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ABC transporters involved in drug resistance in human parasites

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Abstract

The ABC (ATP-binding cassette) protein superfamily is a ubiquitous and functionally versatile family of proteins that is conserved from archaea to humans. In eukaryotes, most of these proteins are implicated in the transport of a variety of molecules across cellular membranes, whereas the remaining ones are involved in biological processes unrelated to transport. The biological functions of several ABC proteins have been described in clinically important parasites and nematode worms and include vesicular trafficking, phospholipid movement, translation and drug resistance. This chapter reviews our current understanding of the role of ABC proteins in drug resistance and treatment failure in apicomplexan, trypanosomatid and amitochondriate parasites of medical relevance as well as in helminths.

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Introduction

ABC (ATP-binding cassette) transporters constitute one of the largest protein families in evolution. They were first discovered and studied in bacteria, but it soon appeared that these transport systems were ubiquitous, being present in all kingdoms of life. The structure of a typical ABC transporter consists of transmembrane segments that compose the TMD (transmembrane domain) and the Walker A and Walker B motifs that together form the NBD (nucleotide-binding domain). The NBD of ABC proteins possesses in addition a short highly conserved sequence just upstream of the Walker B site called the 'ABC signature' or 'C motif', which is characteristic of this superfamily. ABC transporters are either encoded as full transporters (TMD–NBD–TMD–NBD) or as half-transporters (TMD–NBD) that combine as homo- or hetero-dimers to create a functional unit (Figure 1). On the basis of homology in the NBD sequence, eukaryotic ABC proteins have been divided into eight subfamilies (ABCA–ABCH), and an extra subfamily named ABCI was noted in plants. ABC proteins are involved in various physiological activities and usually are involved in the transport of molecules across biological membranes through the hydrolysis of ATP. Known substrates include lipids, steroids, peptides, ions, heavy metals, toxins, nucleotides, carbohydrates and drugs. Some eukaryotic ABC proteins do not have TMDs, however, and are probably associated with functions unrelated to transport. Many ABC proteins in humans are of medical relevance owing to their relationship to genetic diseases either by a mutation or through an altered mode of expression. ABC proteins were also shown to play key roles in cellular detoxification of endo- and xeno-biotics, and the overexpression of a number of ABC proteins has been associated with the MDR (multidrug resistance) phenotype described in

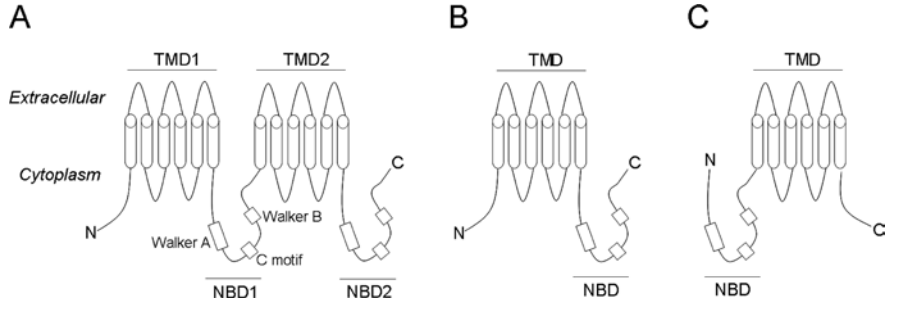

Figure 1. Structural organization of ABC transporters

Transmembrane α-helices are shown as cylinders. The Walker A, Walker B and C motifs are shown as boxes. (**A**) Most transporters of the ABCA, ABCB and ABCC subfamilies contain two TMDs fused to two NBDs (TMD1–NBD1–TMD2–NBD2). Not shown is the additional α-helix and long extracytoplasmic loop found at the N-terminus of ABCA proteins and the extra TMD (TMD0) found in some members of the ABCC subfamily. (**B**) Half-size ABC transporters having an N-terminal transmembrane domain fused with a single NBD (TMD–NBD). (**C**) Half-size ABC transporters displaying a reverse topology with the NBD located at the N-terminus of the protein (NBD–TMD).

cancer cells and in numerous pathogenic micro-organisms. The purpose of this chapter is to give an overview of the ABC transporters that have been formally reported or highly suspected to be involved in drug resistance in parasites infecting humans. Since space does not permit a fully comprehensive coverage of ABC proteins in all human parasites, we emphasize those with sufficient data linking them to drug resistance.

ABC transporters in protozoan parasites

Protozoan parasites are responsible for the majority of the mortality and morbidity associated with parasitic infections in humans. There are over 50 000 species of protozoa, of which one-fifth are parasitic. Protozoal infections are usually spread through faecal contamination of food or water or by the bite of an insect vector. Despite substantial efforts, no vaccine is yet available for preventing any of the major parasitic diseases. Chemotherapeutic drugs therefore constitute the main line of defence, but their efficacies are threatened by the emergence of resistance. The major protozoa causing debilitating sickness and loss of life in human medicine include apicomplexans (*Plasmodium*, *Toxoplasma* and *Cryptosporidium*), trypanosomatids (*Leishmania* and *Trypanosoma*) and amitochondriates (*Trichomonas*, *Giardia* and *Entamoeba*). Drug resistance is widespread in these parasitic infections and ABC transporters have been linked to treatment failure.

Malaria

Malaria is the world's most prevalent parasitic vector-borne disease, with more than 2.4 billion people being at risk in over 100 countries [1]. The infection is caused by *Plasmodium* species and is transmitted by *Anopheles* mosquitoes. Of the four *Plasmodium* species responsible for human malaria (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*), *P. falciparum* is the most deadly, killing over 1 million people each year [2].

