Recent advances in identification of potential drug targets and development of novel drugs in parasitic diseases. Part I: Drug resistance

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ABSTRACT

Important obstacles facing programs involved in eradication of tropical diseases are drug resistance and environmental aspects surrounding the infected population including poverty, inefficient healthy sewage system, and popular habits. Treatment failure is determined by either persistence of clinical manifestations after scheduled drug regimen, or occurrence of disease relapse or new infections. This means that treatment failure in clinical studies does not mean drug resistance because identified parasite’ resistance to a certain drug does not include pharmacokinetics and pharmacogenetics studies as well as host immunological assessment. Coinfections, drug-drug interactions, and environmental factors also contribute in development of drug resistance. Of note, parasite gene mutation is the main mechanism suggested for drug resistance. The present work aims to review commonly reported drugs associated with resistance, and the proposed genetic markers of resistance.

Keywords: drug resistance; gene mutation; genome plasticity, mechanism of resistance; mode of action; pharmacogenetics, pharmacokinetics.

INTRODUCTION

A review[1] was published to clarify the importance of long-term strategies’ required for eradication of malaria, leishmaniasis, and trypansomiasis. Scientific researches should focus on protecting the efficacy of the current therapeutic WHO-recommended regimens against the emergence and spread of parasites resistant to the current drugs. Long-term strategies include scientific perception data regarding mechanisms of action and resistance of clinical isolates and continuous identification of potential novel drug targets accompanied with financial support for the targeted compounds in more advanced stages of clinical trials. This assures designing alternative drugs to overcome the emerging resistant strains, and increase the chemotherapeutic arsenal against neglected tropical diseases[2].

Drug resistance is defined as the ability of a parasite strain to survive and multiply despite administration of a drug in doses equal to those usually recommended. Generally, a patient’s cure is evaluated by clinical examination associated with routine parasitological diagnosis. On the other hand, complete cure or significant treatment outcome should be evaluated using a series of more sophisticated diagnostic methods. Certainly, some endemic tropical diseases require more assays that may be aggressive (e.g., rectal snip in intestinal schistosomiasis, bone marrow biopsy, and splenic aspirate in leishmaniasis), and costly (e.g., PCR techniques). Therefore, drug resistance is not concerned with success or failure of therapy because it is simply a multi-factorial phenomenon related to environmental, drug, host, and parasite factors. Besides, methods utilized to investigate parasites sensitivity or resistance are not inter-laboratory standardized, or tested in in vitro assays, or clinically[3].

Discussing mechanism(s) of drug resistance, scientists should differentiate between two conditions: in vivo drug sensitivity and in vitro parasite susceptibility. In a report, the reviewers[4] claimed that definite scheduled dose and assessment of drug pharmacokinetics and pharmacogenetics in relation to host immune system are conducted in the in vivo studies. Unfortunately, these results are difficult to interpret due to loss of data regarding drug pharmacokinetics and pharmacogenetics. Although in vitro parasite susceptibility studies are expensive and consume much time and labor, results pointed to validated resistance markers if associated with bioinformatics (i.e. transcriptomic, metabolomic and genomic studies). Accordingly, the reviewers concluded that drug resistance is not attributed...
only to emergence of resistant strains after mass drug administration (MDA)\cite{Abaza}. To identify gene mutations responsible for drug resistance in a locality, two steps are recommended: 1) mapping a genetic data base for the known gene mutations; and 2) identification of the putative drug-resistance loci in each gene\cite{Abaza}. Detailed description of loci, i.e. codons, of gene mutations associated with drug resistance was previously reviewed\cite{Abaza}. Detection of drug-resistant strains and their spread was greatly achieved by the discovery of molecular resistance markers. Genome-wide associated studies (GWASs) presented a powerful tool to identify chromosomal regions and their associated genes under high selective drug pressure. To establish a molecular resistance marker for a certain drug, interested laboratories should maintain in vitro reproduction of proliferating resistant strains. This was a hard task for *Plasmodium* artemisinin (ART) derivatives-resistant strains because it requires almost five years to establish their resistance in vitro. However, Ariey et al.\cite{Abaza} was the first to confirm the genetic marker of ART resistance in Cambodian *P. falciparum* strains.

[I] **Anti-malarial drugs**

Environmental factors determining levels of malaria transmission, low, high or seasonal can affect development of drug resistance. While individuals in lower transmission areas possess lower acquired immunity that increases transmission of resistant strains, those in high transmission settings harbor multiple genotypes and resistant strains have to compete with wild-type strains to survive. In seasonal malaria transmission, asymptomatic parasitemia can serve as a reservoir for sensitive strains\cite{Abaza}.

Resistance to anti-malarial drugs is attributed to inability of the resistant strains to uptake the drug due to mutations in the parasite genes encoding transporters or enzymes responsible for drug uptake and metabolism, respectively\cite{Abaza}. Due to strong selection force of anti-malarial drugs, point mutations have spread through *Plasmodium* populations over the last three decades\cite{Abaza}. Continuous dynamics of drug pressure on the selected linked genes increased the spread of mutant *Plasmodium* resistant isolates to new geographic areas. Therefore, frequent population-based studies were strongly recommended to evaluate the efficacy of anti-malarial drugs in a locality\cite{Abaza}. In studies concerning anti-malarial drug resistance, three complementary approaches are utilized nowadays. In vivo assessment of drug efficacy in clinical samples, followed by in vitro assays against *Plasmodium* life cycle stages', and lastly molecular investigations to detect validated gene mutations associated with resistant strains. An important issue was raised for the national and regional authorities to establish a protocol for local anti-malarial drug resistance aiming at maintenance and improvement of the surveillance networks worldwide\cite{Abaza}.

