) approach took advantage of RNA interference to knockdown gene expression and to ask which knockdowns rendered parasites drug resistant. Notably, only one locus in the genome was linked to cross-resistance to both drugs and this locus encoded two related aquaglyceroporins, AQP2 and AQP3 [39].

Aquaglyceroporins are among the major intrinsic protein family and typically facilitate the passive transport of water and small solutes across membranes [55]; Peter Agre was awarded a share of the 2003 Nobel Prize in Chemistry for his discovery of the related aquaporins. AQPs had previously been considered as potential entry routes for small-molecule drugs [56], and several such channels can transport low-molecular-mass arsenite and antimonite [57]. Indeed, *Leishmania donovani* is sensitive to low-molecular-mass antimonials due to uptake via LdAQP1, and drug resistance appears to have emerged in visceral leishmaniasis patients in the Indian subcontinent due to loss of LdAQP1 function [58]; widespread resistance lead to the recommended withdrawal of these drugs in this region. One hypothesis is that exposure to arsenic in drinking water may have resulted in cross-resistance to antimonials [59]. Although evidence supporting this hypothesis has been obtained in animal models [60], definitive clinical epidemiological evidence is still lacking [61].

Although the findings above suggested a drug-transport mechanism, as the name suggests, AQPs typically transport only water and small solutes, such as glycerol, not far larger compounds such as melarsoprol and pentamidine. Nevertheless, a functional assessment of both

AQPs showed that AQP2, which is restricted to the parasite **flagellar pocket**, was specifically responsible for drug uptake [62]. AQPs generally form tetramers and this study also suggested that AQP2 and AQP3, although closely related, form only homotetramers that partition to distinct membrane compartments. Importantly, cells lacking AQP2 remained susceptible to an arsenical that diffuses across membranes, supporting a role in drug transport. AQP2 was also shown to be dispensable with no loss of parasite fitness in cell culture or in a rodent model [62].

T. brucei cells express three aquaglyceroporins (AQP1-3; Figure 2). While AQP1 and AQP3 contain the conserved dual Asn-Pro-Ala (NPA) 'selectivity filter' motifs and the aromatic Arg (ar-R) motif that form prominent constrictions in the solute channel, AQP2 is unusual and has Asn-Ser-Ala (NSA) and Asn-Pro-Ser (NPS) motifs and also lacks a conserved ar-R [63] (Figure 3), potentially explaining the passage of larger substrates. Indeed, a single change, addition of an ar-R within the selectivity filter, blocked drug transport by AQP2 [64]. Further genetic dissection and ectopic expression in *Leishmania mexicana* cells, combined with pharmacological studies, revealed that *AQP2* does indeed encode an activity that is indistinguishable from that of the previously described HAPT [65].

AQP2 Is Mutated in Melarsoprol-Resistant Laboratory Strains

The connection between AQP2 and drug resistance, although unexpected, prompted an assessment of the *AQP2* locus in the various strains selected for melarsoprol or pentamidine resistance in the laboratory. The first cross-resistant laboratory-selected strain analysed was a *T. b. brucei* strain, engineered to lack *AT1* and selected for increased pentamidine resistance. This strain was found to have a chimeric *AQP2/3* gene in place of the two adjacent *AQPs* [62]

