Malaria Combination Therapies: Advantages and Shortcomings

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Abstract: Drug combination therapies have been devised to delay the development and spread of resistant malaria parasites. However, poor design often leads to ineffective combinations. Here, the properties of various drug combinations are reviewed in relationship to drug resistance and their pharmacokinetic compatibility.

Key Words: Malaria, drug resistance, drug combination therapy, artemisinin, quinolines, antifolates.

INTRODUCTION

Malaria is one of the main causes of death in developing countries, particularly in Sub-Saharan Africa claiming an estimated 1.5-3 million lives each year, mostly among children under 5 years [1].

With the lack of an effective vaccine, control of malaria relies heavily on control of the mosquito vector (mainly *via* bednets and insecticides) and on the use of antimalarial drugs. Current treatment strategies are based on three main types of drugs: antifolates, quinolines (quinine and its derivates) and artemisinins (artemisinin and its derivates). The initial use of single drugs as monotherapies has given way in the past decades to combination therapies of two or more drugs due to the rapid spread of drug resistance among parasites worldwide (Table **1**). Most recently, artemisinin combination therapy (ACT) has been put forward as the new main therapeutic treatment for malaria. Resistance to artemisinin and its derivates has not yet been detected in the field, but its combination with quinolines and antifolates makes such combinations heavily reliant on the artemisinin component, especially in areas where resistance to antifolates and quinolines is widespread. Such combinations also suffer from pharmacokinetic mismatches that further jeopardize the efficacy of artemisinins and it may thus be only a matter of time before the first artemisinin-resistant parasites will be recorded. The need for the development of new drugs and combinations is thus especially urgent.

Several reviews have recently focused on the mode of action of and resistance to the main antimalarial drugs [2-5]. This review will present a concise description of the three main families of antimalarial drugs currently in use: antifolates, quinolines and artemisinins. The main combinations in which members of the three families have been used will be presented and discussed with regards to their efficacy and pharmacokinetic compatibility, with emphasis put on artemisinin combination therapies (ACT's). Experimental combinations being currently tested, as well as the use of drug resistance reversers will also be presented.

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Antifolates

Unlike its vertebrate hosts, malaria parasites are able to synthesize folic acid *de novo*. The complete pathway involves various enzymes that are absent from the vertebrate host and thus provide ideal targets for therapy. However, only two are targeted by current antimalarial therapies: dihydrofolate reductase (DHFR) (also present in vertebrates) and dihydropteroate synthase (DHPS).

In *Plasmodium*, the dhfr gene encodes a bifunctional protein, DHFR-TS (thymidylate synthase), although it is in its function as DHFR that it is targeted. DHFR is responsible for reducing dihydrofolic acid to tertrahydrofolic acid, using NADPH as an electron donor [6]. An alternative pathway involving the exogenous acquisition of folate from the host [7] also exists, although its exact mechanism is still unknown and its contribution is thought to be minimal. However, it exerts some influence on drug resistance [6].

DHPS catalyzes the formation of dihydropteroate (the direct precursor of dihydrofolic acid in the folate metabolic pathway) from p-aminobenzoic acid (pABA) and dihydropteridine-hydroxymethyl-pyrophosphate (DHPPP) [8]. Intriguingly, although the parasite mainly acquires pABA through its diet, it has been shown that, like bacteria and plants, it also possesses a shikimate pathway for the endogenous biosynthesis of pABA [9], although the importance of this mechanism *in vivo* is debated.

Several antifolates have been developed to treat malaria, of which the most widely used are: proguanil, chlorproguanil and pyrimethamine, which target DHFR, and dapsone, sulfalene and sulfadoxine (three sulfadrugs), which target DHPS.

DHFR inhibitors

Proguanil (**1a**) (Paludrine©) was the first antisulfate to be deployed to treat malaria in the aftermath of WWII [10].

Chlorproguanil (**2a**) (Lapudrine©) was derived from proguanil through the chlorination of its phenyl ring (Fig. (**1**)). Both proguanil and chlorproguanil act as prodrugs and are metabolized to their trizine forms: cycloguanil (**1b**) and chlorcycloguanil (**2b**), which are active inhibitors of DHFR [11] (Fig. (**1**)).

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Table 1. Main Currently Used Antimalarial Combination Therapies with Advantages and Disadvantages

Drug Combination	Advantages	Disadvantages
Sulfadoxine+Pyrimethamine (SP)	Synergistic activity, inexpensive	Wide-spread resistance, medium-long half lives, moderate pharmacokinetic mismatch
Chlorproguanil+Dapsone	Synergistic activity, good pharmacokinetic match, short half-lives	Cross-resistance with SP
SP+ Amodiaquine	Similar pharmacokinetic profiles	Resistance to both components spreading, medium-long half-lives
SP+Artesunate	Efficacious	Significant pharmacokinetic mismatch, efficacy dependent on level of SP resistance
Proguanil/Dapsone+Artesunate	Efficacious, similar pharmacokinetic profiles, short half-lives	Cross-resistance with SP may limit its application
Artesunate+Mefloquine	Efficacious	Pharmacokinetic mismatch, resistance to mefloquine spreading
Artesunate+Amodiaquine	Efficacious	Pharmacokinetic mismatch, resistance to amodiaquine spreading
Artesunate+Fosmidomycin	Efficacious, excellent pharmacokinetic match, short half-lives	Not suitable for prophylaxis
Artesunate+ Clindamycin	Efficacious, similar pharmacokinetic profiles, short half-lives	Not suitable for prophylaxis, treatment failure observed
Proguanil+Atovaquone	Synergistic activity, efficacious	Moderate pharmacokinetic mismatch, resistance easy to acquire

Pyrimethamine (**3**) belongs to the 2,4-diaminopyrimidine derivate family, originally developed as antitumor drugs [12]. Due to the similarity in structure with proguanil (Fig. (**1**)), it was predicted that such drugs would have antimalarial properties, leading to the development of pyrimethamine. It was briefly used as a monotherapy (notably as Daraprim©) but as in the case with proguanil, resistance developed rapidly [13] and led to its combination with other drugs.

