Journal of Global Antimicrobial Resistance 18 (2019) 1–11

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Review

Drug Resistance in Protozoan Parasites: An Incessant Wrestle for Survival

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ARTICLE INFO

Article history:
Received 30 July 2018
Received in revised form 4 January 2019
Accepted 15 January 2019
Available online 24 January 2019

Keywords:
ABC transporters
Drug resistance
Protozoan parasites

ABSTRACT

Nowadays, drug resistance in parasites is considered to be one of the foremost concerns in health and disease management. It is interconnected worldwide and undermines the health of millions of people, threatening to grow worse. Unfortunately, it does not receive serious attention from every corner of society. Consequently, drug resistance in parasites is gradually complicating and challenging the treatment of parasitic diseases. In this context, we have dedicated ourselves to review the incidence of drug resistance in the protozoan parasites *Plasmodium*, *Leishmania*, *Trypanosoma*, *Entamoeba* and *Toxoplasma gondii*. Moreover, understanding the role of ATP-binding cassette (ABC) transporters in drug resistance is essential in the control of parasitic diseases. Therefore, we also focused on the involvement of ABC transporters in drug resistance, which will be a superior approach to find ways for better regulation of diseases caused by parasitic infections.

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Contents

1. Introduction

The struggle for existence is fierce among every living being in this world. The strategies of micro-organisms to feed and multiply on their respective host bedazzle us. Parasite growth and multiplication within the host are associated with the manifestation of specific diseases, and their survival strategies are coupled

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https://doi.org/10.1016/j.jgar.2019.01.023
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with drug resistance. Diverse pathogens, namely bacteria, protozoa, fungi and viruses, exhibit resistance [1]. Apparently, all life forms are equipped with one or more mechanism(s) for fighting adverse situation(s) that they face which have existed from the time of the 'origin of life'. Despite widespread reports of clinical and experimental drug resistance, we are uninformed on the subject of the evolution of drug resistance in detail. It is therefore a global problem requiring immediate serious attention with versatile programmes and organisations for sharing of scientific knowledge on drug resistance.

Parasitic infections are very patchy in nature, which has a strong influence on the selection of resistance. Over the past several decades, the perilous situation of drug resistance has been noticed with increasing frequency. *Plasmodium*, *Leishmania*, *Trypanosoma*, *Entamoeba* and *Toxoplasma gondii* show intermittent evidence of resistance to their respective clinically available drugs (Table 1) [2–5]. Drug resistance associated with the treatment of human parasitic diseases is widespread. However, in our current situation of ignorance, we usually cannot identify the crucial factors mediating drug resistance. The genes responsible for drug resistance are not yet fully identified in most parasites. Although some drug resistance-associated genes have been identified, sometimes it is complicated to understand the complex life cycles of parasites and vectors as well as vector–parasite interactions. Thus, difficulties arise in predicting the trajectory of the evolution of drug resistance.

ATP-binding cassette (ABC) transporters have a role in parasite drug resistance and are indispensable components for the maintenance of cellular functions in all eukaryotic and prokaryotic species, including parasites. The ABC transporter protein superfamily encompasses a large, ubiquitous and functionally versatile family of proteins that have been well documented in clinically important pathogenic parasites and are also involved in a broad variety of transport processes such as nutrient uptake as well as diverse cellular processes such as maintenance of osmotic homeostasis, lipid trafficking, phospholipid movement and especially in drug resistance machinery. ABC transporters are categorised into several subfamilies and in most eukaryotic classes the typical ABC transporter consists of four structural domains on the same polypeptide chain, as detailed in Section 3. The majority of ABC proteins associated with drug resistance belong to the family of multidrug resistance (MDR) proteins and multidrug resistance-associated proteins (MRPs) [6]. Although the function of ABC transporters has been implicated in the physiological functions of parasites, until now little is known about the role of ABC drug transporters and related proteins in the drug resistance mechanisms of epidemiologically and clinically important parasites. Therefore, the contribution of ABC transporters is now prompting areas of research, mainly in the field of drug resistance.

The subject of drug resistance is exceedingly large, but we have presented it as a mere chapter. Therefore, we will briefly discuss the phenomena of drug resistance in selective parasitic protozoa (*Plasmodium*, *Leishmania*, *Trypanosoma*, *Entamoeba* and *T. gondii)* that illustrate well our current understanding of the resistance issues and the factors, especially ABC transporters, that have been predominantly linked to this event. However, we appreciate that further research, documentation and awareness among people of all classes of society is required not only for combating diseases but also to improve public health.

2. Drug resistance in parasites

Due to lack of effective vaccines, chemotherapy remains the mainstay of defence against parasites responsible for a wide spectrum of diseases in humans. Unfortunately, resistance has developed in the respective parasites for the majority of clinically used drugs against these diseases. Drug resistance mechanisms in parasites are very inconsistent in terms of the nature and life cycle of parasites [7]. In general, mechanisms of drug resistance in pathogenic parasites depend on a variety of mechanisms consisting of augmented drug efflux, reduced drug uptake, mutation events in targeted enzymes, metabolic upregulation, and deficiency of the target sites for antiparasitic drugs, etc. Apart from these issues, various genetic mechanisms including gene deletions, gene mutations and, most importantly, chimerisation of genes also play a crucial role in drug resistance mechanisms [8]. Incidentally, the formation of chimeric genes in drug resistance is not yet well established in all pathogenic parasites; to the best of our knowledge from a literature search, it is greatly studied in *Trypanosoma* compared with other parasites. However, in some parasites resistance appears through many other unknown mechanisms and resistance to most antiparasitic drugs in the majority of parasites is now widespread. The following sections of this review article discuss the drug resistance of the protozoan parasites *Plasmodium*, *Leishmania*, *Trypanosoma*, *Entamoeba* and *T. gondii*.