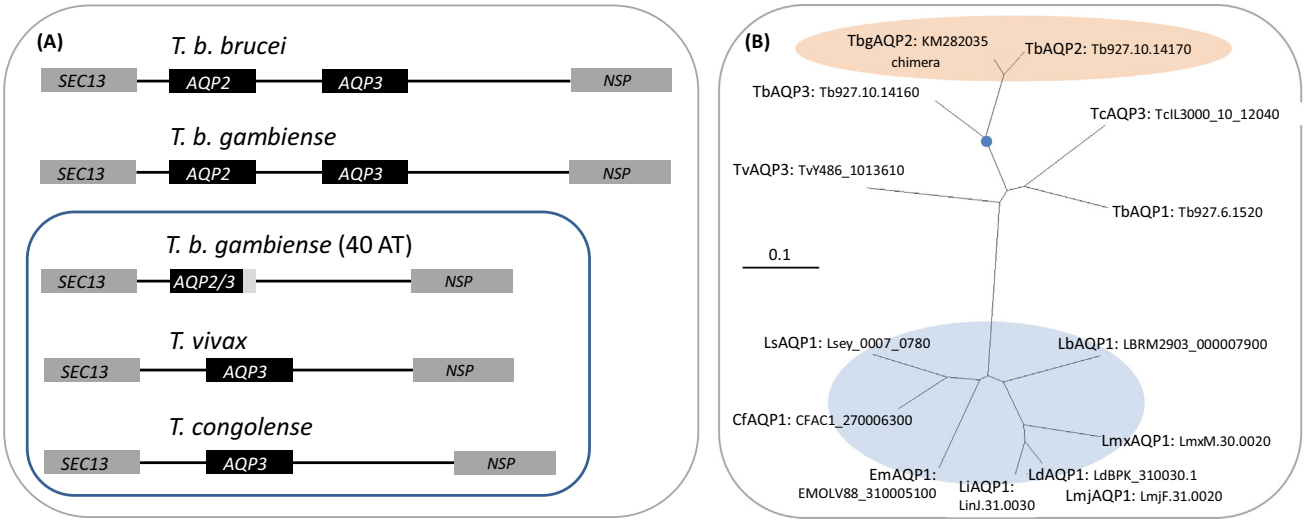

Figure 2. *AQP2*-related Genes in Trypanosomatid Genomes. (A) The African trypanosome *AQP2–AQP3* locus on chromosome 10. The genomes indicated in the smaller box are known to be (40 AT isolate) or are expected to be resistant to melarsoprol and pentamidine since they lack an intact *AQP2* gene. Indeed, the 40 AT *Trypanosoma brucei gambiense* isolate is melarsoprol resistant while *Trypanosoma congolense* and *Trypanosoma vivax* appear to be naturally refractory to melarsoprol. The African trypanosome *AQP1* gene is on chromosome 6 (not shown). *SEC13*, secretory factor; *AQP*, aquaglyceroporin; *NSP*, nuclear segregation factor. (B) Phylogenetic tree showing the relationship between *AQP2* and an *AQP2/3* chimera (orange oval) and other trypanosomatid genes. The *Trypanosoma brucei* (Tb), *T. b. gambiense* (Tbg), *Trypanosoma brucei rhodesiense and Trypanosoma evansi AQP1* and *AQP2* genes are almost identical (only Tb is shown for these genes). A probable duplication event leading to AQP2 and AQP3 is indicated (blue dot). *Leishmania donovani* (Ld) *AQP1* mutation leads to antimonial resistance, and closely related genes are found in other *Leishmania* species and related species (light blue oval). The *Trypanosoma cruzi* genome does not encode syntenic orthologues of these genes. Abbreviations: Tv, *T. vivax*; Tc, *T. congolense*; Ls, *Leptomonas seymouri*; Cf, *Crithidia fasciculata*; Em, *Endotrypanum monterogeii*; Li, *Leishmania infantum*; Lmj, *Leishmania major*; Lmx, *Leishmania mexicana*; Lb, *Leishmania braziliensis*. Scale bar: phylogenetic distance.