It is worth noting that *P. falciparum* possesses unique complex genomic plasticity that encode multiple transporters. To survive, it is able to 1) delete certain portions of genes unessential for its growth; 2) evade host immune response by antigenic variations; and 3) undergo gene mutations in enzymes controlling drug uptake\cite{Abaza}. Cowell and Winzeler\cite{Abaza} discussed another factor, which in conjunction with genome plasticity, contributed in anti-malarial drug resistance. It was observed that high burden of asexual erythrocytic stages increased *Plasmodium* ability to develop drug resistance. American investigators\cite{Abaza} conducted whole-genome microarray analysis and next-generation deep resequencing on several *P. falciparum* clones. The study propagated six cultures of *P. falciparum* asexually for over one year, five were exposed to different concentrations of atovaquone, while the sixth one was cultured without drug pressure. Results revealed higher mutation rates; i.e. more single nucleotide polymorphisms (SNPs) in genes encoding cytochrome complex (Cytc) and multidrug resistance (MDR)-associated proteins in clones cultured with drug pressure\cite{Abaza}. It was concluded that occurrence of gene mutation could happen within few cycles of asexual erythrocytic stages' replication\cite{Abaza}. In contrast to *P. falciparum*, anti-malarial drug resistance in *P. vivax* and *P. ovale* is more difficult to detect due to several reasons: 1) lower relative parasitemia; 2) difficulty to distinguish between relapse and drug resistance; and 3) confirmation with in vitro susceptibility studies is more difficult due to absence of suitable in vitro culture system, as in *P. falciparum*. Absence of clear molecular markers in *P. vivax* was concluded\cite{Abaza}.

1. **Quinolines**

These include aryl amino alcohols (quinine, mefloquine, lumefantrine, and halofantrine), 4-aminoquinolines (chloroquine, amodiaquine, piperaquine, and pyronaridine), and B-aminquinolines (primaquine, and tafenaquine). They act through binding with heme and inhibit hemozoin formation in food vacuoles of intra-erythrocytic stages, i.e., it interferes with parasite’ detoxification (heme → hemozoin transformation)\cite{Abaza}.

• **Aryl amino alcohols:** Utilizing quantitative trait loci analyses to identify genetic mechanisms associated with quinine resistance in *P. falciparum* clinical strains isolated from different areas, the investigators detected *Pfmdr1*, chloroquine (CQ) resistance transporter (*Pfcr*) and sodium’/hydrogen’ exchanger 1 (*Pfne-1*) gene mutations. Being a transmembrane protein in the plasma membrane, NHE-1 is involved in active efflux of protons to maintain *Plasmodium* with neutral pH in response to acidification produced by anaerobic glycolysis, the main source of energy\cite{Abaza}. Another comprehensive analysis of *Pfinhe-1* gene
mutations in *P. falciparum* strains isolated from different areas confirmed its link with quinine resistance in vitro[27]. However, variable results concerning association of mutations in nhe-1 gene and quinine resistance suggested that either another gene, close to nhe-1 physical proximity, was incriminated or nhe-1 gene requires other additional genetic factors for mediating quinine resistance, e.g., adenine triphosphate-4 (ATP4)[30]. Meloquine (MFQ) and lumefantrine (LMF) are used as partner drugs for ART derivatives combined therapy, while MFQ is used alone as prophylactic drug. Increased Pfdmrr1 copy number was associated with LMF-ART combined therapy in *P. falciparum*[19], and MFQ resistance in both *P. falciparum*[20] and *P. vivax*[21].

- **4-Aminoquinolines:** In vitro cross-resistance between 4-aminoquinolines and quinine was observed in several studies suggesting common genetic mechanism of cross-resistance, i.e. Pfdmrr1, Pfert and Pfhe-1 genes[22-25]. A MDR-associated protein (MRAP2), localized in plasma membrane was suggested as CQ, quinine and primaquine transporter[26]. Multiple mutations in cct gene enable *Plasmodium* to efflux CQ out for successful detoxification process[25]. In addition, a homolog of P-glycoprotein, localized on food vacuole membrane and encoded by mdr1 gene was suggested as a drug transporter. Mutations in mdr1 gene were associated with CQ and amodiaquine resistance[27]. In 2017, three studies were conducted in Cambodia to reveal the genomic mechanism of piperquine (PPQ) resistance in *P. falciparum* clinical isolates. A GWAS identified SNPs on genes encoding MDR1, and plasmepsins II and III (aspartyl proteinases), associated with PPQ resistance[28]. Mutation in plasmepsins II encoding gene was further identified, but the mechanism of resistance was not clearly established[29]. In addition, three codons of cct gene mutations were linked with PPQ resistance[30].

- **8-Aminoquinolines:** It was reported that primaquine is administered with CQ to increase its activity against CQ-resistant strain in *P. falciparum*[31], and in treatment of malaria *vivax* and *ovale* preventing relapses[32]. A GWAS was conducted in a clinical isolate from a patient with *P. vivax* relapse despite primaquine treatment, and detected mutations in several putative resistance genes, a result suggestive of no definitive genetic marker[33].

- **Atovaquone (AVQ):** It is a hydroxyl-naphthoquinone developed when the outbreak of World War II caused substantial shortages in quinolines. Its mechanism of action is selective binding to *Plasmodium* Cytbc, a mitochondrial membrane protein responsible for maintenance of the membrane electrochemical potential[34]. It was observed that AVQ resistance began rapidly in *falciparum* malaria when administered as a single agent. Malarone, an AVQ combined with anti-folate drug (proguanil), is recommended nowadays to travelers rather than patients in endemic countries due to its high cost[35]. Single mutations in the genes encoding Cytbc was observed in malarone resistance[36].

2. **Anti-folates**

These include proguanil, and chlorproguanil that metabolize to cycloguanil and chlorcycloguanil, respectively, and both are potent inhibitors of dihydrofolate reductase (DHFR). As *Plasmodium* spp. rely on de novo synthesis of folate, anti-folates used include pyrimethamine, an analogue of folic acid, and sulfa drugs that block folate synthesis[37]. Interest in sulphonamide (sulfadoxine) and sulphone (dapsone) was raised when it was observed that they act as synergized drugs with DHFR inhibitors besides their direct inhibitory potency of dihydropterotide synthase (DHPS)[38].