Like cycloguanil and chlorcycloguanil, pyrimethamine binds to the malaria DHFR in competition with its natural substrate, dihydrofolic acid (**4**). All three drugs target the DHFR enzyme of *Plasmodium* with a higher affinity than the human DHFR [14, 15]. Alternatively, the role of parasite DHFR expression rate has been proposed as a more important cause for the higher lethality in parasites [16], although this has been more recently disputed [17]. In any case, DHFR-TS bears enough structural difference from the DHFR of other organisms for preferential drug targeting to take place, as observed in the case of pyrimethamine and proguanil and its derivates. In fact, the rigid length of the pteridine ring of many folate inhibitors appears to fit between residues 108-54 within the active site of DHFR [18] Unsurprisingly, several mutations associated with antifolate resistance are located within or in close proximity of the active site (see 1.1.3).

DHPS Inhibitors

Three sulfa drugs are currently widely used in combination therapies for the treatment of malaria: sulfadoxine (**5**) (a sulfonamide), dapsone (**6**) (a sulfone) and, to a lesser extent, sulfalene (**7**) (another sulfonamide) (Fig. (**2**)).

Sulfa drugs bear a structural similarity with pABA (**8**) (i.e. a benzene ring substituted with an amino group) (Fig. (**2**)) and target the DHPS enzyme, which, in malaria parasites is encoded by a gene encoding a bifunctional protein (*dhps-pppk*) which also contains a hydroxymethylpterin pyrophosphokinase. There is no mammalian counterpart to *dhps*. Sulfa drugs bind to DHPS in competition with pABA and can thus deplete DHPPP and reduce DNA synthesis [8]. Competition with pABA and parasite uptake of exogenous folic acid also partially explain the low efficacy of sulfa drugs on their own [19, 20].

Antifolate Resistance

Resistance to both pyrimethamine and proguanil spread almost immediately among parasites. The main reason for this was the relative ease with which increasing degrees of tolerance under constant drug exposure could be accumulated by the parasites. In fact, resistant phenotypes are characterised by the presence of single nucleotide polymorphisms (SNP's) in the *dhfr* gene.

Pyrimethamine resistance has been described both *in vitro* and in the field. An initial mutation in position 108 (S108N) was identified *in vitro*, with progressively more resistant clones displaying mutations a positions 51(N51I), 59(C59R) and 164 (I164L) [21]. A further mutation at codon 16 (A16V) associated with S108T was also found. Field studies confirmed the importance of these mutations. The

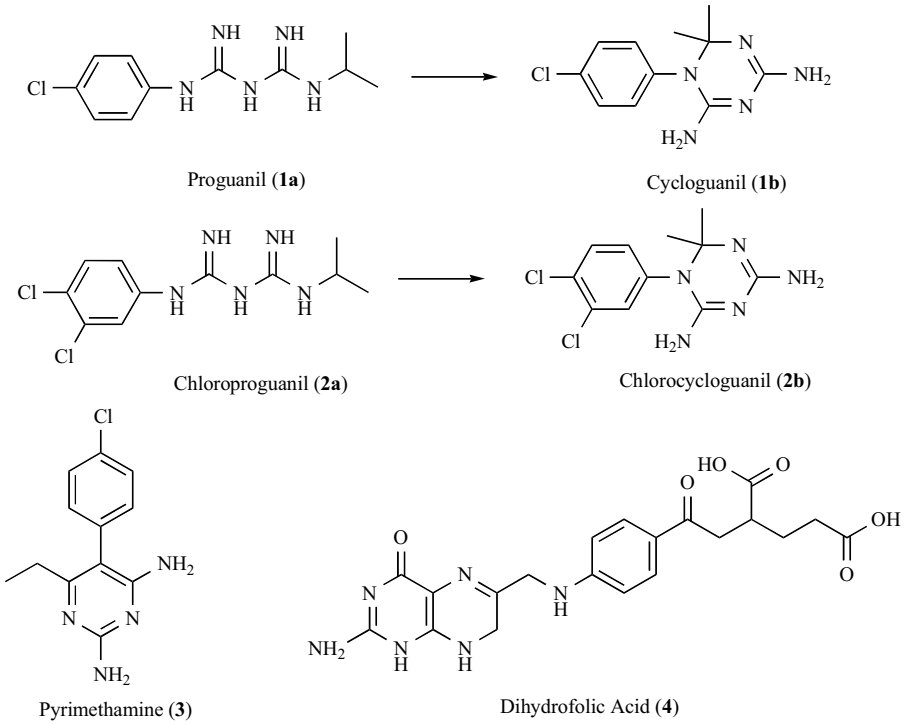


Fig. (1). Chemical structure of the major antifolate drugs targeting DHFR and the natural substrate dihydrofolic acid.

A16V/S108T mutant showed a greater resistance to cycloguanil than pyrimethamine [22]. In the other cases, it was observed that S108N was essential to first establish resistance and that mutant parasites were more resistant to pyrimethamine than cycloguanil, with the exception of treble mutant S108N/C59R/I164L [23]. More recently, a quadruple mutant form (N51I plus C59R plus S108N plus I164L) which is spreading fast in South America and Southeast Asia, was found to be completely refractory to pyrimethamine, as well as being highly resistant to cycloguanil [24].

Resistance to the sulfa component of antifolate combinations is also widespread. Six different mutations affecting four codons of *dhps* were originally identified in sulfonamide-resistant isolates: S436A/F, A437G, A581G, and A613T/S [25]. More recent studies identified a further mutation in position 540 (K540E), which only occurred in association with A437G [26, 27]. As in the case of anti-DHFR antifolates, resistance to sulfonamides tends to increase with the number of mutations. A437G alone conferred the lowest degree of resistance, while the highest degree of resistance was found for either the S436A/A437G/A613T or S436A/A437G/K540E mutations [27]. Codons 436, 437 and 540 line the channel to the active site of DHPS, while codons 581 and 613 are physically close to the channel [8]. The latter two are considered to be compensatory mutations, aimed at re-establishing enzyme efficiency. Mutations in DHPS also conferred cross-resistance to dapsone, indicating the presence of a unique binding site in the active site of the enzyme for all sulfa drugs [27].