Despite intensive research efforts, there are no effective vaccines for the prevention of *Plasmodium* infections. Several strategies were used for the control of malaria, including the use of insecticides to reduce the population of mosquito vectors and the use of CQ (chloroquine), a highly active and inexpensive 4-aminoquinoline, as a first-line treatment. CQ was the first-line anti-malarial drug for much of the 20th Century and its mode of action is well understood. Malaria parasites infect red blood cells, where they degrade haemoglobin present in the cytoplasm of the erythrocyte as a source of free amino acids. Haemoglobin degradation occurs in a specialized organelle of the parasite termed the DV (digestive food vacuole). Within this acidic compartment, the globin portion is hydrolysed to its constituent free amino acids and the potentially toxic haem moiety is dimerized to become a major component of the malarial pigment, also known as haemozoin. The anti-malarial CQ enters the DV by passive diffusion as an uncharged species and becomes trapped in the acidic organelle in its diprotonated form. The binding of CQ^{2+} to free

haem molecules in the DV prevents the crystallization reaction and leads to a massive membrane impairment causing the death of the parasites (reviewed in [3]).

CQ was long an effective drug, but the increase of CQ resistance to epidemic levels has forced many countries to change their national treatment protocols. Today, anti-malarials in clinical use include the CQ structurally related aryl amino alcohols quinine, mefloquine, lumefantrine and halofantrine, the antifolate drugs pyrimethamine, sulfadoxine, proguanil, chlorproguanil and dapsone and, finally, the artemisinin derivatives. Clinical treatment failures resulting from drug-resistant *P. falciparum* have been described for all of these replacement drugs, with the exception of artemisinins, and the use of combination therapies is required to decrease the likelihood of emergence of resistance. Artemisinin-based combination therapy is now the leading treatment regimen in several countries, but a decreased response observed at the Thai–Cambodian border (although without corresponding reductions on conventional *in vitro* susceptibility testing) suggests that treatment guidelines conformity and containment measures are urgently needed in order to preserve the efficacy of this last-resort therapy [4,5].

The mechanisms underlying drug resistance in *P. falciparum* are complex and involve various putative parasite transporters, some being part of the ABC superfamily. The sequencing and annotation of the 23 Mb *P. falciparum* genome in 2002 [6] provided the complete inventory of plasmodial ABC proteins [7] by revealing the presence of 15 *ABC* genes. A role in drug resistance has been described for a few of these ABC proteins and was shown to involve gene amplification, gene overexpression and/or gene mutations. The first *ABC* gene that was suspected to have a role in drug resistance in parasites was *pfmdr1*, a member of the ABCB subfamily localized on chromosome 5 of *P. falciparum* [8,9]. Initial studies indicated that CQ resistance in *P. falciparum* shared common features with the MDR phenotype conferred by the Pgp (P-glycoprotein)/MDR1 in tumour cells and that CQ resistance was associated with a verapamil-sensitive efflux of the drug in resistant parasites [10,11]. The cloning of the *pfmdr1* gene coding for Pgh1 (Pgp homologue 1) in subsequent studies suggested that similar MDR mechanisms were operating in tumour cells and parasites. It is now clear that *pfmdr1* is not the main CQ-resistance gene, however [12,13]. Indeed, the primary determinant of CQ resistance was mapped to chromosome 7 and corresponds to PfCRT (*P. falciparum* CQ-resistance transporter). PfCRT is localized to the food vacuole of *P. falciparum*, and mutations in its gene have been linked to CQ resistance (reviewed in [14]). It has recently been shown that the mutated version, but not the wild-type version, of the protein is able to transport CQ [15] away from its site of action.

A role for the ABC *pfmdr1* gene amplification in drug resistance came from the analysis of laboratory and field isolates in which the levels of cross-resistance between mefloquine, halofantrine and quinine were shown to increase proportionally with gene copy number (reviewed in [16]). In addition,

meta-analyses looking for an association between resistance markers and treatment outcome clearly established that the best overall predictor of mefloquine treatment failure was an increase in copy number of *pfmdr1* [17,18]. It is noteworthy that the susceptibility responses to aryl amino alcohols (including quinine, lumefantrine, mefloquine and halofantrine) and to artemisinins appear to be genetically linked. Indeed, gene-disruption experiments revealed that a 50% decrease in Pgh1 levels resulted in a 50% increased susceptibility to mefloquine, lumefantrine, halofantrine and quinine and to the structurally distinct artemisinin [19]. Interestingly, monitoring for the *pfmdr1* copy number as a surveillance tool for drug resistance in South-East Asia indicated a higher prevalence of *pfmdr1* amplification in parasites in western Cambodia than in eastern Cambodia [17], a finding consistent with a decreased efficacy of mefloquine and artesunate/mefloquine treatments at the Thai–Cambodian border [20], but not in eastern Cambodia [21].

Molecular markers such as gene polymorphisms can serve as useful tools in the surveillance for drug resistance in the field. The analysis of the *pfmdr1* sequence in different drug-sensitive and -resistant *P. falciparum* isolates revealed an association between polymorphic alleles containing key mutations at amino acids 86, 184, 1034, 1042 and 1246 of Pgh1 and drug resistance. Two haplotypes have initially been described, the K1 allele coding for a N86Y mutation in Pgh1 and the 7G8 allele carrying codon changes a positions 184, 1034, 1042 and 1246 of the protein (Table 1). Notably, a molecular epidemiological study correctly predicted the CQ-susceptibility status of a set of 34/36 clinical isolates based solely on the analysis of these polymor-

Table 1. Examples of *P. falciparum* lines displaying *pfmdr1* polymorphic alleles associated with altered drug susceptibilities.

D10, 3D7 and 7G8 are cloned parasite lines. The K1 isolate has been isolated in South East Asia. The CQ susceptibility status of the parasites is based on data from [22].