Several studies documented mutations in genes encoding both DHFR and DHPS as markers for tracking resistance to anti-folates[39-41]. Utilizing genome analyses of field and laboratory-adapted *P. falciparum* strains isolated from Southeast Asia, the investigators identified an amplification surrounding the gene encoding GTP-cyclohydrolase 1 (GCH1), an enzyme in the folate biosynthesis pathway. This was explained as a compensatory mutation by the mutant *Pfdhfr*[42]. Another genetic study was conducted to compare between *P. falciparum* isolated from Thailand (common antifolates use), and Laos (rare antifolates use). Higher levels of copy number variations were identified in *gch1* gene in Thailand isolates[43].

Prolonged use of sulfadoxine-pyrimethamine (Fansidar) in *P. falciparum* clinical isolates from Malawi showed gene duplication in *gch1* promotor, an infrequent mutation in other African countries[44]. An observational cohort study was conducted recently in Zambia investigating fansidar prophylactic efficacy in pregnant women and gene mutations associated with its resistance. As WHO-recommended, SP is administered during antenatal care in *P. falciparum* endemic areas to prevent high risk of related sequences, i.e. improving birth outcome. It was observed that ~one-fifth of the study population showed prophylactic failure with prevalent point mutations in the gene encoding DHPS and DHFR. Mutations showed double Pfldhfr, triple Pfldhfr, as well as quintuple and sextuple mutants among fansidar-resistant isolates[45].

3. **Artemisinin (ART) derivatives**

These include ART, artesunate, artemether, dihydro-ART, and synthetic ART compounds, e.g. OZ439. They are short-acting anti-malarial drugs (half-life 0.5–1.5 h) that showed rapid relief, and quick clearance of parasitemia. Their mechanism of action is inhibition of P2ATP6, also known as sarco/endoplasmic reticulum membrane calcium ATPase (SERCA). The latter is involved in active transport of cytosolic free calcium concentrations into membrane-bound stores, an essential process for cellular survival[46]. Another
study reported that ART derivatives obligate ring stages to enter a dormant phase, facilitating their clearance by the host immune system[47]. Later, it was observed that the essential factor in the efficacy of ART derivatives is the pexoid group that is cleaved by heme. This cleavage produces reactive oxy-radicals that were suggested to alkylate proteins adducting with essential molecules, causing rapid parasite death[46,60]. Subsequently, administration of ART-combined therapies was recommended, i.e., ART derivatives combined with a long-acting anti-malarial drug with a different mode of action. Six partner drugs were assigned in ART-derivatives combined therapy; mefloquine and lumefantrine (aryl amino-alcohols), amodiaquine, piperaquine and pyronaridine (4-aminoquinolines), and fansidar (anti-folate)[52].

Emergence of resistant strains against ART-combined therapy started since 2008 and became prevalent in Southeast Asia[50]. Clinical resistance was manifested by increased gametocytaemia with slow clearance of parasitemia suggesting that the infection requires more time for a long acting anti-malarial to complete cure. In other words, delayed clearance was attributed to the resistance gained against the partner drugs[51]. Two genes were incriminated in ART resistance: cysteine protease falcipain-2 (fp2) and Kelch-domain 13 (kelch13). A single article reported fp2 gene as genetic marker for ART resistance, and the investigators found that this mutation was not prevalent in Uganda[52]. Of note, members (~60) of Kelch family are proteins that have essential cellular response to oxidative stress (organizing and interacting with other proteins), i.e., a regulator of protein quality control[53]. Mutations in Pfkelch13 gene resulted in dysregulation of phosphatidylinositol-3-phosphate levels conferring ART resistance[44]. Investigating kelch13 gene in P. falciparum isolated from patients from Asian and African countries revealed its link with ART derivatives resistance in Bangladesh[52], China[59] and Myanmar[60]. In contrast, its mutation was not prevalent in Africa; Uganda[52], Senegal[59], Kenya[59] and Mali[60]. Interestingly, mutation loci (codons) detected in Southeast Asia were not observed in isolates from Sub-Saharan African countries; instead the investigators detected several novel loci, but with very low frequencies[51].

Another suggested mechanism of ART resistance was activation of the unfolded protein response (UPR). Utilizing population-based transcriptomic studies of 1,000 clinical strains, the investigators identified transcriptional increase of two chaperones linked to unfolded protein response (UPR); reactive oxygen species (ROS), and T-complex protein ring (TRIC). It was hypothesized that increased chaperones expression (ROS and TRIC) alleviated toxic protein aggregates in the endoplasmic reticulum and cytoplasm. Restoring endoplasmic reticulum homeostasis, i.e., maintenance of protein folding quality control was suggested as UPR main function in stress condition[63]. On the other hand, although an orthologue of Pfkelch13 was demonstrated in P. vivax, no ART resistance has been reported yet[55].

Siddiqui et al.[64] reported that ART resistance in Southeast Asia, the hotspot of multidrug resistance, was observed with increased resistance to the partner drugs (mefloquine and piperaquine) in ART-combined therapy. High possibility of trans-continental spread of resistant strains was attributed to the rapid decline of the partner drugs’ efficacy in Southeast Asia[62,64]. Resistance to ART combined therapy accompanied with emergence of kelch13 gene mutations was not observed in other continental areas, e.g., Oceania, Africa, and South America. Accordingly, the reviewers[63] warned of future trans-continental spread, and recommended frequent molecular surveillance of resistance markers to track resistant strains’ origin. Worldwide anti-malarial resistance network (www.wwarn.org) should be notified by the resistance markers in all surveyed localities. They also recommended triple ART combined therapy utilizing a second partner drug with different anti-malarial mechanism of action[44].

**Recent approach to identify genetic markers of ART resistance:** Since reported polymorphisms in several genes were associated with a resistant phenotype, it is difficult to identify the causal genetic marker. A recent American study observed that GWASs missed functional genomics characterization to establish a certain genetic marker as a causal link between parasite genotype and phenotype[67]. Accordingly, they developed a new approach “Integrated selection of allele favored by evolution (iSAFE)” to identify favorable genetic mutations among regional population with a selective spread. The investigators supplied iSAFE system with the data obtained from both GWASs and transcriptomic studies. They established two genetic markers in ART-derivatives, kelch13 gene, and gene products involved in stress response. Surprisingly, they detected favorable mutations in the gene encoding FIKK4.2, an atypical protein kinase with a possible role in host-cell remodeling in ART resistance. Mutations identified in GWASs, e.g. phosphatidylinositol-4-phosphate 5-kinase (PPIPS5K), histone acetyltransferase (PfHAT), and calcium-dependent protein kinase 5 (PfCDPK5) were not observed in iSAFE approach. Instead, they were identified as potential drug targets that required
further validation for development of novel antimalarial drugs[67].