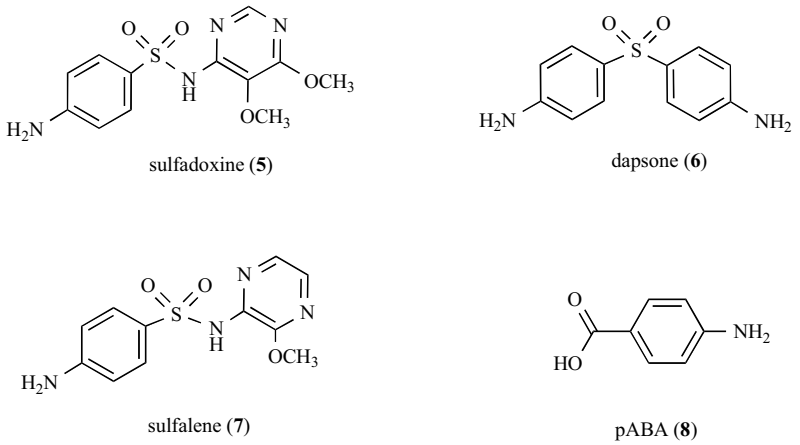


Fig. (2). Chemical structure of the major sulfa drugs and of the natural substrate pABA.

QUININE AND RELATED DRUGS

Quinine, the first drug used specifically to treat malaria was "discovered" by Jesuit priests in Peru in the 17th century who identified the anti-fever properties of the bark of the Cinchona plant which was used in indigenous medicine. Quinine was synthesized in the laboratory for the first time in 1944 and thereafter its use has been widespread, mainly as a last resort drug for the treatment of severe and complicated malaria. Chloroquine is a 4-aminoquinoline derivative of quinine first synthesized in 1934 and has since been the most widely used antimalarial drug. In the past, it has been the choice drug for the treatment of non-severe or uncomplicated malaria and for chemoprophylaxis, although drug resistance has dramatically reduced its usefulness. Amodiaquine is a relatively widely available compound closely related to chloroquine, which has recently been adopted as one of the major partner drugs in ACT. Mefloquine and halofantrine (quinolinemethanol derivatives of quinine) resulted from efforts to find replacements for chloroquine when resistance to this drug became apparent, mefloquine being widely known for its use as a prophylactic agent, especially among travellers worldwide. Both mefloquine and halofantrine are also used as partner drugs in ACT.

Mode of Action

Quinine (**9**) and related drugs have similar structures because they share a common double carbon ring containing one nitrogen atom (N) (Fig. (**3**)) and therefore could be expected to have the similar mechanisms of action. In addition, cross resistance of Plasmodium to more than one drug of this class of drugs is reported to occur. Interestingly, however, chloroquine (**10**) appears to act differently from all other quinine related drugs and, accordingly, also displays dissimilar resistance mechanisms.

Chloroquine is a lysosomotropic and weak base drug, uncharged at neutral pH while it carries a positive charge at acidic pH. Due to this feature, chloroquine is selectively accumulated inside lysosomes. The uncharged compound rapidly diffuses through the plasma and lysosomal membranes, and once charged the compound becomes trapped inside the acidic lysosomal compartment (food vacuole) of the parasite. This may lead to the generation of a concentration gradient of several orders of magnitude. Trophozoites, the active feeding stages of malaria, digest haemoglobin inside the parasitophorous food vacuole. The by-product of haemoglobin metabolization is a molecule known as haem, which is highly toxic to the parasite unless it is polymerized into an

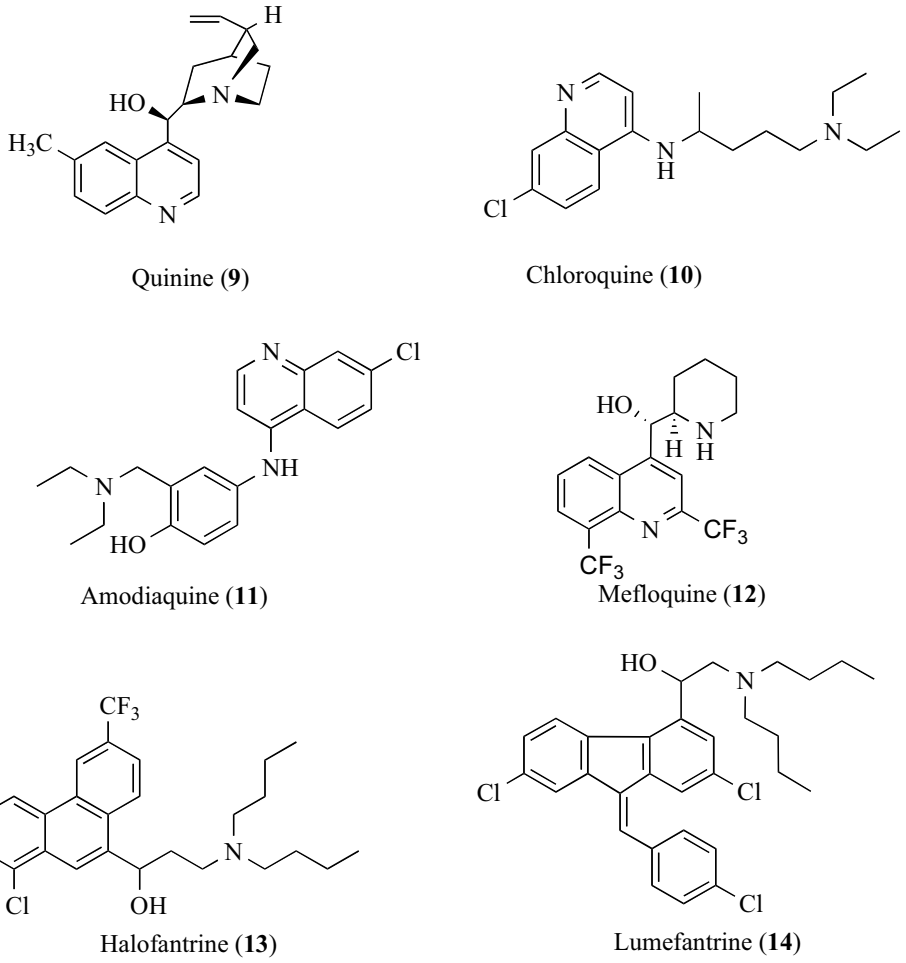


Fig. (3). Chemical structure of the main quinolines.