		Amino acid position in Pgh1				
Isolates	**CQ susceptibility**	**86**	**184**	**1034**	**1042**	**1246**
D10	Sensitive	Asparagine	Tyrosine	Serine	Asparagine	Aspartic acid
3D7	Sensitive	Asparagine	Tyrosine	Serine	Asparagine	Aspartic acid
K1	Resistant	Tyrosine	Tyrosine	Serine	Asparagine	Aspartic acid
7G8	Resistant	Asparagine	Phenylalanine	Cysteine	Aspartic acid	Tyrosine

phisms in the *pfmdr1* sequence [22]. A positive correlation between the N86Y polymorphism and resistance to CQ and amodiaquine was supported by several studies, although numerous studies failed to observe a complete association between the N86Y substitution and resistance to CQ (reviewed in [16]). It has not yet been possible to engineer allelic exchange at the 5′ end of *pfmdr1* for directly assessing the role of the N86Y mutation in susceptibility to anti-malarials [23]. Interestingly, the N86Y mutation in *pfmdr1* increases the resistance to CQ of *P. falciparum* parasites harbouring the K76T mutation in PfCRT and a non-random association between the two mutations was described in parasites from various endemic regions [24–26]. Since the K76T mutation appears to cause detrimental effects to the physiological function of the protein, this suggests either a co-operativity between Pgh1 and PfCRT in determining CQ resistance or a compensatory role for the *pfmdr1* polymorphism following PfCRT mutations. The *pfmdr1* N86Y polymorphism has also been associated with hypersensitivity to aryl amino alcohols and artemisinin in laboratory isolates [27] and clinical samples from Africa [28] and South-East Asia [29], a finding consistent with other studies reporting on the selection of parasites carrying a wild-type *pfmdr1* allele after lumefantrine and lumefantrine/artemether treatments [30–34].

Reverse genetic studies revealed an association between mutations at the 3′-end of *pfmdr1* and altered sensitivities to several anti-malarial drugs. Indeed, the triple Pgh1 mutations S1034C, N1042D and D1246Y were shown to induce hypersensitivity to mefloquine, halofantrine and artemisinins, but to confer resistance to quinine (reviewed in [14]). Clinical studies further correlated the presence of a mutation at positions 1034 and 1042 with an increased sensitivity to mefloquine and lumefantrine [29,35] and confirmed the importance of these substitutions as indicators of drug responsiveness. The heterologous expression of *pfmdr1* in *Xenopus* oocytes [36] demonstrated that the wild-type version of Pgh1 transports CQ and quinine, but not halofantrine, whereas mutant polymorphic Pgh1 variants transport halofantrine, but not quinine or CQ. These experiments clearly established an inverse correlation between certain anti-malarial drugs and suggested that selection for CQ resistance in the field should impair the parasite's ability to survive the use of aryl amino alcohols or artemisinins as an alternative treatment.

Clinical isolates sharing the same *pfcrt* and *pfmdr1* genotypes exhibit different degrees of sensitivity to CQ, suggesting that other genes are involved in CQ response. It has been suggested that multiple transporters could be involved in resistance, and the search for polymorphisms in 49 transporters revealed a significant association between polymorphisms at 11 loci (including PfCRT and the ABC transporters Pgh1, PfABCC1, PfABCB4 and PfABCH2) and resistance to CQ [37]. Other studies failed to support this observation, however [38,39], and the involvement of these transporters in drug resistance will require further investigation. Two point mutations at amino acids 191 (tyrosine to histidine) and 437 (alanine to serine) in the

ABCC transporter PfMRP (*P. falciparum* multidrug-resistance protein) 1 were associated with higher IC_{50} for CQ and quinine in *P. falciparum* field isolates [37]. Gene-disruption experiments have since confirmed a role for PfMRP1 in drug resistance as inactivation of its gene in drug-resistant parasites increased their susceptibility to CQ, quinine, piperaquine, primaquine and artemisinin [40]. Although the localization of PfMRP1 at the plasma membrane of *P. falciparum* [40,41] suggests a role in drug efflux, the precise molecular resistance mechanisms still need to be elucidated. Another polymorphism in PfMRP1 was recently associated with resistance to sulfadoxine/pyrimethamine treatments [42], but whether it has a direct role in sulfadoxine/pyrimethamine resistance remains to be established. A second member of the ABCC subfamily, PfMRP2, might be involved in drug resistance in malaria parasites, at least in *in vitro* cultures. This ABC transporter was localized at the plasma membrane of the parasite [41], and expression of both *pfmdr1* and *pfmrp2* was increased by mefloquine and CQ pressure in laboratory cultures of drug-sensitive and -resistant strains [43].

Finally, the overexpression of a second Pgp homologue, *pfmdr2* on chromosome 14, was linked to the parasite's response to mefloquine [44], and one study has linked the overexpression of *pfmdr2* to CQ resistance [45], although this was not observed by others [46]. The PfMDR2 protein shares homology with the heavy metal transporter HMT1 of *Schizosaccharomyces pombe* and was shown to play a role in heavy metal tolerance in *P. falciparum* [47].