2.1. Plasmodium *spp.*

Among the five *Plasmodium* spp. (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*), *P. falciparum* is the most prevalent species responsible for human malaria worldwide [9]. The global battle against malaria was started in 1898 by Ross and Grassi with the finding that infected mosquitoes act as a vector of the disease [10]. The alkaloid quinine is one of the oldest antimalarial agents isolated from the bark of the *Cinchona* (quina-quina) tree in 1820 [11]. However, several reports have described the emergence of quinine resistance in *P. falciparum*. In 1930, discovery of the synthetic drug chloroquine brought a great revolution in malaria treatment and its use was at its peak in the 1950s to 1990s [12]. However, within a decade chloroquine-resistant malaria arose. Subsequently, sulfadoxine/pyrimethamine, a combination of the sulfa drug sulfadoxine and the antifolate drug pyrimethamine, was launched as a synergistic antimalarial therapeutic in 1967 and effectively replaced chloroquine. However, resistance to sulfadoxine/pyrimethamine spread rapidly and now occurs at a very high frequency in the major malarious regions [13]. Resistance to antifolates is caused by point mutations in the *P. falciparum* genes

Table 1
Diseases associated with protozoan parasites and common drug resistances.

Parasite	Common disease	Drug resistance
Leishmania spp.	Leishmaniasis	Pentavalent antimonials, pentamidine, miltefosine, paromomycin and amphotericin B
Plasmodium spp.	Malaria	Chloroquine, artemisinin, sulfadoxine/pyrimethamine, piperaquine, mefloquine, amodiaquine and atovaquone
Trypanosoma spp.	Trypanosomiasis	Melarsoprol, suramin, nifurtimox, nitrofuran and benznidazole
Entamoeba spp.	Amoebiasis	Metronidazole, trifluoromethionine and emetine
Toxoplasma gondii	Toxoplasmosis	Artemisinin, atovaquone and sulfadiazine

dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthase (*Pfdhps*), which encode the two key enzymes involved in the folate biosynthesis pathway targeted by antifolate drugs. In addition, resistance to sulfadoxine is caused by point mutations in the *Pfdhps* gene [14]. Resistance to the important antifolate drug pyrimethamine is due to the point mutation S108N and is further increased by mutations A16V, N51I, C59R and I164L in the *Pfdhfr* gene [15]. On the other hand, resistance to sulfadoxine is primarily caused by the same kind of mutation event S436A and A437G in the *Pfdhps* gene, which is then augmented by mutations K540E, A581G and A613S/T [14]. Sulfadoxine/pyrimethamine resistance is caused by combined mutations in these two genes. Although chloroquine resistance compels the extensive use of antifolates, antifolate resistance also appeared very quickly in a parallel way. At that point, use of combination therapy came into focus in malaria treatment. Subsequently, artemisinin-based combination therapy (ACT), i.e. an artemisinin derivative plus other long-circulating drugs (artemether, lumefantrine, artesunate, mefloquine, etc.), has been widely used as an effective alternative to treat malaria [16]. In addition, curcumin combined with artemisinin has also been reported as a potential ACT against malaria infection [17]. Artemisinin, a compound derived from *Artemisia annua* L. leaf, depolarises the mitochondrial membrane and generates reactive oxygen species in parasites but not in mammalian mitochondria [18]. Regrettably, resistance to artemisinin and ACTs has also been reported via mutations in *P. falciparum* proteins, e.g. Ca^{2+}-ATPase PfATP6 and PfMDR1, respectively [16,19]. In brief, *P. falciparum* has developed resistance to almost all clinically existing antimalarial drugs [13,20].

Mutations involved in drug resistance to various antimalarials are found in several genes, including chloroquine resistance transporter (*Pfcrt*), multidrug resistance protein 1 (*Pfmdr1*), *Pfdhps*, *Pfdhfr* and cytochrome b1 (*Pfcytb1*). Experimental studies also revealed that mutations in transporter proteins, mainly PfMDR1 (located at chromosome 5) and other transporter proteins such as K13 propeller, also play a role in resistance to antimalarial drugs [13,21]. Mutations in the *Pfcrt* gene (chromosome 7) and genetic polymorphism in ms4760 alleles of the Na^+/H^+ exchanger-encoding gene *Pfnhe1* (chromosome 13) cause quinine resistance [13,22]. Other mutations in the genes *Pfmdr1* and *Pfcrt* (K76T mutation) confer resistance to chloroquine and amodiaquine. Another mechanism implicated in chloroquine resistance is similar mutation events in the protein PfCG2, which significantly acts at the time of haemoglobin uptake [16,23,24]. A similar mutation phenomenon has also been noted in *Pfdhps* and *Pfcytb* genes conferring resistance to sulfadoxine and atovaquone, respectively [23,25]. In another situation, a single mutation in the *Pfdhfr* gene was found to be responsible for pyrimethamine, cycloguanil and proguanil resistance [26,27]. However, therapeutic failure of the available antimalarials has compelled researchers to develop more modified agents for the treatment of malaria infection [28]. Moreover, quinolone-3-diarylethers are being suggested as efficient antimalarial agents as a substitute for atovaquone [29]. Very recently, a solid drug nanoparticle-based formulation of atovaquone was also proven to be efficient in the resistance issue [19]. As malaria parasites frequently develop resistance to the known available antimalarial drugs as well as novel antimalarial agents, the therapeutic strategy of new antimalarials should focus on the mechanisms of action of resistant parasites.