4. Antibiotics

Antibiotics such as clindamycin, doxycycline, and tetracycline were also recommended for combined therapy with other anti-malarial drugs due to their inhibitory efficacy against protein translation inside Plasmodium apicoplast[68]. Clindamycin-resistance was linked with a point mutation in the apicoplast ribosomal RNA (23S rRNA)[69]. It was claimed that no clear markers of doxycycline and tetracycline resistance were identified so far[12].

II] Anti-leishmanial drugs

Drug resistance and its relations to gene mutations in leishmaniasis is a complex phenomenon of distinct genetic diversities. Similar to Plasmodium spp., Leishmania spp. have unique highly plastic genome with increasing potentiality for aneuploidy; i.e., ability to modulate gene expression (amplification or deletion), and to alter its chromosome ploidy in reaction to stress (drug exposure)[70]. This was attributed to variations in local copy numbers of specific loci, and numerous pairs of short repeats flanking groups of genes. Variable genomic flexibilities create increased transcripts number of target genes for adaptive and evolutionary DNA amplification to generate drug resistance[71,72]. Therefore, several resistance markers were investigated such as genes encoding mitochondrial Cybc[73], P-glycoprotein[74], heat shock protein 83[75], kinetoplastid membrane protein 11[76], ornithine decarboxylase[77], topoisomerase I[78], histone H2A[79], γ-glutamylcysteine synthase[80], aquaglyceroporin-1 (AQP1) with ABC transporter MDR-associated protein (MRAP2)[81], and several protein kinases[82].

1. Pentavalent antimonial drugs (PADs)

They are heavy metal compounds used worldwide since the 1920s in spite of their adverse cardiac and renal toxicity. However, due to spread of drug resistance, administration was localized in Latin America and East Africa[83]. Sodium stibogluconate (SBV) achieves its function through biological reduction, i.e., by conversion to active trivalent SBIII that has an apoptopic anti-leishmanial effect[84]. Acidic pH and elevated temperature favor SBV reduction to stage specific SBIII, i.e., it increases in amastigotes than promastigotes rendering amastigotes more susceptible to SBV[85]. After biological reduction, it enters Leishmania via AQP1 membrane carrier, and inhibits trypanothione reductase (TR) which is a crucial survival enzyme. Trypanothione reduction is utilized by the tryparedoxin/tryparedoxin peroxidase system to neutralize ROS produced by the infected macrophages[86].

Several hypotheses were proposed for mechanism of PADs resistance. First, overexpression of ABC transporter MDR protein A2 (MRPA2, previously known as PGPA) localized in membrane vesicles close to the flagellar pocket. The MRPA2 transporter decreases drug efflux by sequestering thiol–metal conjugates in intracellular vesicles[87]. Second, diminished biological reduction decreases drug entry through overexpression of tryparedoxin peroxidase. This was associated with increased levels of reduced intracellular thiols including cysteine, glutathione, and trypanothione. The latter, a unique molecule in pathogenic trypanosomatids, has a high affinity binding with PADs forming thiol-metal conjugates. It was observed that reduced intracellular thiols in PADs-resistant mutants caused partial conversion of the resistance phenotype[88]. Third is the overexpression of enzymes involved in trypanothione synthesis[89]. Fourth, mutant parasites with gene encoding AQP1 and reduced AQP1 expression lead to PADs resistance[90]. Other ABC transporters were identified linked with PADs resistance through contribution in drug efflux as thiol-metal conjugates. They included ABC14[90] and ABCG2[91]. Finally, Leishmania spp. transfected with genes encoding HSPs 70 and 90 were more resistant to SBV and this was attributed to increased tolerance against stress of drug exposure that allowed Leishmania developing more effective resistance mechanisms[92].

Of note, host factors also contribute in PADs resistance. Some infected macrophages increased their ability to extrude PADs via overexpression of host MDR1. The surface of resistant isolates express glycans contributing to cellular events resulting in up-regulation of anti-inflammatory cytokines that provoke host MDR1 overexpression. Regarding this issue, host infections that necessitate administration of particular therapy e.g., anti-inflammatory drugs, to stimulate cytokines production, can diminish Leishmania sensitivity to PADs[93].

A recent study demonstrated involvement of extensive and dynamic lipid remodeling in L. tropica PADs resistance. The investigators suggested possible use of lipid profile as a marker to differentiate between resistant and sensitive strains. Lipidomic profile was assessed in both strains before and after drug exposure. Resistant strains showed significant downregulation of phosphatidyl-cholines (PCs) with increased levels of phosphatidyl-ethanolamines (PEs). In sensitive strains, triglycerides upregulation was observed with increased long-chain fatty acids, and decreased PEs levels. It is worth mentioning that PCs and PEs are the most abundant cell membrane components and are essential precursors of several transporters. The investigators hypothesized that PADs resistance under drug pressure might trigger phospholipid metabolism reducing PCs biosynthesis, as an efficient pathway to optimize the oxidative stress response against drug exposure. Further studies were recommended to validate lipid biomarkers in other Leishmania spp.[94].
2. Pentamidine (PMD)

The aromatic diamidine drug, PMD, used in treatment of African trypanosomiasis, was first used in treatment of PADs-resistant cases of leishmaniasis. Its exact mechanism of action is not well established however, several mechanisms were hypothesized. Due to its disruption of the mitochondrial inner membrane, PMD accumulates with induction of apoptosis through inhibition of respiratory chain complexes. It also causes ROS generation with increased cytosolic calcium concentration enhancing PMD cytotoxicity[96]. Interestingly, calcium channel blockers and P-glycoprotein inhibitor (verapamil) showed inhibitory potency on PMD efflux leading to accumulation of PMD in resistant clinical isolates[96]. Furthermore, PMD targeted Leishmania DNA topoisomerases (TOPs), essential enzymes required during transcription, replication, and DNA repair. This was confirmed when PMD was observed as a selective inhibitor of TOPII[97]. Regarding associated mutation, PMD resistance in Leishmania spp. was linked to ABC transporter P-glycoprotein, and it was termed pentamidine resistance protein 1[98].