Cryptosporidiosis

Cryptosporidiosis is a gastrointestinal disease usually self-limiting in immunocompetent individuals, but with life-threatening importance in immunocompromised patients, caused by the apicomplexan parasite *Cryptosporidium*. These parasites are transmitted either by the direct faecal–oral route or by the ingestion of food or water contaminated with parasite oocysts. Several drugs active against other apicomplexa were tested against *Cryptosporidium* spp., but none appeared to be effective, except for the aminoglycoside paromomycin and the PFOR (pyruvate–ferredoxin oxidoreductase) inhibitor nitazoxanide, albeit with modest benefit in treating this protozoal infection [48]. Different factors might explain the intrinsic drug resistance of *Cryptosporidium* spp. Indeed, the drugs currently available may not be able to target the divergent biochemical pathways of this parasite, whereas its unique biological niche inside the parasitophorous vacuole (an intracellular attachment zone separated from the host cytoplasm by an extensively folded membrane structure) in enterocytes may prevent drug access to their targets. The membrane of the parasitophorous vacuole is thought to protect the parasite against toxic drugs, and a role in drug extrusion for ABC transporters located at the host/parasite interface has been suggested [49]. Accordingly, the genome of *Cryptosporidium parvum* was searched

for ABC protein-coding genes in order to better understand the transport pathways operating between the parasite and its host. Bioinformatic analyses suggested the presence of 33 *ABC* genes in the genome of *C. parvum*, with only two ABCB proteins (CpABC3 and CpABC4) and two ABCC proteins (CpABC1 and CpABC2) characterized so far [50].

CpABC1 has been localized to the host/parasite boundary [51], which suggests a possible involvement in drug resistance or in metabolic interactions occurring between the parasite and its infected host cell during parasitic maturation. However, its ability to efflux drugs has not been fully addressed, mainly due to the lack of a stable transfection protocol for *C. parvum* (only transient transfections are possible at the moment). The gene *CpABC2* encodes a putative 1587-amino-acid protein [50] and was shown to be expressed at the RNA level only in sporozoites. CpABC2 is located at the apical end of sporozoites and could possibly be involved in invasion and/or in the very early stages of the establishment of infection [50]. Because of similarities with MRPs (multidrug-resistance proteins), it is thought that both CpABC1 and CpABC2 could be organic anion transporters and may be involved in the transport of endogenous and xenobiotic glutathione conjugates. Paromomycin was shown to significantly up-regulate the transcript levels of CpABC4 and CpABC1 [52], and it was postulated that these two ABC proteins, and especially CpABC4, might be key effectors in the intrinsic drug resistance of *C. parvum* [53]. However, a clear role for any ABC protein in drug resistance in *C. parvum* still needs to be demonstrated.

Leishmaniasis

Leishmaniasis is endemic in 88 countries in northern Africa, Asia, Latin America and the Middle East. More than 350 million people are at risk of infection, with ~500 000 new cases reported annually. The disease is spread by the bite of female sandflies in which the parasite survives and replicates as promastigotes. The promastigotes are transferred in humans from the bite of the sandfly and become ingested by macrophages, where they transform as amastigotes and multiply inside the phagolysosome. Clinical manifestations include three main groups of disorders: visceral leishmaniasis (kala-azar) that may lead to the individual's death if left untreated, cutaneous leishmaniasis (oriental sore) which often heals spontaneously, and mucocutaneous leishmaniasis which is often disfiguring. Since successful vaccination against *Leishmania* still remains a distant reality, chemotherapy is required, especially for the visceral form which is lethal if left untreated.

Treatments based on pentavalent antimonials (Sb^V) such as SSG (sodium stibogluconate) and meglumine antimoniate are the primary means against leishmaniasis and have been used for more than 60 years, despite their renal and cardiac toxicity. As a consequence of this long usage history, drug resistance to antimonials has reached epidemic levels in certain countries, particularly in northern Bihar (India) where more than 60% of patients do not

respond to antimonials [54]. There are few alternatives when treatment failures occur, namely amphotericin B and its various lipid preparations, paromomycin, and the phosphocholine analogue miltefosine. Miltefosine (under the brand name Impavido®) is currently the only effective oral treatment, but has to be given in combined therapy, since the development of resistance in monotherapy is readily achieved, at least *in vitro* [55]. Pentamidine represents another therapeutic option for leishmaniasis refractory to Sb^V treatments. A Phase 3 clinical trial has been successfully completed for the aminoglycoside paromomycin [56] which may become the only affordable drug in endemic countries in the near future. Also currently in clinical trials, the primaquine analogue sitamaquine would potentially represent the second orally effective anti-leishmanial drug, but will also need to be given in combined therapies because of resistance issues.

The availability of the genome sequence of *Leishmania* [57] has allowed an inventory of ABC transporters in this parasite, with 42 transporters belonging to all subfamilies described in eukaryotes (A–H) [58]. To date, only ABC transporters related to the ABCA, ABCB, ABCC and ABCG subfamilies have been functionally described in *Leishmania* spp. The *ABCA4* and *ABCA8* genes belonging to the ABCA subfamily of lipid traffickers have first been reported in *Leishmania tropica* and were localized to the plasma membrane and internal vesicles of the parasite [59]. These ABC proteins are involved in lipid translocation and infectivity, but not in resistance [60].

Mechanisms of resistance prevailing in clinical *Leishmania* isolates are only beginning to be unravelled [61,62], and consequently most of our knowledge is derived from studies performed with *in vitro*-selected resistant strains. Resistance to antimonials in *Leishmania* involves at least one member of the ABC superfamily, namely MRPA (ABCC3) alias PgpA [63]. MRPA is a member of the ABCC subclass which contains proteins involved in the transport of an array of structurally diverse compounds including organic anions such as glucuronide, glutathione (γ-glutamylcysteinyl glycine or GSH), sulfate and drugs conjugated to GSH (reviewed in [64]). Although GSH is present in *Leishmania*, TSH (trypanothione), which is composed of two GSH molecules linked by a spermidine moiety, is the main intracellular thiol [65]. The relationship between MRPA and the TSH biosynthesis pathway was derived from the analysis of *in vitro*-selected arsenite (As^{III})- and antimonite (Sb^{III})-resistant mutants in which an amplification and/or overexpression of two TSH biosynthetic enzymes [γ-GCS (γ-glutamylcysteine synthetase) and ODC (ornithine decarboxylase)] was observed (reviewed in [66]). The resulting increase in TSH levels in resistant parasites was shown to work synergistically with MRPA in conferring resistance to antimony by co-transfection experiments. Two other ABCC proteins in *Leishmania* (ABCC4 and ABCC5) were shown to confer a small increase in antimonial resistance when overexpressed, but their role in resistance seems to require a specific genetic background [67].