inert pigment known as haemozoin [28-30]. In normal circumstances malaria parasites are able to polymerize haem avoiding its cytotoxic effects [31] but chloroquine is able to prevent haem polymerisation inside the vacuole by blocking the sequestration of toxic haem into hemozoin. This causes accumulation of toxic levels of haem that ultimately result in parasite killing. This premise may be however an oversimplification of chloroquine's mode of action since the quantity of free haem remaining in the lysosome does not seem to be enough for complete parasite elimination. Accordingly, it has been demonstrated that approximately 80% of haem diffuses out to the cytosol where it is then degraded by reduced glutathione [32]. In light of the above, the synthetic theory on the mechanistic grounds of chloroquine's toxicity supports that the effect of chloroquine is dependent on the formation of a haem-chloroquine complex that inhibits the degradation of haem by reduced glutathione [32]. Amodiaquine (**11**) is structurally related to chloroquine (Fig. (**3**)), but retains a high degree of efficacy against chloroquine-resistant clones [33].

Mefloquine (**12**), halofantrine (**13**) and lumefantrine (**14**) are aryl-amino alcohols structurally similar to quinine (Fig. (**3**)). All four drugs are monoprotic bases and are therefore expected to accumulate in the acidic parasitophorous vacuole less efficiently than their counterpart chloroquine. They appear to share similar modes of action, the most studied of which is mefloquine's. Mefloquine is a 4-quinolinemethanol derivative with the specific chemical name of (R*, S*)-(+)-α-2-piperidinyl-2, 8-bis (trifluoromethyl)-4-quinolinemethanol hydrochloride. It is a 2-aryl substituted chemical structural analog of quinine (Fig. (**3**)). In this context, it has been suggested that mefloquine uptake into the food vacuole is facilitated by the action of an active transporter [34]. Recent studies suggest that mefloquine has the ability to interfere with haemozoin formation, resulting in the accumulation of free haem which is toxic to parasites [35]. In addition, it has been demonstrated that mefloquine is able to bind peptides present on the surface of *P. falciparum*-infected erythrocytes, suggesting that these peptides may be involved in the uptake of the drug or may represent direct targets of action [36]. Alternatively, it has also been proposed that these drugs might act by disrupting membrane trafficking events involved in the uptake of metabolites essential to the parasite [37]. More recent work with *P. falciparum* lends weight to this view; having shown that mefloquine strongly inhibits endocytosis of essential macromolecular nutrients into the parasite food vacuole [38].

Resistance

Chloroquine resistance can be partially reversed by calcium channel antagonists, such as verapamil (**24**) [39, 40] which is known to interact with P-glycoproteins (ATP-binding cassette transporters) that are able to pump out a number of chemically unrelated cytotoxic drugs in chemo resistant cell lines [41]. A homologue of cancer P-glycoprotein has been identified in *P. falciparum*, encoded by the *multi-drug resistance gene 1* (*pfmdr1*) which has been implicated in chloroquine resistance [42] to some extent as demonstrated by allele replacement experiments and field observations which have suggested that some alleles of the gene are able to modulate the parasite's susceptibility to the drug [43]. However, numerous observations where chloroquine resistant parasites lacked mutations in the *pfmdr1* gene have led to the search for alternative modulators of chloroquine responses. To this purpose, a genetic approach has subsequently allowed the identification of another gene denoted *pfcrt* (*P. falciparum* chloroquine resistance transporter), in which a mutation at codon 76 encoding a K to T amino acid switch, segregates perfectly with low chloroquine resistance among field parasite isolates of *P. falciparum* [44]. A number of subsequent field studies and allelic replacement experiments revealed that *pfcrt* K76T and a number of other mutations in this gene not only played a central role in chloroquine resistance, but could also modulate its levels [45]. The peptide product of this gene, PfCRT is an integral membrane protein with 10 predicted transmembrane domains, located on the membrane of the intra-erythrocytic parasite's digestive vacuole, where chloroquine acts [46]. Mutations in the *pfcrt* gene in resistant parasites thus suggest a mechanism through which PfCRT mediates the transport of protonated chloroquine through the digestive vacuole membrane [47].

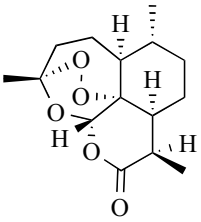
Interestingly, and in contrast with chloroquine responses, mutations and/or altered expression of the *pfmdr1* gene are highly associated with resistance to mefloquine, quinine, and halofantrine. Thus the plasmodial P-glycoprotein (Pgh 1) plays a role in mefloquine resistance and may also be the target of action of this drug [48]. Independent laboratory studies on *P. falciparum*, which involved the selection of parasite lines that are more resistant to mefloquine, resulted in an increase in copy numbers of *pfmdr1* and/or overexpression of the peptide product of the *mdr1* gene, Pgh-1 [49-51]. Interestingly, the mefloquine selected parasites also presented concomitant resistance to quinine and halofantrine, suggesting similar resistance mechanisms.