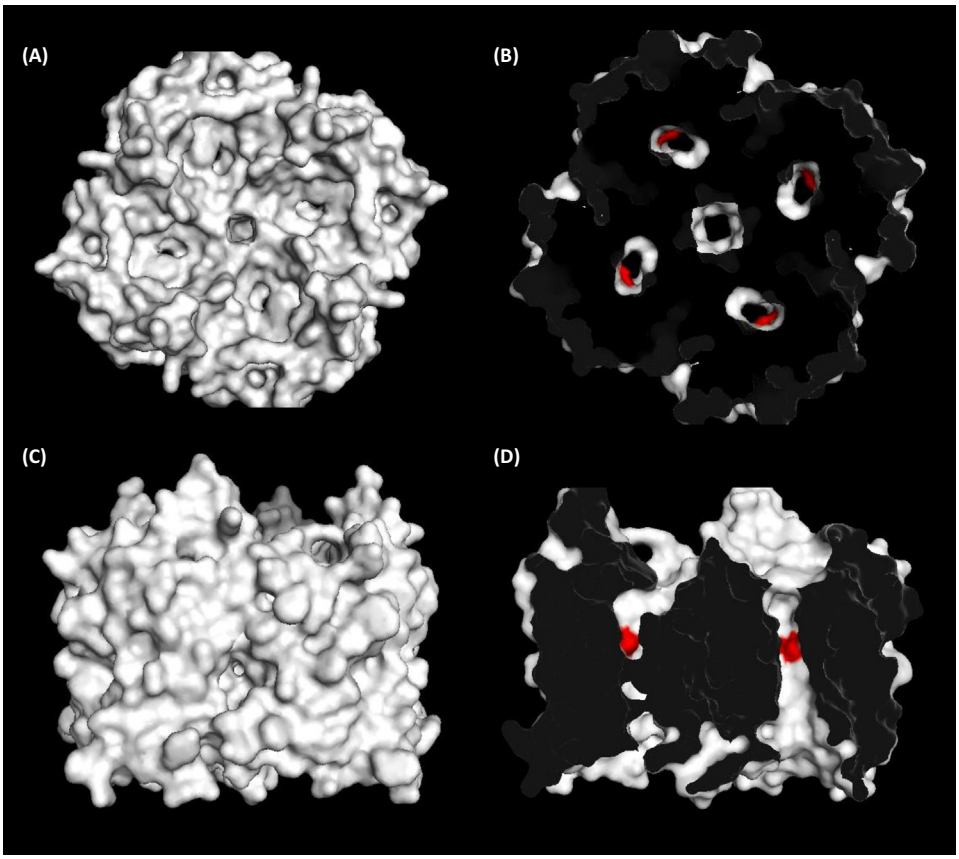

Figure 3. Structure Prediction of the Tetrameric AQP2 Membrane Transporter. The panels show top (A,B) and side (C,D) views. The cut-away views (B,D) show the location of a leucine reside (red) in place of the more usual aromatic arginine found in other AQPs. A central potential pore (visible in A,B) is not thought to be involved in drug uptake [64]. Images created using SWISS-MODEL[i] and PyMOL.[ii]

(see Figure 2A). These studies were extended to additional *T. b. brucei*, *T. b. gambiense*, and *T. b. rhodesiense* cross-resistant laboratory-selected strains, which all revealed either chimeric *AQP2/3* genes (Figure 2A) or complete loss of *AQP2* [65,66]. Notably, *AT1* was also absent or mutated in the strains above. It should be noted here that both genes may be prone to deletion/disruption due to their positions in the genome; *AT1* is located in a notoriously fragile, subtelomeric region, and *AQP2* is in tandem with *AQP3*. In the latter case, a DNA break can be repaired by recombination between adjacent and shared blocks of sequence identity, resulting in gene fusion or deletion. These studies extended the drug-resistance connection with *AQP2* and *AT1* to human-infective *T. b. gambiense* and *T. b. rhodesiense* parasites, to strains selected for either arsenical or amidine resistance [65,66], and also established that parasites lacking both transporters were both viable in a rodent model and could be transmitted by tsetse flies [65–67].

Loss of AQP2 Function Explains Melarsoprol Resistance in Patients

Melarsoprol remained effective in patients for some time but treatment failures were widespread by the late 1990s; relapse rates following treatment for gHAT were reported at 20–50%

in Angola [68], the Democratic Republic of the Congo [69], South Sudan [70], and Northern Uganda [71], and resistant isolates from rHAT patients were also reported in Tanzania [72] (Figure 1B). On the one hand, resistance could have emerged due to the drug pressure applied since the 1940s, either from mass pentamidine chemoprophylaxis or as a result of widespread melarsoprol treatment. On the other hand, treatment failure does not necessarily reflect the presence of drug-resistant parasites [73,74].

The laboratory studies above showed that uptake of both melarsoprol and pentamidine by trypanosomes is under the control of both the P2 adenosine transporter and AQP2 [65]. This drug transport certainly improves drug efficacy and potency against parasites versus host since similar transport mechanisms are not thought to operate in mammalian host cells. However, this comes with a risk of resistance because the transport mechanisms appear to be dispensable for parasite viability [51,62]. Thus, both *AQP2* and *AT1* emerged as candidates that may underpin drug resistance in patients, and the more recent emergence of AQP2 as a candidate presented an opportunity to revisit clinical isolates for further analysis.

Work with clinical isolates is generally more challenging than work on laboratory (adapted) strains since many trypanosomes from patients fail to grow readily in standard culture medium, some standard laboratory manipulations may be less efficient, and there are additional safety concerns; the laboratory strains that we typically work with are *T. brucei brucei,* which, unlike *T. b. gambiense* and *T. b rhodesiense*, are sensitive to lytic factors in human serum [75]. Thus, an important step in understanding clinical drug resistance is to assemble a panel of strains for analysis in the laboratory, which was carried out for *T. b. gambiense* strains from patients before treatment and after melarsoprol treatment failure [76].