2.2. Leishmania *spp.*

Leishmania is a genus of trypanosomes that are the causative agent of the complex disease leishmaniasis. In humans, clinical manifestations of the disease range from simple self-healing cutaneous lesions to life-threatening visceral leishmaniasis (VL) [30]. For over than the last 60 years, antimonial compounds, including meglumine antimoniate and sodium stibogluconate, remained the mainstay of treatment for leishmaniasis. However, the mechanism of action was not fully clear until recently. In 1920, Prof. U.N. Bramhachari first synthesised pentavalent antimony, which was applied as a chemotherapeutic agent for the treatment of Indian VL patients [31]. The pentavalent form of antimony does not have efficacy to prevent *Leishmania* spp. growth. However, reduction of the pentavalent form to the trivalent form occurs inside macrophages using thiols from the parasite and host cell surface, and the trivalent form has parasiticidal properties [32]. Therefore, thiol metabolism and increased intracellular thiol levels play significant roles in emerging antimonial resistance. Although pentavalent antimonials are still first-line drugs against leishmaniasis in several countries, they are no longer recommended on the Indian subcontinent owing to the appearance of drug resistance to antimony [33]. Despite the toxicity of the second-line drugs, e.g. pentamidine and amphotericin B (AmB), they are used in the treatment of antimony-unresponsive and human immunodeficiency virus (HIV)–*Leishmania* co-infected subjects [34,35]. AmB has been established as a very successful chemotherapeutic agent against VL in India. However, due to its frequent use, drug resistance issues have emerged. In addition, more extensive use of lipid formulations of AmB, which have a longer half-life, could increase resistance associated with this drug [36]. A report by Purkait et al. suggested that altered membrane composition, upregulated thiol metabolic pathway and ABC transporters are factors responsible for AmB resistance in clinical *Leishmania* isolates [37]. Among several new drugs that are still under study, the plant-derived carbazole alkaloid mahanine has very recently been well established against *Leishmania* parasites [38]. AmB resistance is due to a defect in the transmethylation process of the enzyme S-adenosyl-l-methionine:C-24-Δ-sterol methyltransferase (SCMT), which is responsible for sterol methylation at the C-24 position [39]. Alteration of the intracellular level of the drug as well as the effectiveness of the drug to affect the target site is commonly observed in a wide variety of organisms, including the parasite *Leishmania*. A number of bases consisting of ABC transporters, upregulated thiol metabolism and changes of membrane composition also play a pivotal role in conferring resistance to AmB in clinically isolated *Leishmania donovani*. Excepting these issues, AmB resistance has also been found to emerge in *Leishmania* owing to lack of the appropriate target site for action [8]. AmB preferentially binds to ergosterol, the major sterol present in the membrane of the parasite *Leishmania*. Resistance to AmB is significantly correlated with alteration of the sterol composition of the parasite membrane. In resistant *Leishmania*, changes in the sterol profile of the parasite plasma membrane are caused by the replacement of cholesta-5,7,24-trien-3β-ol and consequently result in decreased AmB uptake [37,40]. Very recently, innovative approaches consisting of a high-throughput analysis strategy are allowing a better understanding of parasite biology and detection of novel sites for drug targets as well as primary resistance mechanisms for improved treatment against *Leishmania* infection [41].

In geographical areas with antimony resistance, use of the first oral drug miltefosine results in high cure rates against *Leishmania* infection especially in cases of visceral and cutaneous leishmaniasis. Miltefosine, a simple molecule, is reasonably safe and is highly efficient when used properly. However, major drawbacks of miltefosine are its teratogenic potential and significantly long half-life, which increases the probability of the rapid emergence of resistance. The leishmanicidal mode of action of miltefosine is achieved by intracellular drug accumulation. Accordingly, decreased drug accumulation results in miltefosine resistance by two independent mechanisms, including an increase in drug efflux by

overexpression of the P-glycoprotein (P-gp) ABC transporter and a decrease in drug uptake by inactivation of the miltefosine transporter protein LdMT [39,42]. In addition, inactivation of LdRos3 (a β subunit of LdMT) also confers miltefosine resistance following the reduction of miltefosine accumulation. Another important leishmanicidal agent is methotrexate, which significantly acts as an antimetabolite cum antifolate drug and inhibits the enzyme dihydrofolate reductase (DHFR) responsible for conversion of dihydrofolate to the active form tetrahydrofolate. *Leishmania*, being a folate auxotroph, depends on uptake of folate from the environment. Folate and pterin metabolic pathways are significantly associated with methotrexate resistance machinery in *Leishmania* [43]. Leishmanial resistance to the antifolate drug methotrexate can be explained by several mechanisms, including diminished uptake of drug owing to modulation of the expression of a series of folate transporters (FTs), point mutation of dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) and amplification of the *ptr1* gene encoding the enzyme pteridine reductase (PTR1). Notably, in *Leishmania* the PTR1 enzyme carries out a salvage pathway of folate synthesis by pteridines and thus PTR1 is used to bypass the classical pathway for folate metabolism [44]. Hence, blocking the enzyme DHFR-TS by methotrexate, another pathway of folate metabolism, i.e. tetrahydrofolate synthesis by pteridine reductase, is initiated [44]. Thus, methotrexate resistance in *Leishmania* is caused by reduced drug accumulation through upregulation of an alternative metabolic pathway [8]. *Leishmania* contain numerous well documented pteridine transporter genes and some of these genes are found to be deleted in methotrexate-resistant *Leishmania*. It is also reported that the biopterin transporter BT1 in *Leishmania* was found to be overexpressed in all methotrexate-resistant cells with markedly reduced folate uptake [43]. This highly overexpressed BT1 can selectively transport biopterin and folate into cells but does not transport methotrexate. However, transporters BT1 and FT1 are well documented in in vitro resistance to methotrexate. In addition, alteration to the degree of polyglutamylation of folates as well as methotrexate extensively contributes to methotrexate resistance in *Leishmania*, followed by efflux of the drug with reduced retention [45]. Now, most of the clinically available antileishmanial drugs have reported resistance in *Leishmania* spp.