Not all mefloquine resistant isolates of *P. falciparum* contain amplified Pgh-1 suggesting the involvement of other mechanisms in determining the resistant phenotype. In fact, apart from changed expression, there is also evidence to suggest that single nucleotide polymorphisms in the *pfmdr1*gene may modulate parasite susceptibility to quinine, mefloquine and halofantrine. A mutation at codon 86 (N84Y) was associated with increased mefloquine sensitivity in *P. falciparum* from Thailand [52, 53] and the Gambia [54]. Genetic crossing experiments have shown that polymorphisms at codon 184 (F to Y) and 1042 (D to N) in the Pgh-1 protein are associated with increased sensitivity to both mefloquine and halofantrine [55]. These amino acid substitutions, predicted to lie in transmembrane domains, may alter the configuration of the Pgh-1 peptide decreasing its efficiency by changing the substrate specificity of the pump. Finally, genetic transfection studies have also strongly suggested that point mutations in the *pfmdr1* nucleotide sequence which encode polymorphisms at codons 1034 (S to C), 1042 (N to D) and 1246 (D to Y) may cause *P. falciparum* parasites to accumulate more mefloquine and halofantrine [43]. Cross-resistance between lumefantrine and mefloquine has also been reported [56].

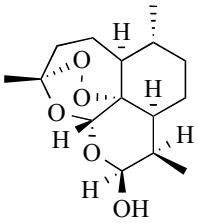
ARTEMISININS

Artemisinin (**15**) and its derivatives are currently the recommended drugs for malaria therapy. Artemisinin was suc-

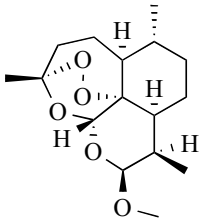
cessfully isolated in the early 1970's from the sweet wormwood plant (*Artemisia annua*) [57]. Several semisynthetic derivatives were developed since to improve solubility and pharmacokinetics. Of these, the most frequently used in therapy (beside artemisinin itself) are: dihydroartemisinin (**16**), artemether (**17**), arteether (**18**) and artesunate (**19**) (Fig. (**4**)).



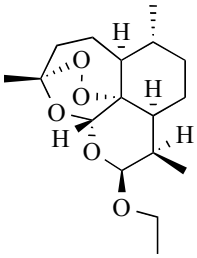
Artemisinin (**15**)



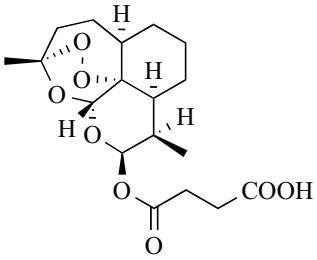
Dihydroartemisinin (**16**)



Artemether (**17**)



Arteether (**18**)



Artesunate (**19**)

Fig. (4). Chemical structures of artemisinin and its derivatives.

Artemisinin and its derivatives are sesquiterpene trioxane lactones and contain an endoperoxide bridge which is essential for antimalarial activity (Fig. (**4**)). This was shown by the substitution of the endoperoxide bridge with a single oxygen atom, which abrogated the antimalarial activity of artemisinin [58].

Artemisinins have short half-lives, with artemisinin itself between 2-5 h, artesunate <1 h and arthemeter between 3-11 h [59]. Artemether and artesunate are also rapidly converted into dihydroartemisinin, which has a very short half life (approximately 45 min) [60]. The only exception is represented by arteether, with a half-life of >20 h, but also the lowest bioavailability [59].

Mode of Action

The mode of action of artemisinin has not yet been fully characterised and several mechanisms have been proposed. The most studied involves the production of free radicals following artemisinin activation. Several studies have indicated that the production of free carbon-centred radicals following the cleavage of the endoperoxide bridge by either free iron ions or ferrous haem (although the role of the latter has been disputed, [61, 62]) plays a crucial role in killing the parasite [60]. This was demonstrated in *in vitro* studies where iron chelators inhibited the activity of artemisinin [63].

The free radicals thus generated may then have different effects within the parasite. One is the generation of oxidative stress. *In vitro* studies indicated that artemisnin, albeit at significantly higher concentrations required for antimalarial effects, caused oxidative stress as well as a reduction in reduced glutathione levels [64]. *In vivo* work in rodents showed that reduction of Vitamin E (an important antioxidant) levels in the diet increased artemisinin activity [65]. Artemisinin also inhibited the activity of glutathione-S-transferase (an enzyme family which can use glutathione to detoxify products of oxidative stress) in *Plasmodium knowlesi*, a monkey malaria parasite [66]. However, it has been proposed that, rather than an unspecific oxidation within the parasite, free radicals may actually bind specific targets [67]. Indeed, alkylation of haem, as well as parasite proteins, such as cytochrome c, catalase and translationally controlled tumor protein (TCTP), by alkyl radicals has been shown to take place *in vitro* and, for haem, also *in vivo* [69,70]. The favoured alkylation of protein-bound haem in haemoglobin over free haem has led to the conclusion that its alkylation had the dual effect of stopping haemozoin formation by binding to haem and creating a more reducing environment unfavourable to haemozoin formation, thus killing parasites [71]. Controversially, a recent *in vitro* study, where culture conditions where artificially manipulated to inhibit haem iron reactivity indicated that not only haem iron played no role but also that free radicals were not required for toxic activity [72].

A specific molecular target of artemisinins was identified in 2003 by Eckstein-Ludwig and colleagues [62]. By identifying a structural similarity between artemisinin and thapsigargin, a highly specific inhibitor of sacroplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA), an ion pump required for maintaining of calcium ion concentrations within a cell, the group proceeded to identify a homologue in *P. falciparum* (PfATP6) and express it in *Xenopus* oocytes. Upon exposure to artemisinin, the activity of PfATP6 was exclusively abolished. Notably, no inhibition occurred when the endoperoxide bridge was removed. It was concluded that artemisinin bound specifically to PfATP6 and, upon activation by Fe^{2+}-ions present in the cytoplasm, it specifically alkylated PfATP6 in order to exert its parasiticidal activity. It was also demonstrated that the artificial introduction of a single amino acid substitution in transmembrane segment 3 of PfATP6 as well as other mutations in the *Xenopus* model abolished the activity of artemisinin [73]. These mutations were shown to induce conformational changes near the hydrophobic binding cleft which prevented the non-covalent binding of artemisinin to PfATP6.