After nearly 20 years of *in vitro* studies punctuated with few reports describing work in clinical isolates, a global model for antimonial resistance in *Leishmania* was proposed (Figure 2). It is generally accepted that all pentavalent antimonials are prodrugs that require biological reduction to their trivalent form (Sb^{III}) in order to acquire anti-leishmanial activity [68]. It was later shown that AQP1 (aquaglyceroporin 1) localized at the parasite surface is responsible for the cellular uptake of the reduced trivalent forms of arsenic (As^{III}) and antimony (Sb^{III}) [69] and that a lower activity of this protein resulted in increased resistance [69,70]. Another resistance mechanism implicates the ABC transporter MRPA which confers resistance by sequestering drug–TSH conjugates within an intracellular organelle near the flagellar pocket, from where the antimonial target(s) are probably absent [71]. It is thought that the sequestered drugs are then expelled from the parasite through exocytosis occurring at the flagellar pocket. Finally, a protein localized at the parasite

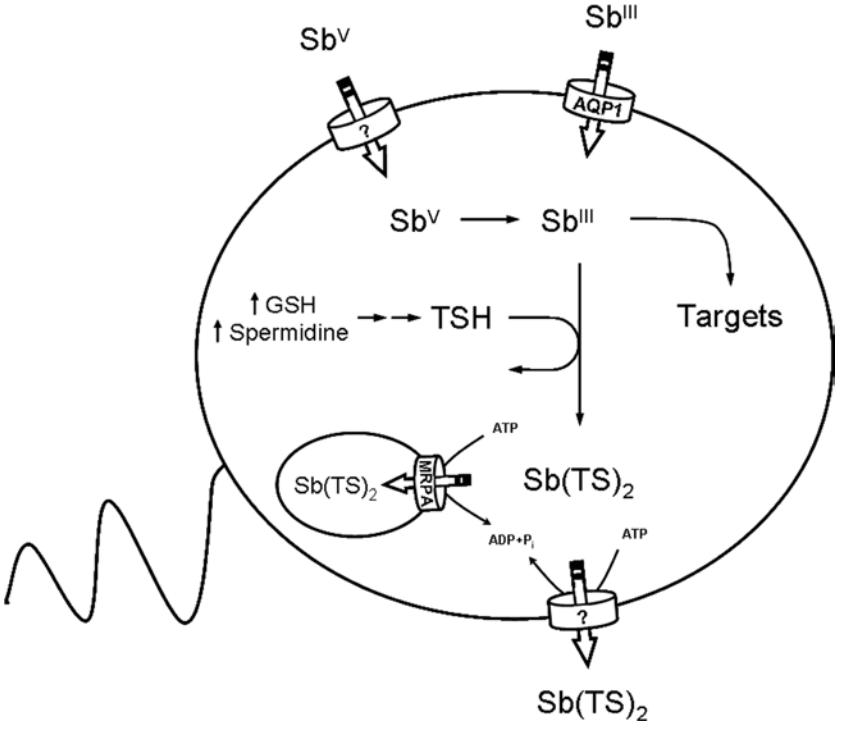

Figure 2. Mechanisms of resistance to antimony in *Leishmania*
AQP1 is responsible for the uptake of Sb^{III} in *Leishmania*, whereas the mode of entry of Sb^{V} is unknown. Once inside the cell, Sb^{V} is reduced to its biologically active trivalent form (Sb^{III}) by reductases. TSH levels are increased in antimony-resistant parasites due to the increased expression of the γ-GCS and ODC enzymes, respectively responsible for the rate-limiting steps of GSH and spermidine (a polyamine) synthesis. Sb^{III} is conjugated to TSH before being transported inside an intracellular detoxification vesicle by the ABC transporter MRPA or before being expelled by a plasma membrane thiol-X pump of unknown identity.

cell surface was reported to be responsible for the active efflux of conjugated antimonial compounds outside the parasite [72], although the identity of this plasma membrane thiol-X-pump remains elusive. It is important to note here that part of the *in vitro* antimonial-resistance model presented in Figure 2 was recently confirmed in natural antimony-resistant *Leishmania* clinical isolates recovered from patients unresponsive to SAG (sodium antimony gluconate). Indeed, an active role for MRPA, γ-GCS and ODC in resistance was confirmed [73,74], which tends to corroborate the resistance model proposed for *in vitro* resistant strains. However, mechanisms other than those described here may also operate in field isolates [62].

The drug pentamidine was used as an alternative in patients refractory to Sb^V [75]. Since resistance is now frequent and because better alternatives such as amphotericin B are available, pentamidine is no longer used against visceral leishmaniasis, although it is sometimes used against cutaneous and mucocutaneous forms. The mode of action of pentamidine is not clear, but it appears to interact with the parasite's single mitochondrion leading to disintegration of the kinetoplast DNA. The reduced accumulation of pentamidine in the mitochondrion [76] and/or increased efflux from the cell were proposed to be involved in pentamidine resistance. Molecularly, pentamidine resistance was shown to involve the overexpression of PRP1 (pentamidine-resistance protein 1; ABCC7), a member of the ABCC subfamily [77,78]. PRP1 was localized to an intracellular tubulovesicular compartment of the parasite that could connect to the mitochondrion, the target site of pentamidine. It was proposed that PRP1 may transport pentamidine into intracellular vesicles that would be later exocytosed through the flagellar pocket [79]. A second ABC transporter, the MDR1 (ABCB4) protein, was also localized to the multivesicular compartment [80], but this protein increased parasite susceptibility to pentamidine when overexpressed and might indirectly import pentamidine within the mitochondrion through the multivesicular element [81]. A lower import activity of MDR1 may therefore decrease parasite susceptibility to pentamidine. Interestingly, a novel synthetic flavonoid dimer called 'compound 9d', was shown to reverse pentamidine (as well as antimony, i.e. SSG) resistance, presumably by inhibiting some ABC-type transporters others than MDR1 [81,82]. It remains to be tested whether compound 9d could interfere with the activity of PRP1 or with other putative ABC proteins not yet characterized in order to reverse resistance.