3. Miltefosine (MIL)

The major limitations of its therapeutic use as efficient anti-parasitic drug include teratogenicity, hepatic and renal toxicity side effects, high potentiality for drug resistance, and its elevated cost[99]. The anti-cancer drug, MIL, has a direct anti-leishmanial effect through interfering with phospholipids biosynthesis and alkyl-lipids metabolism. It also causes mitochondrial depolarization through its effect on cytochrome c oxidases, and decreases ATP intracellular levels beside its effect as an apoptosis-like cell death[99]. It targets P-type ATPase Leishmania transporter responsible for phospholipids translocation within the plasma membrane. Mechanism of MIL action starts when it binds to a specific B subunit of Leishmania MIL transporter termed LROS3[100]. Although LROS3 and its cofactor (LROS2) were linked to MIL resistance in L. donovani, scarcity of definite in vivo drug-resistant clinical isolates obstructed full identification of MIL pharmacogenetics[101]. Later, a study identified alterations in the gene encoding LROS3 and the investigators attributed these alterations to the role played by P-type ATPase Leishmania transporter in lipids arrangements within Leishmania plasma membrane[102].

Utilizing whole-genome and RNA sequencing in MIL-resistant strains showed mutations in genes encoding pyridoxal kinase and α-adaptin. Results also showed up- and down-regulation of genes specifically associated with stress, and folate metabolism[103]. Overexpression of Leishmania ABC-like transporters (P-glycoproteins), including ABCB4, ABCG4, and ABCG6, reduced MIL intracellular accumulation because of increased drug efflux through Leishmania plasma membrane[24]. Lipids alterations and sterol biosynthesis in Leishmania plasma membrane controlling drug-membrane interactions, also contributed in MIL resistance in L. infantum[104]. Interestingly, several compounds were developed to overcome MIL resistance such as flavonoid derivative[105], and β-aminoquinoline (sitamaquine)[106] through inhibition of ABC-like transporters’ overexpression.

4. Amphotericin B (AmB)

Besides being expensive, the main AmB side effect is acute nephrotoxicity that requires close monitoring during the first month of administration. Therefore, a liposomal formulation (LAmB) with reduced toxicity was developed[106]. The anti-fungal agent, AmB is a natural product produced by Streptomyces nodosus and used as anti-leishmanial drug since the 1960s. Similar to fungi, Leishmania spp. use ergosterol as a primary membrane sterol source and AmB has high specificity to bind with plasma membrane’ ergosterol-related sterols. Of note, AmB has amphipathic nature, i.e., both hydrophilic and hydrophobic moieties. Within Leishmania plasma membrane, it spontaneously gathers with membrane lipids using its hydrophobic moiety, followed by sterols sequestration using its hydrophilic moiety. This leads to local membrane thinning that allows aqueous pores’ formation for ions exchange resulting in Leishmania death[107]. Another mode of AmB action was proposed through induction of oxidative stress and Leishmania ability to prevent this oxidative damage might reduce AmB sensitivity[108].

Sole AmB resistance was linked to mutations of the gene encoding sterol 14α-demethylase[109]. Reports of liposomal AmB resistance in leishmaniasis are scarce since treatment failure was reported only in HIV-Leishmania coinfection in France[110], and in an immunocompromised patient in Switzerland[111]. On the other hand, since it was observed that AmB resistant clinical isolates develop with normal ergosterol levels, an American reviewer[112] suggested that the main mechanism for AmB resistance was by protection against the ROS-induced oxidative damage. This protection blocked the rat sarcoma virus protein (Ras)-ATPases signaling pathway triggered by aqueous pores formed within Leishmania plasma membrane, leading to increased ROS production. Functionally, Ras proteins are molecular switches controlling intracellular signaling pathways, and it was hypothesized that local reduced membrane thickness caused functional activity changes of several membrane proteins, among them small lipid-anchored Ras ATPases[113]. This hypothesis was based on three evidences: First, several Ras isoforms were reported with AmB-induced apoptosis observed in oxidative damage of fungus[112]. Second, AmB as a fungalicidal drug stimulates host immune response triggering toll-like receptors (TLRs) through CD14 expression leading to production of pro-inflammatory cytokines[114], in addition to TLRs activation that was attributed to local reduced membrane thickness[115]. Third, although lack
of ergosterol was reported in L. donovaniAmB resistant clinical isolates, proteomic analysis demonstrated possession of exogenous cholesterol incorporated within their plasma membrane[116].

5. Paromomycin (PMM)

It is an antibiotic orally administered for a short time and is well tolerated with rare side effects, beside its low cost. It inhibits protein synthesis through binding with 16S ribosomal subunit leading to conformational RNA changes[117]. Other mechanisms of action were suggested such as modifications in membrane fluidity and lipid metabolism, decreased mitochondrial membrane potential, and respiratory dysfunction[118]. Its uptake is facilitated by binding to Leishmania surface proteins, e.g., para-flagellar rods (1D and 2D prohibitin), and a P-type ATPase transporters[119]. Its insufficient concentration requires efficient delivery system, therefore liposomal PMM formulation[120] or albumin PMM microspheres[121] were recommended.

Surprisingly, no gene mutations were reported associated with PMM resistance in Leishmania spp. since resistant strains demonstrated mechanisms involved in decreased PMM uptake[121]. This caused a decreased PMM accumulation with reduction in the initial PMM binding to Leishmania surface. Accordingly, several hypotheses of decreased PMM uptake were suggested; modulation of translation rate, interaction with vesicle-mediated trafficking, increased energetic metabolism through glycolysis, and effective protection by stress-related proteins[122,119,122].