Another activity for artemisinin seems to involve the interference with the mitochondrial electron transport in the parasite. Indeed, artemisinins were shown to inhibit the res-

piratory chain of both sexual and asexual stages of *P. falciparum* [74]. Antimalarial activity might be triggered by the presence of an iron group in the cytochrome centre, which could induce artemisinins cleavage and free radical formation. It is worth noting that cytochrome c was one of the proteins identified as targets of artemisinin-mediated alkylation [68].

Artemisinins have also been shown to have other activities, such as the inhibition of parasite endocytosis (possibly caused by SERCA inhibition), and to have effects on the host's immune system. These are discussed in greater detail in a recent review by Golenser *et al.* [5].

Resistance to Artemisinins

Although no case of resistance to artemisinins in the field has been reported, increased tolerance has been observed [52, 75]. In one case, increase in tolerance was associated with a point mutation in PfATP6, as discussed above [75]. However, other genes have also been implicated in potential artemisinin resistance.

A field study from Thailand reported an association between *mdr-1* (multi-drug resistance-1) gene copy numbers and decreased susceptibility to artesunate [52]. Decreased suscrptibility with increasing copy numbers was also found *in vitro* [76], while most recently disruption of a *mdr-1* gene copy, resulted in an increased susceptibility to artemisinin, as well as other drugs [77]. Point mutations [54] have also been found to be associated with susceptibility to artemisinins. These findings may reflect the pivotal role of *mdr-1* in general drug resistance modulation, and rather than being driven by artemisinins themselves may merely reflect the parasite's adaptation to the widespread use of mefloquine, lumefantrine and other quinolines.

Pfcrt (chloroquine resistance transporter) mutations have also been implicated in conferring increased sensitivity to artemisinins [78]. Overexpression of *tctp*, whose protein is also alkylated by artemisinins, was implicated in transient resistance in a rodent model [79].

More recently, stable resistance to artemisinins in two *in vivo* rodent malaria models was achieved [80, 81]. Analysis of the major candidate genes for artemisinin resistance (*atp6*, *tctp , mdr-1* and the equivalent of *Pfcrt*) in the *P. chabaudi* resistant clones revealed no mutation associated with the resistant phenotype [80].

DRUG COMBINATION THERAPIES

Antifolates, quinolines and artemisinins have been deployed in various combination therapies with each other to both increase the efficacy of treatment as well as delaying the insurgence of drug resistance.

Two aspects are important when choosing drug combinations with the aim of containing the development and spread of drug resistance: different yet synergistic modes of actions and a good pharmacokinetic match. As recently discussed by Hastings and Watkins [82], mismatched drug combinations do not significantly impact the spread of resistance and may even risk jeopardizing the efficacy of components against which resistance has not yet been recorded (such as artemisinins). Accordingly, drug combinations need to have similar half-lives in order to keep a constant dual pressure on current and reinvading parasites. Ideally, drugs with short half-lives should be preferred, in order to reduce the exposure of reinvading parasites to suboptimal drug levels which may induce the selection for tolerance and eventually the development of resistance.

Here we will limit our analysis to the most promising combinations involving artemisinin combination therapy (ACT), as well as mentioning alternative strategies involving antifolate/quinoline combinations and drug resistance reversal.

Antifolate-Antifolate Combination Therapies

Pyrimethamine was combined with several sulfa drugs, as discussed in more detail by Nzila [3]. Of those only three combinations, Fansidar© (Sulfadoxine), Metakelfin© (sulfalene) and Maloprim© (dapsone) were commercially developed. Maloprim© however suffered from the different half-life of its components. While pyrimethamine has a relatively long half-life of approximately 95h [83], dapsone is considerably shorter lived (approximately 24h [84]). This resulted in a decreased synergy after the second day post treatment and thus low efficacy [85]. While both sulfadoxine and sulfalene have longer half-lives (180h and 65h respectively [83]), it is Fansidar© that found wide application in the treatment of malaria.

Proguanil has been combined with dapsone and found to be effective in some studies [86]. However, the more recent combination of chlorproguanil and dapsone (Lapdap©) has proved more efficient in treating malaria, due to chlorcycloguanil's higher efficacy [87]. Unlike pyrimethamine, proguanil and chlorproguanil have shorter half-lives (12-21h and 12-24 h respectively [88, 89] that closely match the half-life of dapsone and are thus better suited partner drugs for dapsone rather than sulfadoxine or sulfalene.

Other Antifolate Based Combinations

Antifolates have been combined with various quinolines in the past. Most combinations, such as pyrimethamine/sulfadoxine (PYR/SDX) with chloroquine or mefloquine, did not prove very effective, mainly due to the wide spread of resistance to antifolates and quinolines [90-92]. There were also issues with the different half-lives of the two components, with quinolines having considerably longer half-lives (with the exception of quinine). A combination that has shown some potential is PYR/SDX with amodiaquine. Although not recommended in areas where resistance to both drugs is now overwhelming (such as South East Asia or South America), it has shown some degree of success in some parts of Africa [92, 93]. However, due to the rapid spread of resistance, it is likely to remain only a short-term solution. Quinine has also been tried as a potential partner for PYR/SDX. With a relatively short-half life (16-18 h) [94], it represented a better partner from a pharmacokinetic point of view than other quinolines. However a potential use appears to be limited to areas where the efficacy of both drugs has not yet been compromised by resistance [95-97].

Proguanil has successfully been combined with atovaquone (**20**) as an effective combination (Malarone©) in the

treatment of malaria, including hepatocytic stages. The combination does not, however, rely on the antifolate activity of proguanil. Instead proguanil appears to potentiate the activity of atovaquone, which inhibits the parasite mitochondrial electron transport [98]. Atovaquone (31-72h, [99]) and proguanil (12-21h) are only slightly mismatched in their half-lives. In spite of this, resistance has already been recorded in Africa and is associated with a point mutation in the cytochrome b gene of the parasite's mitochondrion [100]. The ease with which the mutation can be acquired does not make Malarone© a suitable treatment for populations in endemic areas.