Two independent mechanisms can confer miltefosine resistance in *Leishmania*: (i) a decrease in drug uptake, which is easily achieved by the inactivation or at least a decrease in the expression of any one of the two proteins known to be responsible for the miltefosine uptake, i.e. LMT (*Leishmania* miltefosine transporter) and its β-subunit LdRos3 that are co-expressed as a functional machinery at the parasite cell surface (reviewed in [83]); and (ii) an increase in drug efflux, mediated by the overexpression of membrane proteins such as ABC proteins [83,84]. Two members of the ABCG subfamily

were reported to be involved in miltefosine resistance in *Leishmania*, namely ABCG4 and ABCG6, whose localization is mainly to the parasite plasma membrane and flagellar pocket [84–86]. Both genes were also shown to be involved in sitamaquine resistance [84,85] and recently, ABCG6 was also implicated in chemoresistance to camptothecin (an uncompetitive topoisomerase 1B inhibitor), due to rapid efflux at the plasma membrane [86]. The physiological roles of ABCG4 and ABCG6 remain to be established, but the two half-transporters were reported to be involved in phospholipid trafficking at the plasma membrane (transbilayer lipid movement) as they were shown to reduce the accumulation of short-chain phospholipid analogues when overexpressed [85]. Since ABCG4 and ABCG6 show similar spectra of activity (i.e. similar substrates) and both proteins are half ABC transporters co-localizing mainly at the plasma membrane, it was speculated that these two ABC proteins may not only homodimerize, but also heterodimerize together to confer resistance or to extend the substrate specificity of each individual homodimer [85]. Finally, in an *L. tropica* strain selected for daunomycin resistance, MDR1 was overexpressed and this cell line was cross-resistant to miltefosine, suggesting that MDR1 may also contribute to miltefosine resistance, although this was not formally established.

Two ABC proteins in *Leishmania* were reported to cause resistance to drugs normally not used in anti-*Leishmania* regimens. The first one is ABCB2 (alias MDR2) which was identified in *Leishmania amazonensis* [87]. Indeed, transfection of ABCB2 in promastigotes showed significant resistance to 5-fluorouracil, but not to typical MDR1 (ABCB4) substrates. Resistance to 5-fluorouracil was due to a reduced accumulation of the drug, suggesting that ABCB2 is involved in extrusion of xenobiotics.

Trypanosomiasis

Trypanosomiasis is caused by *Trypanosoma brucei* or *Trypanosoma cruzi*, respectively responsible for HAT (human African trypanosomiasis) or 'sleeping sickness' in Africa and 'Chaga's disease' in Latin America. Genome projects of *T. brucei* and *T. cruzi* were completed in 2005 [88,89] and revealed multiple *ABC* transporter genes in both pathogens, although a role for members of this protein superfamily in drug resistance has been formally reported only in *T. brucei*.

HAT, or sleeping sickness, is transmitted to humans through the bites of infected tsetse flies that propagate the two causal *T. brucei* subspecies, *T.b. gambiense* and *T.b. rhodesiense*. The statistics of HAT in sub-Saharan Africa are grim: over 50 000 deaths per year and 60 million people at risk. In the early stage of HAT, parasites are found in both the blood and the lymphatic systems, with minor symptoms such as fever and malaise. When parasites cross the blood–brain barrier and reach the CNS (central nervous system), the disease then enters the later encephalitic stage characterized by neurological symptoms with severe headaches, somnolence and alterations to the sleeping

pattern. Without treatment, patients eventually fall into a coma or die within a few months. For early stages, pentamidine is used against *T.b. gambiense* and suramin against *T.b. rhodesiense* (pentamidine is ineffective against *T.b. rhodesiense*). For late-stage 'Gambian' HAT, eflornithine (a selective and irreversible inhibitor of ODC) or melarsoprol are equally effective. For patients with *T.b. rhodesiense* infections, late-stage cases need to be given the toxic arsenical melarsoprol, since eflornithine is ineffective.

Melarsoprol is still a useful drug, but, over the last decade, an increase in the rate of treatment failures has been observed [90]. Some of the possible causes of resistance have been elucidated. The first mechanism involved a decreased in import through the loss of the aminopurine transporter P2, encoded by the gene *TbAT1*. Deletion or inactivation of the *TbAT1* gene results in increased resistance to melarsoprol, both in culture and in the mouse model [91]. The second mechanism of melarsoprol resistance, at least under *in vitro* conditions, is conferred by the overexpression of the ABC transporter TbMRPA which confers 10-fold resistance to melarsoprol [92]. TbMRPA is a putative thiol-conjugate transporter localized at the plasma membrane that may function by pumping drug–TSH moieties out of the parasite. The role of TbMRPA in mediating resistance in a clinical context is still open to debate, however [93]. Overexpression of another ABC transporter, TbMRPE, gives 2–3-fold resistance to suramin, a marginal resistance to melarsoprol and confers sensitivity to pentamidine [92]. TbMRPE was localized in an intracellular compartment between the nucleus and kinetoplast, and thus probably confers resistance by a sequestration mechanism [92].