2.3. Trypanosoma *spp.*

Trypanosoma, a haemoflagellate unicellular parasitic protozoa belonging to the group of kinetoplastids, causes numerous diseases in vertebrates, so-called trypanosomiasis. Among different *Trypanosoma* spp., *Trypanosoma brucei* and *Trypanosoma cruzi* are the most crucial with respect to human health. In humans, trypanosomiasis includes human African trypanosomiasis, also known as African sleeping sickness, and Chagas disease caused by *T. brucei* and *T. cruzi*, respectively. Primary chemotherapeutics for sleeping sickness depends on the melaminophenyl arsenical derivatives melarsoprol, suramin and diamidines such as pentamidine. Unfortunately, resistance to the most clinically effective and well-known drugs melarsoprol and diamidines has appeared in *T. brucei* [46]. Among the antitrypanosomal drugs, the mechanism of resistance to melarsoprol is the most studied in the laboratory. The drug resistance scenario associated with the use of diamidines and pentamidine in the treatment of trypanosomiasis was recently reviewed in great detail [47,48]. On the other hand, the most frequently used drugs for the treatment of Chagas disease are nitroheterocyclic compounds such as nifurtimox, nitrofuran and benznidazole [49]. Recently, combination of benznidazole and posaconazole has been considered as an efficient therapy against *T. cruzi* infection [50]. Various types of ABC transporters play a key role in drug resistance in *T. cruzi*. Apart from role of ABC transporters in *T. cruzi*, the proline transporter TcAAP609 confers resistance to nifurtimox and benznidazole [51]. The *T. brucei* P2 aminopurine transporter encoded by the *TbAT1* gene has been associated with the transport system. Aside from the transport of adenosine and adenine, *T. brucei* P2 aminopurine transporter has also been implicated in the transport of trypanocidal diamidine drugs, e.g. pentamidine, diminazene and the melaminophenyl arsenical derivative melarsoprol [52,53]. In *T. brucei*, aquaglyceroporins (TbAQPs) are well established to transport water and glycerol with participation in the drug resistance mechanism [54]. TbAQP2 is restricted to the flagellar pocket of the parasite and is accountable for the high-affinity uptake of melarsoprol and pentamidine in *T. brucei* [54,55]. The mechanism behind resistance is mutation in *TbAQP2* and especially chimera formation between *TbAQP2* and *TbAQP3* genes. Indeed, it is supposed that point mutation of the *TbAT1* gene at the *TbAQP2* locus opposes melarsoprol accumulation resulting in treatment failure against these parasites [56]. Chiefly, different types of chimera gene formation by *TbAQP2* and *TbAQP3* in different trypanosomal strains have been found to be involved in melarsoprol–pentamidine cross-resistance [57]. Alternatively, pentamidine resistance in the TbR25 cell line (pentamidine-resistant clonal cell line) is due to deletion of *AQP2* in one allele as well as the formation of the *AQP3*/*AQP2* chimera in the other allele [58]. Likewise, in *T. brucei* strain B48, formation of the chimeric gene *TbAQP2-3*$_{(569\text{-}841)}$ was found to be responsible for inactivation of transport and sensitivity to the drug pentamidine, thus resulting in resistance to this drug. Furthermore, another chimeric gene *TbAQP2-3*$_{(814)}$ has been found in clinically isolated endemic melarsoprol-resistant strains [55]. Recently, the nifurtimox resistance mechanisms in *T. brucei* have also been evaluated using a genome-scale RNA interference target sequencing (RIT-seq) screen [59]. A total of 75 genes were identified as being associated with nifurtimox resistance, with 8 genes found to have a strong association [60]. The resistance ability of *T. cruzi* against the drug benznidazole was demonstrated by generating resistant clones [61,62]. In view of this, the mechanisms of action of clinically available trypanocides should be sought more against resistant *Trypanosoma*.

2.4. Entamoeba histolytica

Entamoeba histolytica is the protozoan parasite responsible for human amoebiasis. Metronidazole derivatives and emetine drugs are mainly used to treat and control amoebiasis [63]. Although drug resistance in amoebiasis was previously very rare, in 1985 the first drug-resistant mutants of *E. histolytica* were isolated [64]. Metronidazole has also been widely used to treat anaerobic or microaerophilic micro-organisms causing infectious diseases [63]. The multidrug resistance mechanism in *E. histolytica* is mostly due to expression of the multidrug resistance proteins EhPgp1, EhPgp5 and EhPgp6 [65,66]. The antibiotic paromomycin was also found to be effective against *E. histolytica*, although its mechanism of action is more complex [67,68]. In *E. histolytica*, flavodoxin and ferredoxin act as electron acceptors of pyruvate:ferredoxin oxidoreductase to convert pyruvate to acetyl coenzyme A (acetyl-CoA) [64]. Drug enters the cell through passive diffusion, where a nitro group is consequently reduced by ferredoxin or flavodoxin to reactive cytotoxic nitro radicals. Metronidazole resistance in *E. histolytica* is strongly connected with the upregulation of iron-containing superoxide dismutase and peroxiredoxin and this resistance property is also linked with the downregulation of ferredoxin 1 and flavin reductase [69]. Presently, trifluoromethionine has been revealed as an effective amoebicide against *E. histolytica* [70]. However, its mechanism of resistance in *E. histolytica* has also been established [71,72]. The mechanisms of action of flavonoids, e.g. kaempferol, as anti-amoebic agents were also studied by Bolaños

et al. in 2015 who demonstrated their anti-amoebic effects via alteration of cytoskeletal function [73]. Therefore, more mechanisms of action and molecules implicated in *E. histolytica* resistance should be considered to overcome treatment failure against human amoebiasis.