Artemisinin Combination Therapies

ACT has become the first treatment of choice in many countries. The principal combinations being adopted consist of a pairing of an artemisinin with a quinoline or an antifolate drug. Combinations with other drugs have also been tested, but not yet adopted on a wide scale.

The combination of artemisinins with quinolines has given mixed results. While the different mode of actions of the two components should guarantee a good synergy, the pharmacokinetic half-life differences between the short-lived artemisinins and the long-lived quinolines is a cause for concern regarding the long-term development and spread of drug resistance.

Artemisinins have been most prominently combined with the quinolines amodiaquine, mefloquine and lumefantrine. Artesunate-mefloquine has proved particularly effective in field trials [101]. However, the combination is expensive and presents a pharmacokinetic mismatch (mefloquine having a half-life between 14-21 days) [102] which could drive the spread of mefloquine resistance and jeopardize the efficacy of artemisinins in the long-term. Furthermore it has already shown significant failure rates in areas of high mefloquine resistance [56]. Pharmacokinetically the combination of artemether and lumefantrine (half-life: 3-6 days) [103] is better matched. However, it is also rather expensive and appears to be slightly less efficacious than artesunate-mefloquine in areas with a high prevalence of multidrug resistant parasites [104, 105]. Artesunate-amodiaquine has been recently introduced as an inexpensive combination therapy (ASAQ®) directed especially at Sub-Saharan Africa. ASAQ® also suffers from a pharmacokinetic mismatch due to the long half-life (9-18 days) of its active metabolite (desethylamodiaquine) [94]. Furthermore, some African studies have already indicated a significant failure rate [106, 107, 108] due to the presence of amodiaquine resistance. Resistance is likely to spread more rapidly as the combination is being adopted as the main treatment in several African countries.

The combination of artemisinins with antifolates also suffers from the widespread level of resistance to antifolates in various parts of the globe. The combination of artesunate with pyrimethamine/sulfadoxine was assessed in Africa, but gave mixed results [108-110], probably depending on the level of antifolate resistance present. Furthermore, the high rate of reinfection following treatment recently observed in a study in Uganda [111] is an indication of the poor pharmacokinetic match of this combination. It has been proposed that combining artesunate with the considerably shorter lived and more efficacious (chlor)proguanil/dapsone may present a better strategy in delaying the appearance and spread of resistant mutants [82], although trials using proguanil/dapsone with artesunate in Thailand showed a significant failure rate due to cross-resistance with pyrimethamine/sulfadoxine [112].

Artemisinins have also been combined with antibiotics with antimalarial properties. Clindamycin (**21**), a short lived antibiotic (2-4h) [113] targets the apicoplast and mitochondrion of malaria parasites through an unidentified mechanism and causes a delayed parasite death [114]. It has been combined with artesunate in a field trial resulting in a cure rate of 87% [115]. Fosmidomycin (**22**), a phosphonic acid originally developed as an antibiotic agent, inhibits isoprenoids biosynthesis in *Plasmodium* parasites by targeting 1-deoxy-D-xylulose 5-phosphate reductoisomerase of the nonmevalonate pathway located in the apicoplast [116]. Fosmidomycin has been tested both in rodent models and in a field trial in Africa [116, 117]. Its use as a monotherapy led to a recrudescence due to its short (1,5-2h) half-life and thus to its subsequent combination with other antimalarial drugs. In combination with artesunate, which is an ideal partner from a pharmacokinetic point of view, it resulted in complete clearance of parasitaemia in patients during a field trial [118].

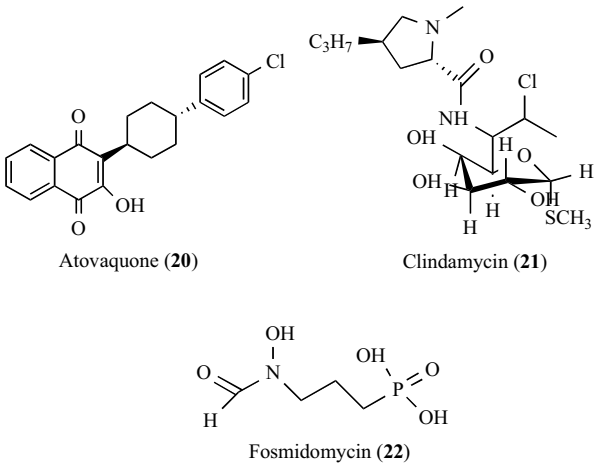
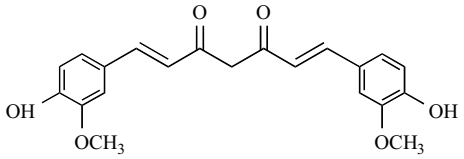


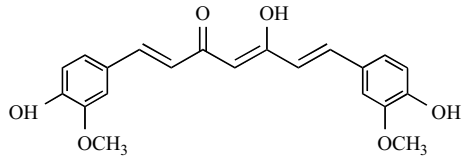
Fig. (5a) Other antimalarial partner drugs for artemisinins.

A recent experimental combination has involved the use of curcumin. Curcumin (**23**) is a natural polyphenolic compound extracted from the spice turmeric. It has been used in anti-tumour therapy and shown to have also anti-inflammatory and anti-oxidant properties [119]. It has also been shown to have anti-malarial properties [120]. Its target in malaria parasites has not yet been identified, although pfATP6 has been proposed as a target, while its pro-oxidant activity and its suppression of histone acetyltransferase has been recently elucidated [121]. In cancer cells, curcumin has also been shown to inhibit the proteasome function [122, 123]. Curcumin has shown a good synergy with artemisinin both *in vitro* and in *in vivo* animal trials [124]. With a short half-life (1-2h) closely similar to that of artemisinins, curcu-

min is also a potentially good partner drug for artemisinins. However, its poor bioavailability poses a serious issue which ought to be addressed before curcumin or a derivate can be seriously considered for antimalarial therapy.