Amoebic dysentery/trichomoniasis/giardiasis

Over 1 billion individuals worldwide are infected with anaerobic or microaerophilic protozoa, with the most clinically important ones being *Entamoeba histolytica*, *Trichomonas vaginalis* and *Giardia lamblia* (syn. *duodenalis* or *intestinalis*) causing respectively amoebic dysentery, trichomoniasis and giardiasis. *E. histolytica* mainly invades the gut and liver, causing dysentery and liver abscesses, but can also invade the brain, lungs, skin and genitals. A high percentage of infected people worldwide are asymptomatic, but actively spread the infection. *T. vaginalis* is the causative agent of the most common non-viral human sexually transmitted disease, infecting the urogenital tract of both sexes. The infection causes vaginitis in women and urethritis/prostatitis in men with 25–50% of infected people being asymptomatic (World Health Organization data, 1995). *G. lamblia* is highly contagious and is still the most common cause of chronic diarrhoea in travellers. This parasite evades the host's immune response by undergoing antigenic variation, a process that exacerbates the development of chronic and recurrent infections. *G. lamblia* causes a variety of diarrhoeal diseases,

malabsorption problems and growth retardation in children, but many individuals are asymptomatic.

These three parasites are all susceptible to 5-nitroimidazole drugs such as MTZ (metronidazole) and tinidazole. MTZ is administrated as a prodrug and apparently enters the parasites by passive diffusion. Once inside the cell, the prodrug gains electrons at the nitro group and is converted into toxic nitro or nitroso anions or intermediates, such as hyroxylamines. Once generated, nitroso free radicals interact primarily with DNA, RNA and intracellular proteins, causing DNA strand breakage and disrupted transcription, ultimately leading to parasite cell death. Cross-resistance between all the currently used 5-nitroimidazole drugs is reported worldwide. Fortunately, prevalence rates of MTZ resistance in *E. histolytica* and *G. lamblia* are still low. Indeed, although MTZ resistance can be easily generated *in vitro*, MTZ-resistant parasites have been reported almost exclusively in acute *E. histolytica* infections and for some *Giardia* isolates purified and grown from patients with MTZ treatment failure. In contrast, resistance to MTZ is a major problem among *T. vaginalis* clinical strains and has been noted for years [94]. Decreased drug uptake or increased efflux were proposed as putative resistance mechanisms and may suggest a possible role for ABC transporters in protecting the parasites against MTZ.

The extent of MTZ resistance among clinical isolates of *T. vaginalis* probably comes from the scarcity of alternative treatments for patients suffering from recalcitrant trichomoniasis, a problem not found for *Entamoeba* infections that can be treated by other drugs such as emetine. The selection for emetine resistance in *E. histolytica* is associated with a verapamil-sensitive decrease in drug accumulation [95] and suggests a role for Pgp-like proteins in the drug-resistance phenotype [96]. Six Pgp-like genes have been described in *E. histolytica* (*Ehpgp1–Ehpgp6*), two of which are pseudogenes containing stop codons in the coding sequence (*Ehpgp3* and *Ehpgp4*) [97]. *EhPgp5* and *EhPgp6* gene expression is induced by emetine [98] and gene-transfection experiments have confirmed the role of EhPgp1 in conferring emetine resistance [99]. Intriguingly, trophozoites secrete EhPgps which are located in the plasma membrane and in vesicles [100], but the biological reasons for this are unknown.

In 2007, the genome of *T. vaginalis* was published [101] and its annotation revealed a large set of ABC transporters with 88 *ABC* genes. One of these genes, a half-transporter termed *TvPGP1*, is in two copies in the genome of *T. vaginalis*, but only one was detected in four of seven drug-resistant strains in one study [102]. Furthermore, *TvPGP1* was found to be overexpressed at levels ranging from 2- to 20-fold (although without gene amplification) in several MTZ-resistant strains isolated from patients infected with *T. vaginalis* that were unresponsive to recurrent treatments [102]. However, no correlation was found between the *Tvpgp1* mRNA level and the level of MTZ resistance [102], although more studies are needed to exclude the involvement of *Tvpgp1* in drug resistance.

ABC transporters in parasitic helminths

More than 2 billion people are infected with parasitic roundworms or flatworms, collectively known as helminth parasites. People become infected by coming into contact with soil, water or food that contains the eggs or larvae of young worms. Treatment for helminthic infections depends almost exclusively on three or four drugs: macrocyclic lactones (e.g. avermectins and milbemycins), benzimidazoles, imidazothiazoles (e.g. levamisole) and PZQ (praziquantel). The problem of anthelmintic resistance is much more acute in animals than in humans, but there are anecdotal reports describing a decrease in susceptibility or tolerance to anthelmintics in human parasites [103,104]. Although the genetics of drug resistance in helminths infecting humans is still at an early phase of research, studies point towards some ABC proteins, particularly Pgps which are much more abundant in helminths than in their mammalian counterparts. This section overviews two prevalent helminth infections for which some ABC members have been linked or highly suspected to contribute to the clinical resistance phenotype.

Schistosomiasis

Schistosomiasis is caused by parasitic blood flukes of the genus *Schistosoma*. This flatworm kills 280 000 people annually in sub-Saharan Africa alone, but the disease is also prevalent in the Middle East, in parts of South-East Asia and in Latin America. Schistosomiasis is probably the most important helminth infection in term of human morbidity; nearly 200 million people are infected and a further 800 million or so are at risk. Schistosomiasis infections occur through contacts with food or water contaminated with larvae. In the absence of treatment, internal haemorrhage may cause death. The current drug of choice against schistosomiasis is PZQ, a low-cost pyrazinoisoquinolone compound that is active against all schistosome species, although its mode of action has not been defined with certainty. Most evidence points to a role in the disruption of Ca^{2+} homoeostasis within the worm, but other mechanisms have also been proposed (reviewed in [105]). PZQ is the sole commercially available effective treatment against schistosomiasis and this precarious situation makes the prospect of emerging resistance particularly troubling. In fact, PZQ-resistant parasites can be easily selected for in the laboratory, and there were some reports within the last few years describing an increase in drug tolerance to PZQ in the field (reviewed in [105]).