2.5. Toxoplasma gondii

The sporozoan parasite *T. gondii*, a well-known tissue parasite in humans, belongs to the phylum Apicomplexa [74]. It can cause severe life-threatening disease, particularly in immunocompromised patients and congenitally affected children [75]. The most effective and available treatments against *T. gondii* include pyrimethamine and sulfadiazine [68]. First-line therapy against toxoplasmosis consists of the combination of sulfadiazine and pyrimethamine, with the addition of leucovorin to avoid haematological toxicity [76]. However, due to severe side effect of these agents, limitations arise in their application. Several treatment failures have been reported against toxoplasmic encephalitis, toxoplasmic chorioretinitis and congenital toxoplasmosis [77]. Currently, artemisinin derivatives have also been established as new and efficient anti-toxoplasmosis therapeutics [68]. Pyrimethamine and sulfadiazine, respectively, inhibit dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) enzymes, providing a synergistic blockade of the folate biosynthetic pathway [76]. These two enzymes are responsible for the synthesis of folate compounds that are crucial for survival as well as replication of the parasite [77]. Studies have revealed that sulfonamide resistance is also associated with mutations in the *dhps* gene [78]. Therapeutic failure of toxoplasmosis has been reported especially in cases of immunocompromised individuals as well as congenital transmission. These therapeutic failures followed by the development of drug-resistant parasites may be associated with host factors such as malabsorption or intolerance of the drug and/or parasitic factors such as variability in drug susceptibility between genetically different parasite strains [78]. Apart from the drug exposure issue, mutations in the *dhps* gene also confer resistance in *T. gondii*. Although resistance to sulfonamides emerges through mutations in the *dhps* gene, research is also ongoing to find alternative mechanisms of drug resistance. Such genetic information of atovaquone resistance has not yet been determined in clinical isolates, but in vitro resistance with chemically induced mutations by means of chemical mutagenesis has been observed [79]. Some evidence suggests several inconsistencies in the susceptibilities of *T. gondii* strains both to atovaquone and pyrimethamine, but no such clear drug resistance evidence was observed [78]. Doliwa et al. developed two sulfadiazine-resistant strains (RH-R^{SDZ} and ME-49-R^{SDZ}) to illustrate sulfadiazine resistance mechanisms in *T. gondii* [80]. The mechanism of resistance to sulfadiazine in *T. gondii* is not clear owing to the absence of overexpression/polymorphism in genes in the case of drug resistance and ABC transporters [81]. However, polymorphisms in *dhps* exons have been reported recently in the case of sulfadiazine-resistant strains [82]. Hence, the governing mechanism for this evolution is still under study. Owing to drug resistance, researchers switched their attention recently to parasitophorous major surface antigens, namely SAG1, *T. gondii* dense granule antigen (GRA) 4 and GRA 7, and *T. gondii* rhoptry protein (ROP) 2 as innovative and suitable candidates for the development of a vaccine against toxoplasmosis [83,84].

3. ATP-binding cassette (ABC) transporters in parasites

The ABC transported superfamily is a cluster of proteins belonging to one of the largest classes of membrane transport proteins [85]. Transport proteins responsible for resistance are commonly named multidrug resistance (MDR) proteins, most of which belong to the ABC family. Generally, ABC transporters are prevalent in all kingdoms of life and are organised by two transmembrane domains (TMDs) and two conserved nucleotide-binding domains (NBDs), which comprises three characteristic motifs such as Walker A, Walker B and motif C (Fig. 1). In eukaryotic parasitic protozoa, drug transporters of the ABC superfamily contribute to the development of resistance to their respective drugs, and the involvement of ABC transporters in this route is reported extensively in laboratory as well as field isolates, as discussed briefly below.

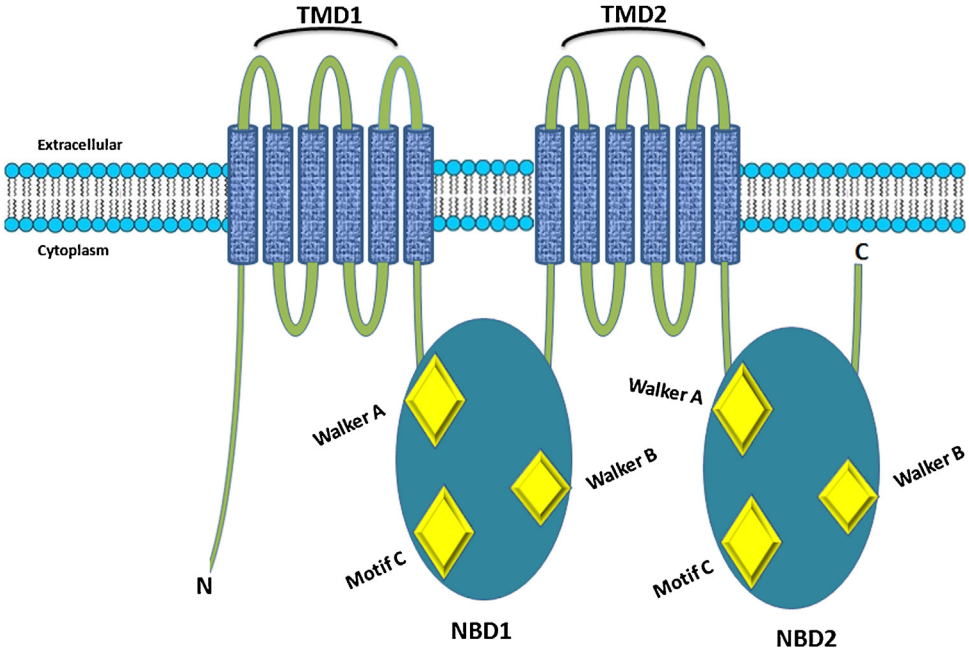

Fig. 1. Typical structure of an ATP-binding cassette (ABC) full transporter. Schematic representation of the ABC full transporter (TMD-NBD-TMD-NBD) organised as two transmembrane domains (TMDs) containing six transmembrane segments and two nucleotide-binding domains (NBDs) consisting of the motifs Walker A and Walker B and motif C.