Finally, a recent review[123] discussed an important issue in anti-leishmanial drugs resistance due to the different genetic markers among Leishmania spp. Proteomic analyses of sensitive and resistant strains for PAds identified different overexpression levels of enzymes between L. infantum and L. braziliensis. Iron superoxide dismutase A overexpression was associated with PAds resistance in L. infantum, and not in L. braziliensis[124]. Similar results were obtained for pteridine reductase as genetic marker in L. braziliensis, but not in L. infantum[125]. In addition, the Brazilian reviewers claimed that PMM sensitivity or resistance was observed among different Leishmania spp.[123].

[[III] Anti-Schistosomal drugs]

The pyrazino-isouquinoline derivative, PZQ, is the drug of choice in schistosomiasis for the last four decades. During its use, PZQ proved a safe drug inducing only mild and transient side effects. Despite forty years of administrated PZQ, its exact parasite target is not established yet. Actually, PZQ causes calcium influx and disrupts schistosomes’ homeostasis resulting in muscle contraction with tegmental alterations[126]. Three other hypotheses were proposed. First, upregulation of genes encoding aerobic metabolism and cytosolic calcium regulation. The obtained results suggested transcriptomic response similar to that observed during oxidative stress[127]. Second, transcriptomic analysis revealed upregulation of genes encoding MDR transporters, calcium regulation, and stress response as well as apoptosis-related proteins in juveniles compared to adults exposed to PZQ in vitro[128]. Third, impairment of reproduction and activity of sodium/potassium-ATPase in female and male adults, was reported[129].

Doubtless, relying on a single drug while there is considerable concern regarding resistance development, renders rapid search for alternatives a critical issue. Although only two studies reported three schistosomiasis haematobium cases who failed to respond to PZQ treatment[140,113], several studies observed resistance of S. mansoni and S. japonicum in field and clinical studies[112,113,115]. Due to absence of an exact PZQ target, its mechanism of resistance remains undetermined yet. Therefore, the reviewers[116] claimed that since PZQ has a single drawback, i.e., inefficiency against juveniles, appearance of resistant strains could be solved. Sufficient and efficient understanding of PZQ mechanisms of both action and resistance as well as its metabolism in field studies would accelerate development of novel targets or improvement of efficacy in treatment of schistosomiasis[116].

Recently, a systematic review published in 2020 analyzed other factors contributing to reduced PZQ efficacy[137]. The British reviewers discussed the few cases with incomplete cure after PZQ treatment that were attributed to decreased drug efficacy or emergence of resistant strains. They hypothesized that there are other factors influencing host PZQ level that participate in causing low cure rates. They concluded that host cytochrome P450 (CYP) enzymes is-incriminated in PZQ reduced efficacy ascertained by three evidences. First, CYPs genetic polymorphisms are linked with inter-individual response variation to metabolism of other drugs. In other words, efficacy of drugs tested in Europe may not have similar efficacy in Africa[138]. Second, host CYPs were shown mediating PZQ metabolism[139]. Third, since CYPs are essential sites for drug-drug interactions (DIDs), and since PZQ is metabolized by several CYPs, hence DIDs within CYP pathways might lead to metabolic products accumulation, and subsequent reduction of PZQ therapeutic efficacy[140]. Drug factors also contribute to understanding the mechanism of PZQ resistance. Only PZQ (R) enantiomer has anti-schistosomal activity, while its (S) enantiomer might participate in PZQ’s side effects and resistance[141,142]. Based on these evidences, the British reviewers[137] proposed host factors and PZQ formulation in PZQ incomplete cure.

[[IV] Anti-filarial drugs]

Lymphatic filariasis elimination relies mainly on MDA-WHO recommended regimen that was based on once-yearly administration of single doses of two drugs given together for at least 5 years to at least
Drug resistance

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65% of residents in the endemic area. In coinfection with onchocerciasis, ivermectin (IVM) and albendazole (ABZ) are recommended; while IVM is replaced by diethylcarbamazine (DEC) in other areas. In contrast to anti-schistosomal drugs, currently available anti-filarial drugs have microfilaricidal potency with low effects on adult filarial worms.[142].

Anti-filarial drug resistance is a common phenomenon attributed to MDA programs implemented in endemic areas. A Spanish researcher[143] reviewed the mechanisms of drug resistance and claimed that control or elimination of lymphatic filariasis is difficult due to anti-filarial drug resistance, ineffective vector control programs and unavailability of protective vaccine candidate. The reviewer observed that MDA in endemic countries created a strong selective pressure leading to emergence of resistant strains, and subsequent unsuccessful complete treatment. Moreover, drug pressure has significant influence on genetic variation among the parasite population; i.e., an additional factor for development and spread of resistant strains. The reviewer concluded that understanding the mechanisms of drug resistance due to genetic variation is an essential step to determine the existence of drug-resistant strains, and to search for new filarial genetic markers for drug resistance[143].

1. Diethylcarbamazine (DEC): A piperazine derivate, DEC exerts its effects on filarial polyunsaturated fatty acids’ production that block the cyclooxygenase pathway. Their cytotoxic activity produces vasoconstriction and endothelial adhesion leading to microfilaria immobilization and death[144].

2. Albendazole (ABZ): Notably, ABZ is a broad-spectrum anthelmintic drug that selectively binds to the cytoskeletal protein β-tubulin blocking microtubule matrix formation and stops cell division. It blocks glucose uptake in microfilaria and adults, leading to reduction of ATP with parasite immobilization and death. Prolonged suppression of W. bancrofti reproduction was also suggested as an ABZ mechanism of action[145]. Genetic variation associated with SNPs in the gene encoding β-tubulin in microfilaria-ABZ-resistant strains was suggested[146].

3. Ivermectin (IVM): Although IVM is a broad-spectrum nematocidal drug, its exact mechanism of action is still unknown. Because IVM in vitro effects required higher concentrations than in vivo effects, it was suggested that IVM caused reduction of excretory/ secretory proteins that modulated host immune responses in vivo[147]. Later, it was observed that IVM has a high affinity to glutamate-gated chloride channels, and neurotransmitters, causing increased cell membrane permeability that affects parasite motility and reproduction. Genomic and transcriptomic analyses of B. malayi showed expression of glutamate-gated chloride channel subunits in female and to lesser extend in male reproductive tracts as well as microfilaria embryonic stages. Accordingly, the investigators suggested sterility as IVM mechanism of action[148]. Up to date, no confirmed resistance to IVM in lymphatic filariasis was reported. However, IVM resistance associated with genetic variations in O. volvulus was observed in genes encoding MDR1[149], ABC transporter[150], P-glycoprotein-like protein[151], and cytochrome P[152].