The strains above were categorised as either melarsoprol-susceptible or melarsoprol-resistant, and the *AQP2* locus was then assessed, revealing specific loss of *AQP2*, or a novel chimeric *AQP2/3* gene (Figure 2A) in every resistant strain [77]. Notably, the *AT1* gene was intact in these isolates [77]. A subsequent study, with a larger number of clinical isolates, also linked chimeric *AQP2/3* genes, but not *AT1* mutation, to melarsoprol resistance, with chimeras in isolates dating back to 1974 [73]. Another important step was to demonstrate that reintroduction of *AQP2* into a clinical resistant *T. b. gambiense* isolate fully restored susceptibility to melarsoprol (and pentamidine) while the *AQP2/3* chimera failed to do so [78]. These experiments confirmed that, in patients, the *AQP2* gene alone can account for drug sensitivity, and for resistance when defective.

Studies on AQP2 illustrate the steps required to unequivocally demonstrate a clinical impact for a genetic mutation. First, parasite samples must be isolated from patients, ideally before and after they have relapsed after treatment. These isolates must then be propagated in the laboratory (may require adaptation both *in vivo* in a rodent model and *in vitro*) and shown to be resistant (compared to the initial isolate or a related susceptible isolate). A defect in the candidate gene must then be identified (in its expression or protein-coding sequence). The defect must then be corrected genetically and shown to restore drug sensitivity. This full set of criteria have been satisfied for the *AQP2* gene using a melarsoprol-relapsed patient isolate from the Democratic Republic of the Congo (see Figure 2A, 40 AT [after treatment] isolate, [78]). Distinct *AQP2/3* chimeras found in clinical isolates suggest that resistance arose independently on multiple occasions [73].

The situation has remained unresolved in the case of *AT1*. *AT1* is disrupted in some resistant strains selected in the laboratory [66] and appears to be mutated in some resistant clinical

isolates [54,73], but several resistant clinical isolates have wild-type copies of the *AT1* gene [53,73,77]. Thus, selection for melarsoprol or pentamidine resistance in the laboratory and melarsoprol resistance in clinical isolates always involves loss of AQP2 function. Current findings suggest no associated loss of virulence in patients or in transmission competence in the tsetse fly vector. Thus, *AQP2* status is a valuable genetic marker for predicting the presence of drug-resistant parasites. It may not be a reliable genetic marker for predicting clinical outcome, however, as a number of other factors can also lead to treatment failure [73,74].

Impact on Other Therapies

Fortunately, safer treatment options are now available for HAT, and a 2013 World Health Organisation report stated that 'today, there is no place for melarsoprol in the treatment of gambiense HAT, apart from the treatment of relapses' [79]. Results of trials of pairwise combinations of melarsoprol, eflornithine, and nifurtimox in Uganda were reported in 2006 [80], and nifurtimox–eflornithine emerged as the favoured combination [8]. Melarsoprol-resistant parasites do not display cross-resistance with eflornithine or nifurtimox, and the probability of resistance emerging to this combination would appear to be low. The evidence indicates, however, that prior drug pressure had a major impact on the prevalence of parasites that lack AQP2 circulating in patients (Figure 1B), which may impact other therapies. For example, since cross-resistance is typically observed in the laboratory, we might expect that AQP2-defective, melarsoprol-resistant parasites would also be associated with pentamidine treatment failure. This connection is not as straightforward as it might seem, however, since pentamidine failure may be due to parasites reinvading the bloodstream from the CNS. By contrast, the gHAT cure rate for pentamidine has remained at around 95% for decades [79]. This suggests that both stage diagnosis is effective and also that parasites lacking AQP2 are still effectively eliminated by pentamidine, likely because this drug is maintained at sufficiently high levels in serum to remain effective. We suspect that melarsoprol also remains effective against non-CNS parasites lacking AQP2. Thus, a low safety index can mean that even a small shift in resistance has a major clinical impact (such as in the case of melarsoprol against CNS-resident parasites) while a substantial shift in resistance can be overcome if a drug has a higher safety index (such as in the case of pentamidine against non-CNS-resident parasites).