3.1. ABC transporters in Plasmodium resistance

The fundamental reason for therapeutic failure against *Plasmodium* involves many of the plasma membrane proteins that actively pump out a wide range of structurally and functionally diverse amphipathic drugs, resulting in drug resistance following a decrease in the intracellular drug concentration [85]. Drug resistance mechanisms in one of the most severe malarial agents *P. falciparum* are very intricate. Various putative parasite transporters, some being part of the ABC superfamily, are extensively involved in this resistance phenomenon. Studies of antimalarial drugs have revealed that PfMDR1 belonging to the ABC superfamily is involved in resistance [13]. The *P. falciparum* ABC family includes 16 ABC members that have been categorised on the basis of phylogenetic analysis of the primary or secondary structures of the conserved NBDs into eight different subfamilies (A–H) [85,86]. In addition, the 3D7 isoform of PfMDR1 was sequenced and was found to be 1419 amino acids in length and contain approximately 12 TMDs [16]. PfMDR1 is mainly localised to the parasite's digestive vacuole membrane and, to a lesser extent, the plasma membrane [87]. Variation in mRNA expression level and polymorphism of the *Pfmdr1* gene has also been involved broadly in the resistance mechanism to various antimalarials and the emergence of multidrug-resistant parasites [88]. Various studies reported that the *Pfmdr1* gene was amplified in the case of some chloroquine-resistant isolates, signifying that it might be associated with chloroquine resistance in *P. falciparum* [89]. Mutations in the *Pfmdr1* gene at positions N86Y, Y184F, S1034C, N1042D and D1246Y have been implicated in determining susceptibility to chloroquine, quinine, mefloquine, halofantrine, lumefantrine and artemisinin [16,90]. A previous clinical study suggested that in vivo amodiaquine resistance in parasites is mediated by mutations in both the *Pfcrt* (Lys76Thr) and *Pfabcb1* (Asn86Tyr) genes [81]. In addition, a systematic review and meta-analysis of published data showed that *Pfmdr1* polymorphism was correlated with chloroquine, mefloquine and amodiaquine treatment failure [91,92].

3.2. ABC transporters in Leishmania resistance

There are 42 *ABC* genes recorded in the *Leishmania* genome sequence with representative members from all eight different subfamilies (ABCA to ABCH) of ABC transporters generally described in eukaryotes [93]. Some members of the ABC transporters have been well characterised and also implicated in drug resistance in *Leishmania*. Up to now, ABC transporters in *Leishmania*, including ABCA, ABCB, ABCC and ABCG subfamilies, have been functionally well described [94]. The *ABCA4* and *ABCA8* genes belonging to the ABCA subfamily of lipid traffickers were first reported in *Leishmania tropica* and were localised to the plasma membrane and internal vesicles of the parasite [95]. These ABC proteins are involved in lipid translocation and infectivity, but not in resistance. At least two transporters of the ABCC family, including ABCC3 and ABCC7, confer antimony resistance. ABCC3 (PGPA), also named MRPA, from the ABCC subclass plays a major role in antimony resistance. Generally, MRPA is localised in membrane vesicles that are near to the flagellar pocket, familiar as the site of endocytosis and exocytosis in the parasite [96]. Two other ABCC proteins (ABCC4 and ABCC5) in *Leishmania* were shown to confer a small increase in antimony resistance when overexpressed, but their role in emerging resistance requires a specific genetic background [97]. Based on several scientific reports and on existing knowledge from various literature, a universal model of antimony resistance in *Leishmania* was proposed (Fig. 2). It is generally accepted that all pentavalent antimonials (SbV) are prodrugs requiring biological reduction to their active trivalent form (SbIII) to gain leishmanicidal properties [98]. Moreover, the *mdr* gene expresses P-gp 170, a P-glycoprotein molecule acting as an efflux pump that is found mostly on the cellular membrane of