In humans, PZQ has been shown to be an inhibitor of mammalian Pgps [106], and therefore a putative link between the activity of some schistosome ABC transporters and PZQ resistance was hypothesized. The annotated genomes of *Schistosoma mansoni* and *Schistosoma japonicum* have been released [107,108] and revealed the presence of several *ABC* genes. One of these, *SMDR2* in *S. mansoni*, was shown to be modulated both at the RNA and protein levels in response to sublethal PZQ exposure [109,110]. PZQ was

also able to modulate SMDR2 expression in several PZQ-selected laboratory strains [111] and the protein was expressed at higher levels in at least one schistosome isolate with reduced PZQ susceptibility [110]. These data argue for a role of SMDR2 in the development of resistance to PZQ in the field, although this needs to be confirmed by direct experimentation. A second Pgp-like gene in *S. mansoni*, *SMDR1*, has been described [112], but the encoded gene product does not seem to contribute to PZQ resistance.

Onchocerciasis

Onchocerciasis, also known as 'river blindness', is the second leading cause of blindness worldwide, after trachoma. The disease is caused by the filarial species *Onchocerca* and is spread by blackflies (genus *Simulium*), mostly in Africa. Infective larvae are inoculated into the skin during the bite of blackflies and migrate mainly through the skin to invade eyes. Of the 37 million people infected worldwide with this parasite, approximately 270 000 are blind and an additional 500 000 are visually impaired. IVM (ivermectin, a member of the avermectin class of drugs) is used as a safe drug for mass treatment of onchocerciasis, and MOX (moxidectin, a member of the milbemycin class of drugs) is currently being evaluated. The avermectins/milbemycins bind to glutamate- and GABA (γ-aminobutyric acid)-gated Cl^- channel subunit proteins, irreversibly opening the channels and causing a hyperpolarization of nerve or muscle cells, leading to worm paralysis [113]. Although highly suspected in patients failing to respond to IVM treatment or presenting suboptimal responses, the emergence of IVM resistance in *Onchocerca volvulus* was only unequivocally demonstrated recently in patients from Ghana that had received a long-term mass treatment regimen of IVM (for 6–18 years) under the scope of the national onchocerciasis control programme [114]. Concomitant with the acquisition of the IVM resistance trait, a lower reproductive rate in the female *O. volvulus* parasite was observed [115], as well as several genetic changes and polymorphisms in a number of genes, namely in the genes encoding the glutamate- and GABA-gated Cl^- channels, in the β-tubulin gene and in multiple *ABC* transporter genes [116]. In particular, specific alleles of the half-sized Pgp-like protein OvPLP in *O. volvulus*, were selected under IVM pressure [117]. In patients having received 13 IVM recurrent treatments, a significant amino acid change from valine to isoleucine has been described in the OvPLP protein [118]. Apart from OvPLP, IVM also seems to select for certain alleles in other ABC transporters [119,120]. Pgp inhibitors such as verapamil were shown to enhance the activity of IVMs/milbemycins [121] at least in the sheep nematode *Haemonchus contortus*, reinforcing the link between Pgp-like ABC proteins and resistance to these anthelmintics. IVM and MOX treatments select for a constitutive or inducible overexpression of at least five Pgps (*PgpA*, *PgpB*, *PgpC*, *PgpD* and *PgpE*) in adult *H. contortus* worms [116]. The constitutive overexpression

is likely to be the result of mutations in the regulatory sequences of these genes, whereas the inducible overexpression could be related to the induction of an oxidative stress by the drugs. Although the findings in veterinary helminths cannot be extrapolated directly to humans, the chances that similar phenomena might occur in *O. volvulus* are real. Similarly, in the model nematode *Caenorhabditis elegans*, increased expression of some ABC proteins (members of the MRP and Pgp subfamilies) was clearly associated with IVM resistance [122]. The heritable IVM resistance trait is now present in certain human nematode populations and may propagate rapidly under constant drug pressure. This could have dramatic consequences for the control of onchocerciasis, since there is currently no alternative drug available for community mass treatment of this parasitic disease.

Conclusion

Micro-organisms resistant to multiple anti-infective agents have increased around the world in the last decades. A multiplicity of resistance mechanisms has been detected in protozoa and helminths, an important one being transporter mutations and amplification/overexpression of transporter genes including members of the ABC superfamily. In this non-exhaustive review, we have described a number of pathogenic parasites in which ABC proteins were involved in the resistance strategies developed to counteract the action of current chemotherapies. Since parasites have the ability to become less susceptible to drugs when drug pressure is present, it is expected that the pharmacopoeia of anti-infectives will have to be continuously expanded if we wish to win the battle against parasites.

Summary

- *Eukaryotic ABC proteins have been divided into eight subfamilies (ABCA–ABCH), all of which are found in parasitic protozoa.*
- *Drug resistance is widespread in parasitic infections. A multiplicity of resistance mechanisms has been detected in protozoa and helminths, with an important one being transporter mutations and amplification/overexpression of transporter genes, including members of the ABC superfamily.*
- *ABC proteins have been involved in clinical drug resistance in parasitic protozoa, including CQ resistance in* Plasmodium falciparum *and antimony resistance in* Leishmania.
- *A decrease in susceptibility or tolerance to anthelmintics in human parasites has been observed in the last decade and studies point towards some ABC proteins, particularly Pgps. Pgps are suspected to be implicated in resistance to PZQ in* Schistosoma *and IVM in* Onchocerca.

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