AQP2 has an impact on another class of potential drugs, the trypanosome alternative oxidase (TAO) inhibitors. In contrast to the situation with pentamidine, parasites lacking AQP2 are hypersensitive to these inhibitors, due to the toxic accumulation of glycerol [81]. Indeed, the major livestock parasites that cause nagana are susceptible to the TAO inhibitor ascofuranone [82], and the absence of *AQP2* in *T. vivax* and *T. congolense* (Figure 2) may provide an explanation. This, combined with the absence of a functional homologue of the P2 adenosine transporter in *T. congolense* [83] may also explain why this parasite is naturally refractory to melarsoprol. Indeed, the *AT1* gene is found only in the *T. brucei* subspecies, and the absence of the P2 adenosine transporter in *T. congolense* and *T. vivax* likely also explains reduced sensitivity to diminazene, a veterinary drug typically used against these parasites [84].

Concluding Remarks and Future Perspectives

Melarsoprol and melarsoprol resistance have had a huge impact on HAT control over the past 70 years. Indeed, melarsoprol resistance represents the only example of widespread drug resistance in HAT patients, and the genetic mechanism has been established only recently. Several questions remain regarding melarsoprol's mode of action and potential resistance mechanisms, however (see Outstanding Questions), and we highlight some of them below.

Outstanding Questions

Can essential drug-uptake mechanisms be exploited to deliver more durable therapies?

Are all clinical cases of melarsoprol resistance due to *AQP2* defects? Does *AT1* make a contribution?

How do melarsoprol and pentamidine interact with AQP2? Do they pass through the channel or are they taken up by a mechanism involving endocytosis?

What are the resistance mechanisms to the previous clinically used arsenicals such as tryparsamide?

How do trivalent aromatic arsenicals induce rapid cell death by membrane lysis? Could this lead to a new target for drug discovery?

Will AQP2-defective and melarsoprol-resistant parasites differ in their susceptibility to new antitrypanosomal drugs?

Will rational combination therapies be used in the future to minimize, delay, or prevent drug resistance?

First, a general question that emerges from our understanding of melarsoprol resistance is whether drugs can be designed to be more durable if they enter parasites via essential transport processes; unfortunately, AQP2 is dispensable, as is *AT1* [51] and the eflornithine transporter [85]. Endocytosis is a potential delivery route and is essential for parasite survival. Indeed, suramin is taken up by receptor-mediated endocytosis [39,86], and endocytosis can be co-opted for the delivery of other drugs, even circumventing resistance mechanisms [87]. Second, relatively few melarsoprol-resistant clinical isolates have been examined in detail to date and it remains unclear whether *AT1, UBP1* [66], or other genes contribute to resistance. Further functional dissection of *AT1* mutations [88] and genetic screening of clinical isolates, combined with drug-sensitivity testing, will help to provide an answer to this question. Third, the question as to how melarsoprol and pentamidine are taken up into parasites by AQP2 persists. These drugs may be taken into parasites by transit through the AQP2 pore or by AQP2-mediated endocytosis. Both mechanisms have been proposed for pentamidine [64,84]. An observation potentially consistent with uptake by receptor-mediated endocytosis is potent inhibition of glycerol transport, involving pentamidine binding to AQP2 [64]. Unfortunately, membrane-associated proteins typically present greater challenges for structure determination. Currently, we can model the AQP2 structure (Figure 3) but direct structural determination, possibly with ligands bound, would help to resolve this question. Transport assays combined with expression in heterologous systems may also be informative.

To conclude, insights into drug mode of action and resistance mechanisms facilitate drug discovery and development and the design of optimal (combination) therapies and dosing strategies [89]. This knowledge can also be used for surveillance and, potentially, to tackle resistant parasites when they arise; studies on melarsoprol resistance have effectively illustrated these principles. We should now ensure that we also develop our understanding of the mode of action and potential resistance mechanisms for other current and new drugs under development against HAT, such as fexinidazole [90–92] and the oxaborole, acoziborole or SCYX-7158 [93–95], as well as for oxaboroles for the treatment of nagana [96]. We believe that this information will help to minimize, delay, or prevent future resistance in the clinic and will facilitate efforts to ultimately eliminate trypanosomiasis as a public health problem.

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Resources

[i]https://swissmodel.expasy.org

[ii]https://pymol.org/2/

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