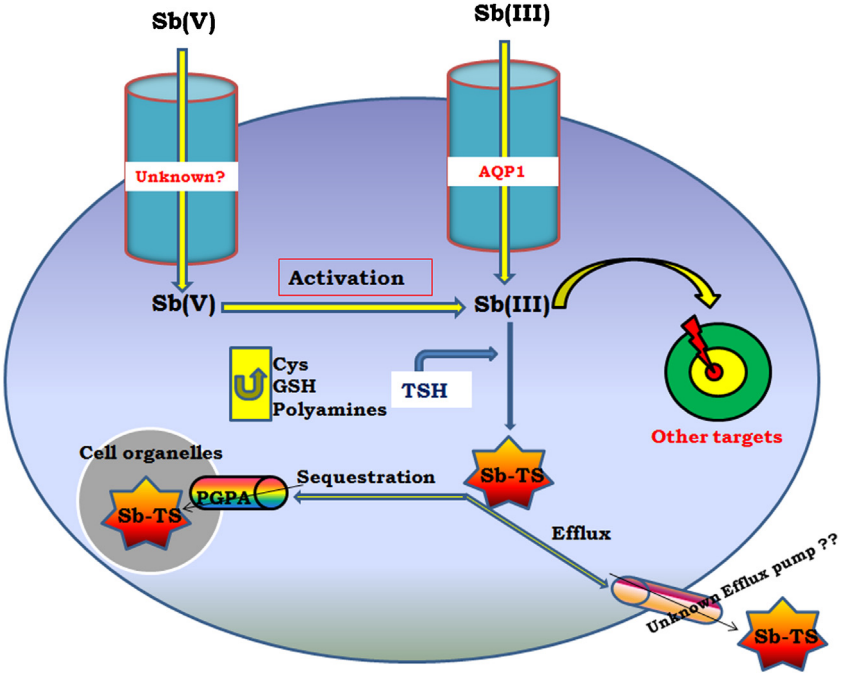

Fig. 2. Predicted model of antimony resistance in *Leishmania*. Trivalent antimony [Sb(III)] is transported into *Leishmania* via aquaglyceroporin 1 (AQP1), whereas pentavalent antimony [Sb(V)] enters the cell by an unknown mechanism. Within the cell, Sb(V) is reduced to its active form Sb(III) by reductase. Increased levels of intracellular thiols such as cysteine (Cys), glutathione (GSH), trypanothione (TSH) and polyamines conjugate with antimony and form antimony–thiol complexes (Sb-TS). These complex molecules are subsequently sequestered in a cell organelle via the ABC transporter PGPA or are effluxed from the cell via an unknown efflux pump in the plasma membrane, resulting in a reduced intracellular antimony concentration.

the parasite and poorly on intracellular membranes of organelles [99]. This efflux pumps play a key role in the drug resistance phenomenon.

Pentamidine was the first clinically approved drug in cases of patients refractory to SbV [100]. Since resistance is very frequent, AmB has been acknowledged as a superior alternative to treat leishmaniasis. Although pentamidine is sometimes used for the treatment of cutaneous and mucocutaneous leishmaniasis, it is no longer used against visceral leishmaniasis. The mechanism of action of pentamidine is still unclear, but it involves interaction with the parasite's single mitochondrion causing crumpling of the kinetoplast DNA. Decreased pentamidine accumulation in the mitochondrion with increased efflux from the cell have been implicated in the emergence of pentamidine resistance [101]. Molecularly, ABC transporter PRP1 (pentamidine resistance protein 1; ABCC7), a member of the ABCC subfamily, has been involved in resistance to antimony [102]. PRP1 is predominantly localised to the parasite's intracellular tubulovesicular compartment that connects to the mitochondrion, the target site of pentamidine. It was proposed that PRP1 accelerates the transport of pentamidine into intracellular vesicles that would be later exocytosed through the flagellar pocket [103]. ABCC7 was shown to confer resistance to pentamidine in the *Leishmania major* promastigote form [104]. It is also reported that the *Leishmania* ABC transporter PRP1 (ABCC7) also confers resistance to pentamidine in the amastigote stage of the parasite [105]. Molecularly, pentamidine resistance also involves PRP1 overexpression. Another ABC transporter protein, MDR1 (ABCB4), is also localised to the multivesicular compartment [106]. This protein increases parasite susceptibility to pentamidine when overexpressed and might indirectly assist in the importation of pentamidine inside the mitochondrion through the multivesicular element [107]. Therefore, lower importation activity of MDR1 may consequently decrease parasite susceptibility to pentamidine. The dominant mechanism of miltefosine resistance in *Leishmania* is associated with an increase in drug efflux attributed to overexpression of ABC transporters [108]. In addition, two members, i.e. ABCG4 and ABCG6, belonging to the G subclass of ABC transporters in *Leishmania* were reported to be involved in miltefosine resistance and whose localisation is mainly to the parasite plasma membrane and flagellar pocket [109]. Both genes encoding ABCG4 and ABCG6 are also involved in resistance to sitamaquine [110]. BoseDasgupta et al. have suggested that overexpression of the ABCG4 transporter is involved in miltefosine resistance [111]. Recently, ABCG6 has also been implicated in chemoresistance to camptothecin (an uncompetitive topoisomerase 1B inhibitor) owing to rapid efflux at the plasma membrane [112]. Accompanied by the protein phosphatase 2A, overexpression of the ABC transporters MDR1 and MRPA increases paromomycin resistance [113]. Several members of the MRP family belonging to ABC transporters were extensively found to confer resistance to methotrexate [43]. PGPE, an important MRP protein, has been recognised to be overexpressed in methotrexate-resistant *L. tropica* [114]. Although the diversity of resistance mechanisms in *Leishmania* has been reported experimentally as well as in field isolates, experimental reports regarding the mechanism of action against resistant *Leishmania* parasites are scarce. Therefore, to protect mankind from the second most alarming parasitic disease, leishmaniasis, it should be observant for mechanisms of drug resistance governed by ABC transporters.

3.3. *ABC transporters in* Trypanosoma *resistance*

The most common resistance mechanism associated with changes in net drug accumulation is energy-dependent extrusion of the drug by ABC transporters, including P-gp (ABCB1) and MRPs. *Trypanosoma brucei* and *T. cruzi* contain 22 and 28 *ABC* genes, respectively [81]. Two ABC transporters in *T. brucei* (TbMRPA and TbMRPE) are deeply connected with drug resistance of *Trypanosoma* against antitrypanosomal drugs [81,115]. TbMRPA and TbMRPE are localised to the plasma membrane and in an intracellular compartment (between the nucleus and kinetoplast), respectively [116]. This localisation of TbMRPE possibly confers resistance via a sequestration mechanism. Moreover, an increased level of trypanothione and glutathione in *T. brucei* results in a slight increase of TbMRPA-mediated melarsen resistance through overexpression of ornithine decarboxylase or γ-glutamyl cysteine synthetase, whereas in melarsen-resistant cell lines trypanothione or glutathione levels are unchanged [117,118]. Therefore, in *T. brucei* the level of endogenous glutathione/trypanothione is sufficient to conjugate melarsen as well as other drugs. On the other hand, overexpression of MRPA is inadequate in emerging in vivo melarsoprol resistance, and MRPA is not overexpressed in melarsoprol-resistant trypanosomes [119]. In addition, in the case of *L. major*, overexpression of an ABC transporter confers pentamidine resistance, although pentamidine retains efficacy against *T. brucei* [120]. Moreover, it is also reported that point mutations in the adenosine transporter gene *TbAT1* are the culprit for drug resistance in *T. brucei* [121]. An in vitro report by Shahi et al. revealed that overexpression of the ABC transporter TbMPRA causes melarsoprol resistance [122]. In *T. cruzi*, mutation in the nitroreductase-encoding gene *TcNTR* causes resistance to benznidazole and nifurtimox [123]. In addition, the *T. cruzi* ABCG-like transporter gene *TcABCG1* is also implicated in benznidazole resistance [124,125].