Three issues were observed in resistance to anti-filarial drugs. First, spread of ABZ resistance was higher when it was combined with DEC than when combined with IVM. Accordingly, higher MDA program coverage in a population accelerated the spread of ABZ resistance[153]. Second, presence of IVM resistance increased ABZ resistance rate, and vice versa[154]. Third, high genetic diversity in W. bancrofti populations utilizing gene encoding cytochrome oxidase subunit 1 was observed. Eleven haplotypes with several polymorphic sites were identified in 15 out of 16 strains collected from patients with lymphatic filariasis[155].

[V] Anti-toxoplasmosis drugs

1. Combined pyrimethamine (PYR), and sulfonamides (SFDs): It was reported that PYR and SFD inhibit essential enzymes for pyrimidine biosynthesis, i.e., DHFR and DHPS, respectively. Accordingly, combined therapy has a synergistic inhibitory activity on T. gondii survival and replication. Using in vitro mutagenesis, a single point mutation detected in DHFR-thymidylate synthase encoding gene and was associated with PYR-resistant RH strain compared to types II and III[156]. Another mutation was identified in gene encoding DHPS associated with SFDs resistance using direct sequencing of PCR products[157]. In contrast, a French study investigated the previous mutations in 17 T. gondii isolates in vitro and observed variable susceptibilities with neither clear evidence of drug resistance, nor defined mutations in the studied genes. Besides, they detected three strains resistant to SFDs, with several identical mutations in dhps gene[158]. Another two studies denied the link between SFDs resistance and polymorphisms or overexpression in dhfr, dhps and ABC transporter genes family. They recommended further studies to investigate mechanism of SFDs resistance in toxoplasmosis and the associated gene mutations[159]. Similarly, no mutation was detected in Tgdhps gene in five samples with SFDs-resistance isolated from newborns with congenital toxoplasmosis in Brazil[160].

2. Atovaquone (AVQ): It is administered in toxoplasmosis acting against chronic bradyzoites through inhibition of mitochondrial electron transport chain. However, its administration failed to prevent toxoplasmosis in hematopoietic cell transplant recipients[162]. Similar to AVQ-resistance in anti-malarial drugs resistance, mutation in the genes encoding Cytochrome C oxidase subunit 1 was associated with AVQ resistance in T. gondii[162].
3. Spiramycin, clindamycin, and azithromycin: Similar to clindamycin resistance as an anti-malarial drug, resistance to these antibiotics was associated with mutation in rRNA encoding genes involved in apicoplast protein synthesis[163].

4. Artemisinin (ART) derivatives: Similar to other apicomplexans, calcium hemostasis was suggested as the mechanism of ART action in toxoplasmosis[164]. Although they are not commonly used in treatment of toxoplasmosis, they proved effective against T. gondii in vitro and in vivo[165]. Mechanisms of ART derivatives' resistance was previously discussed in anti-malarial drugs.

5. Dinitroanilines: They inhibit T. gondii microtubules, and α-tubulin point mutations were identified in resistant strains[166].

[VI] Other anti-parasitic drugs

1. Melarsoprol (MEL): In treatment of human African trypanosomiasis (HAT), the most toxic but with poor ability to cross the blood brain barrier (i.e. suitable for first phase) are pentamidine for HAT gambiense, and suramin (SUR) for HAT rhodesiense. For the second phase, melarsoprol (MEL) was the only drug available until the 1990s, to be later combined with nifurtimox-eflornithine therapy. Among them, MEL is the only drug with widespread drug resistance where the genetic mechanism of resistance was established[167]. It is worth noting that SUR targets glycolysis pathway, competitively inhibiting 6-phosphogluconate dehydrogenase required for pentose phosphate pathway, and impaired cell division. Multiple SUR targets might explain lack of its resistance in humans in spite of use for almost a century[168]. However, under laboratory conditions, emerging resistant strains were obtained with high expression of a variant surface glycoprotein protecting trypanosomes against hosts' immune responses[169].

Notably, MEL is a trivalent arsenical compound intravenously administered in HAT. Due to its ability for crossing the blood brain barrier, MEL might cause a fatal reactive encephalopathy side effect[170]. Two transporters; P2 adenosine and AQP2 were incriminated in MEL uptake that resulted in a competitive inhibitor of the trypanothione reductase. Cell lysis due to impairment in glycolytic pathway and mitosis inhibition were suggested as MEL mode of actions[171].

Gene encoding P2 adenosine transporter (That1) showed mutations in 50% of T. gambiense MEL resistant isolates. However, several patients with relapse after MEL therapy had the wild-type That1 gene. The investigators suggested that That1 gene mutation was not the only mechanism behind MEL resistance or failure[171]. Two years later, the same investigators confirmed contribution of AQP2 transporter in P2 adenosine transporter activity[172]. Later, it was confirmed that selection for MEL resistance occurred with cross-resistance to PMD and vice versa[173]. Mutations in both transporters are responsible for low MEL uptake, and both mutations were demonstrated in MEL-PMD cross resistance in field[174] and clinical isolates[175]. Additionally, overexpression of the ABC transporter MDR protein A (MRPA) was found to increase MEL efflux and was postulated as mechanism of MEL resistance in vitro[176].