Curcumin (Keto form) (**23**)



Curcumin (Enol form) (**23**)

Fig. (5b). Other antimalarial partner drugs for artemisinins.

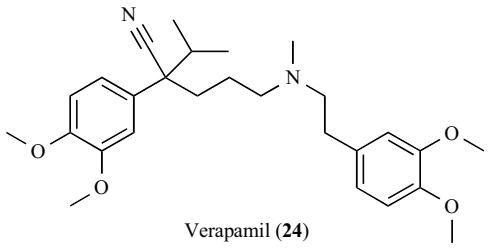
DRUG RESISTANCE REVERSAL

The availability of various quinoline and antifolate resistant clones has led to several studies on the chemical reversal of resistant phenotypes. An extensive amount of research has been done on using compounds to reverse resistant phenotypes to quinolines and in particular to chloroquine. Among these are calcium channel inhibitors (such as verapamil (**24**)), antihistamic drugs and calmodulin inhibitors. These compounds share a structural similarity in the form of two hydrophobic aromatic rings and the presence of a hydrogen bond acceptor [2]. The binding with their target has been proposed to involve an interaction between the hydrogen bond acceptor (usually a nitrogen atom) and the drug-resistant haplotype (CVIET) residues of PfCRT. This suggests a direct competition between chloroquine and resistance-reverser for CRT-binding, with the resistant genotype showing a greater affinity for reversers [2].

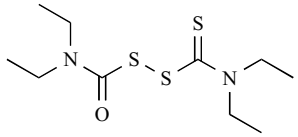
However, not all chloroquine-resistance reversers share this structure. Another group of compounds that have been used to reverse chloroquine resistance are antioxidant inhibitors, such as disulfiram (**25**), indomethacin (**26**) and acetaminophen (**27**) [125]. These compounds increase chloroquine activity by depleting the parasite's glutathione level, thus making it more susceptible to the oxidative stress causes by chloroquine and other quinolines. *In vivo* experiment in rodent models showed some synergy between these compounds and both chloroquine and amodiaquine [125]. The combination treatment did not, however, result in complete phenotype reversal, with morbidity and mortality still being present. It is possible that pharmacokinetic incompatibility played a role as well, as all drugs used had comparatively short half-lives (4-5 h for indomethacin [126], 2-3h for acetaminophen [127] and 2-5 days for disulfiram [128], compared with 1-2 months for chloroquine in humans).

NP-30 (**28**) and probenecid (**29**) also act as quinoline-resistance reversers. NP30 has a general effect in combination with several quinolines including chloroquine, quinine and mefloquine [129]. Probenecid, a uricosuric drug used to treat gout, was shown to chemosensitize chloroquine-resistant clones of *P. falciparum in vitro* by increasing chloroquine accumulation levels [130]. As probenecid functions as an inhibitor of organic anion transporters, the mechanism likely involved an inhibition of chloroquine efflux.

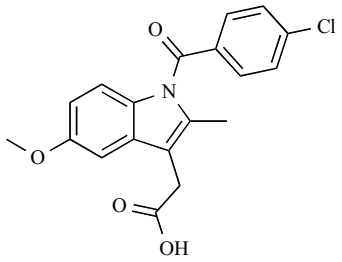
Antifolate resistance reversal has also been recently attempted by using probenecid, which was shown to inhibit folate and possibly pABA uptake from the parasite. *In vitro* studies showed a synergy between probenecid and pyrimethamine, sulfadoxine, chlocycloguanil and dapsone [130], while a field trial showed some interaction with pyrimethamine/sulfadoxine [131]. The latter, however, showed only a modest, short-term increase in treatment efficacy, which



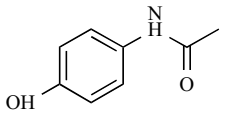
Verapamil (**24**)



Disulfiram (**25**)

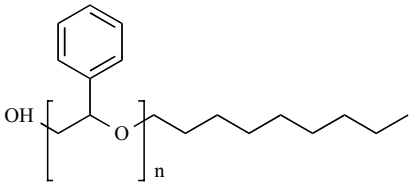


Indomethacin (**26**)

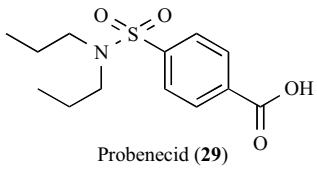


Acetaminophen (**27**)

Fig. (6a). Quinoline and antifolate resistance reversing agents.



NP-30 (**28**)



Probenecid (**29**)

Fig. (6b). Quinoline and antifolate resistance reversing agents.

might be partly explained by the considerably shorter half-life (4-9h) [132] of probenecid compared to pyrimethamine/sulfadoxine.

CONCLUSION

Current malaria treatment relies mainly on the use of three families of drugs with different modes of actions. Antifolates target to folate biosynthesis pathway of the parasite, quinolines induce oxidative stress by interfering with haemozoin metabolism, while the mechanism of artemisinins is still not fully understood, but appears to involve the production of free radicals and the selective alkylation of parasite proteins.

Resistance to both antifolates and quinolines is now common in several parts of the globe and seriously compromising the efficacy of those drugs. Several of the combinations that have been developed to counteract this trend are also losing their efficacy and are being replaced by ACT. However even ACT suffers from drawbacks, in spite of the fact that resistance to artemisinins has no yet been recorded in the field. In particular combination with partner drugs against which resistance is already widespread and pharmacokinetic incompatibility are a cause of concern and may not only prove ineffective at delaying the spread of drug resistance but may even compromise the efficacy of artemisinins. It is thus paramount to develop effective and compatible combinations in order to control the development and spread of drug resistance. Such combinations may also involve the use of drug resistance reversers, especially when those are inexpensive and have low toxicity to the patient. It is also worth remembering that mode of action, pharmacokinetic properties and good drug design can become irrelevant when proper standards for drug use are not observed and further compromised by sub-standard products, as is common in many parts of the developing world [133-135].

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