3.4. *ABC transporters in* Entamoeba histolytica *resistance*

ABC transporters are involved in *E. histolytica* resistance, but a clear mechanism is not well established. A total of 21 *ABC* genes are present in *E. histolytica*, lacking *ABCD* and 2 pseudogenes of *ABCB* [82]. Drug resistance in *E. histolytica* has been linked with the energy-dependent efflux pump P-gp, which is organised in two homologous halves, with each homologous half comprising a TMD with six transmembrane segments followed by a NBD. Multidrug resistance phenomena are contributed by overexpression and amplification of P-gp. In *E. histolytica*, six P-gp-like genes (*EhPgp1*, *EhPgp2*, *EhPgp3*, *EhPgp4*, *EhPgp5* and *EhPgp6*) have been well studied, cloned and sequenced up to now. Four (*EhPgp1*, *EhPgp2*, *EhPgp5* and *EhPgp6*) are clearly expressed in drug-resistant lines, whereas the remaining two (*EhPgp3* and *EhPgp4*) are pseudogenes [82,126]. In addition, transcriptional regulation of the *EhPgp5* gene is triggered by emetine stress. This transcriptional activation of the multidrug resistance gene *EhPgp5* is regulated by a heat shock element (HSE) in the presence of emetine [127]. Although overexpression of ABC transporters contributes to resistance to several hydrophobic drugs, it does not contribute to resistance to the anti-amoebic drug metronidazole [128]. Overexpression of a P-gp homologue was also found in another mutant resistant to the anti-amoebic drug emetine [129]. These emetine-resistant parasites were not resistant to the hydrophilic drug metronidazole, and consequently it not transported via P-gp pumps [129].

3.5. *ABC transporters in* Toxoplasma gondii *resistance*

Treatment failures in toxoplasmosis have been deeply connected to ABC transporters. The first report of the classification of ABC transporters and domain organisation related to ABC proteins in *T. gondii* was successfully performed in 2006 by Sauvage et al. [130]. There are 24 ABC open reading frames (ORFs) in the *T. gondii* genome sequence and the amino acid sequences of these ORFs show all of the distinct biochemical features similar to the ABC superfamily [130]. Among the 24 ORFs, 15 ABC transporters of *T. gondii* cluster into five of the seven families of human ABC proteins:

6 belonging to ABCB, 2 to ABCC, 1 to ABCE, 1 to ABCF and 5 belonging to ABCG. The remaining nine include four ABCHs, four structural maintenance of chromosomes (SMC) proteins and one member from unclear origin, mRNA export factor (Elf1 protein). The absence of genes encoding ABCA and ABCD members appeared to be a remarkable characteristic of the *Toxoplasma* ABC superfamily [81]. In addition, in 2004 Sauvage et al. also demonstrated that P-gp and MRP inhibitors modulated the accumulation as well as efflux of xenobiotics from parasites and also the activity of P-gp inhibitors on efflux in *T. gondii* [131]. Furthermore, expression of 24 genes in the *T. gondii* genome sequence associated with ABC was observed both in the infectious-stage tachyzoite as well as the bradyzoite for the three genotypes (I, II and III) [113]. P-gp and MRP are widely reported to export xenobiotics leading to drug resistance in protozoan parasites as well as in tumour cells [132]. Two P-gp members, namely TgABC.B1 and TgABC.B2, were identified in *T. gondii*. In 2013, Doliwa et al. sequenced and studied the expression levels of two key enzymes for parasite survival, namely DHPS and DHFR, as well as three ABC transporters, i.e. TgABC.B1, TgABC.B2 and TgABC.C1, in sulfadiazine-sensitive and −resistant *T. gondii* strains to identify genotypic and/or phenotypic markers of resistance [80]. Furthermore, the role of ABC transporters in *T. gondii* resistance is not fully clear, therefore it should focus more on ABC transporter-mediated resistance mechanisms.

4. Conclusion

Chemotherapeutic failure in the treatment of parasitic infections has become a worldwide challenge due to drug resistance. This review describes a number of protozoan parasites, wherein, except many other contributing factors to resistance, the involvement of ABC transporter is huge. The biological intricacy associated with resistance sometimes results in variation from one parasite to another because of their complex life cycles. Therefore, each disease system has unique features as well as a unique approach to the emergence of resistance. Hence, the therapeutic strategy of new antiparasitic drugs is imperative on mechanisms of action against existing resistant parasites. However, more biochemical studies are essential on the structure and function of parasitic transport proteins involved in resistance for a better understanding of parasitic drug resistance mechanisms in order to improve the treatment of drug-resistant parasites.

Funding

Department of Biotechnology (DBT-Govt. of West Bengal) [No: 75 (sanc) BTP/Budget/RD-38/2017]; DST-FIST program (Phase-III) [Sl. No. 185 dated 13.08.2015]; UGC-SAP (Phase-II) [No: F5-3/2018/DRS-II]; Indian Council of Medical Research, Govt. of India [No: Fellowship/21/2018-ECD-II]; and University of Kalyani.

Competing interests

None declared.

Ethical approval

Not required.

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