2. Triclabendazole (TCBZ): It inhibits Fasciola microtubule-based processes, i.e., feeding and reproduction, through β-tubulin binding. In their review, Fairweather et al.,[177] claimed that TCBZ binds with β-tubulin at another different site than the other benzimidazoles. Because there is no reported evidence for β-tubulin mutations to date, the reviewers concluded that its mechanism of resistance is not fully understood. Accordingly, the reviewers discussed other possibilities for TCBZ resistance that altered drug uptake and/or metabolism[177]. In TCBZ resistance, investigators observed that overexpression of P-glycoprotein, a membrane transporter, reduced TCBZ intracellular concentration via drug efflux pumps. A SNP was identified in its encoding gene in resistant strains isolated from England[178]. However, this SNP was not identified from TCBZ resistant strains isolated from Australia[179], or Latin America[180]. Increased levels of inert metabolites (TCBZ sulphoxide and sulphur dioxide) from excessive TCBZ metabolism was also observed in resistant strains[181]. Glutathione S-transferase is involved in TCBZ metabolism, and its activity was higher in TCBZ-resistant than -sensitive flukes[182]. Finally, the reviewers concluded that TCBZ resistance in F. hepatica is polygenic in nature. Its mechanism of resistance is much complicated due to subsequent impact of TCBZ metabolites in its mode of action. They recommended further studies to elucidate any genetic markers for TCBZ resistance[177].

3. Metronidazole: It targets the reductive anaerobic pathways. The nitro group of MTZ is reduced by ferredoxin produced by parasites' enzymes; pyruvate ferredoxin oxidoreductase, nitroreductase 1 and dihoredoxin reductase. This reduction leads to production of the active metabolite that binds to DNA macromolecules causing oxidative stress and death[183,184]. Several reasons were proposed in persistence of giardiasis, intestinal amoebiasis, and trichomoniasis in spite of MTZ treatment; drug resistance, parasite virulence, reinfection, and immunosuppressant diseases or therapy[185]. Orozco et al.[186] reviewed MDR in amoebiasis, and it was attributed to mutation of E. histolytica P-glycoprotein-like gene. Muller et al.[187] suggested increased Giardia tolerance to oxidative stress through upregulation of heat-shock proteins. However, it was shown that isolates of G. lamblia, E. histolytica and T. vaginalis had different combinations of gene mutations either
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similar to H. pylori or Bacteroides fragilis in the genes encoding enzymes involved in MTZ activation or deactivation, respectively\(^\text{189}\). In two Iranian studies, the investigators detected point mutation at ITS1 fragment\(^\text{190}\), and ferredoxin encoding gene\(^\text{190}\) of their T. vaginalis isolates. After discussing studies with MTZ-refractory giardiasis, the reviewers\(^\text{191}\) claimed that mechanisms underlying MTZ resistance are not clearly understood yet. Accordingly, they recommended further studies to establish the molecular and/or genetic basis for MTZ resistance in clinical isolates\(^\text{191}\).

On the other hand, an American study conducted recently reported that although T. vaginalis virus was detected in 40% of their study population, it had no influence in clinical manifestations, reinfections and MTZ resistance\(^\text{192}\). In addition, based on the results obtained from two recently conducted studies\(^\text{193,194}\), German reviewers\(^\text{195}\) attributed MTZ resistance to over-expression of various effector molecules without specific target gene mutations, i.e., epigenetic or post-translational modifications without a recognized pattern in proteomic assays. They hypothesized that these modifications had essential roles in the parasite DNA with more significant effects than mutational base exchange\(^\text{195}\).

**CONCLUDING REMARKS**

1. Emergence of drug resistance is a multi-factorial phenomenon. Ability of certain strains for gene mutations is the main factor. Targeting non-essential parasite molecules, poor pharmacokinetic properties, and quality (falsified drugs) are drug factors. Unsuccessful treatment compliance (pharmacogenetics), immune status and coinfections are the host factors. Environmental factors facilitate spread of resistant strains (under drug pressure) to new geographical endemic area.

2. Genetic mechanism of drug resistance arises early with monotherapy, drugs with fast action, as well as drugs acting on a single target. Combined therapy with different mechanisms of action would decrease emergence of drug resistance.

3. Molecular genetic markers are utilized to screen for emergence of drug resistance. Only P. falciparum and Leishmania spp. have unique plastic genome encoding multiple transporters with high potentiality to aneuploidy, and gene expression modulation (gene amplification) in response to drug exposure. Hence, anti-malarial and anti-leishmanial drugs resistance are associated with established definitive genetic markers.

4. All quinolones resistance, except for atovaquone, are associated with gene mutations in the gene encoding transmembrane proteins (mdr1, crt and nhe-1). Atovaquone is associated with Cytb, and anti-folates with Pdhfr, and Pdhps encoding genes. Resistance to ART derivatives is linked either to mutation in kelch13 gene or upregulation of unfolded protein response.

5. In anti-leishmanial drugs, ABC transporters were linked with antimonial drugs (MDR protein A2, and AQP1), pentamidine (P-glycoprotein), and miltefosine (P-glycoprotein-like transporter, ROS3). Resistance to AmB is not common and attributed to Leishmania protection against the ROS-induced oxidative damage. Similarly, paromomycin (PMM) resistant strains showed mechanisms involved in decreased PMM uptake due to effective protection by stress-related proteins.

6. In contrast, neither anti-schistosomal nor anti-filarial drugs are associated with genetic markers. Host cytochrome P450 (CYP) enzymes, and drug formulations are incriminated in PZQ reduced efficacy. In the latter, MDA created a strong selective pressure with a significant influence on development and spread of resistant strains. In O. volvulus, IVM resistance was associated with several genetic variations, e.g., MDR1, ABC transporter, and P-glycoprotein-like protein.

7. No established genetic markers were reported also for anti-toxoplasmosis drugs however, several single point mutations were identified. They included DHFR (pyrimethamine), DHPS (sulfonamides), Cytbc (Atovaquone), rRNA (clindamycin), and Kelch13 (ART derivatives) encoding genes.

8. In African trypanosomiasis, ABC transporters (P2 adenosine, AQP2, and MDR protein A) are involved in increasing MEL efflux and decrease its efficacy.

9. Mechanisms of triclabendazole resistance in treatment of hepatic flukes and metronidazole in treatment of giardiasis, amoebiasis and trichomoniasis are much complicated. Subsequent influence of TCBZ metabolites in its mode of action was suggested for triclabendazole resistance, while post-translational modifications of various effector molecules without a specific point gene mutation was suggested for metronidazole.

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