LITERATURE REVIEW: ARTEMISININ

The Discovery of Artemisinin

In the 1960s, during the Vietnam War, US and Vietnamese soldiers were heavily affected by malaria due to the emerging drug resistance to CQ. The possession of a highly effective antimalarial drug became the strategic factor of both armies. The United States failed to find the antimalarial wonder drug, while Vietnam sought help from China. The Chinese initiated major initiatives to develop safe, new and highly effective antimalarial drugs (Kuhn & Wang, 2008). A very important part of Traditional Chinese Medicine (TCM) is the use of herbs for the treatment of a disease (Ho, 2004). Consequently, the Chinese government began a systematic investigation of the plants used in TCM. More than 40 000 medicinal plant extracts and compounds were screened. In 1971, it was discovered that the ether extraction from the leaves of Qinghao – *Artemisia annua* L. – also called sweet wormwood or annual wormwood, displayed outstanding antimalarial activity (Kuhn & Wang, 2008; Ho, 2004).

A. annua grows in temperate climates, and is most widespread in China and Vietnam, but is also found and grown in East Africa, the United States, Russia, India and Brazil. The plant reaches an average height of two meters, has a single stem with alternating branches, fernlike leaves (Figure 1A), bright yellow flowers (Figure 1B) and a camphor-like scent. It is cross-pollinated by the wind or insects. Qinghao has been used since ancient times for the treatment of fever and chills, which was then understood by the Chinese as "hot and cold due to intermittent fever illness", as well as for internal heat conditions (Kuhn & Wang, 2008).

A   **B**

Figure 1 The leaves (A) and bright yellow flowers (B) of *Artemisia annua* L.

Physical and Chemical Properties of Artemisinin

Artemisinin (**1**) forms fine, colourless, needle-shaped crystals and has a melting point of 156-157 °C. It has no absorption in the UV range and a very low solubility in water (8,4 µg.mL^{-1} at 25 °C) and oil. Artemisinin is stable in neutral solvents, but is sensitive to acid and base treatment (Ho, 2004).

The structure of artemisinin was elucidated through chemical, spectroscopic and elemental techniques. The crystal structure of artemisinin was determined by X-ray crystallography. The internal peroxyl ketal acetal lactone makes the structure of artemisinin quite unique (Figure 2). The active pharmacophore for the antimalarial activity is the 1,2,4-trioxane system containing a peroxide bridge with two contiguous oxygen atoms linked *via* a carbon atom to a third non-peroxide oxygen atom (Kuhn & Wang, 2008; Ho, 2004).
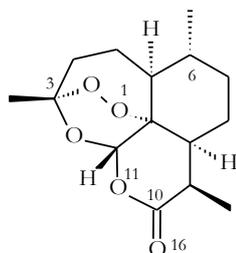

Figure 2 The chemical structure of artemisinin (**1**) with molecular formula, $C_{15}H_{22}O_5$.

Semi-Synthetic Analogues of Artemisinin

The first chemical modifications on artemisinin (**1**) were started on the lactone group, which is the most accessible functionality for chemical intervention, leaving the pharmacophore intact (Kuhn & Wang, 2008).

First-Generation Artemisinin Analogues

Dihydroartemisinin (DHA) (**2**) was prepared by reduction of the lactone carbonyl at C-10 of artemisinin with sodium borohydride in methanol (Figure 3) (Muraleedharan & Avery, 2009). The reduction generates the lactol hemi-acetal as a mixture of diastereomers. The hydroxyl group is either in the "alpha" (α) or "down" as drawn (Figure 3) or "beta" (β) or "up" as drawn (Figure 3). The α- and β-forms of DHA are referred to as epimers. These epimers undergo equilibration in solution (depending on the solvent), by ring opening, which involves an intermediate aldehyde.

The pure β-compound may be obtained by selective crystallisation from the mixture of epimers in solution (Luo *et al.*, 1984; Haynes *et al.*, 2002).
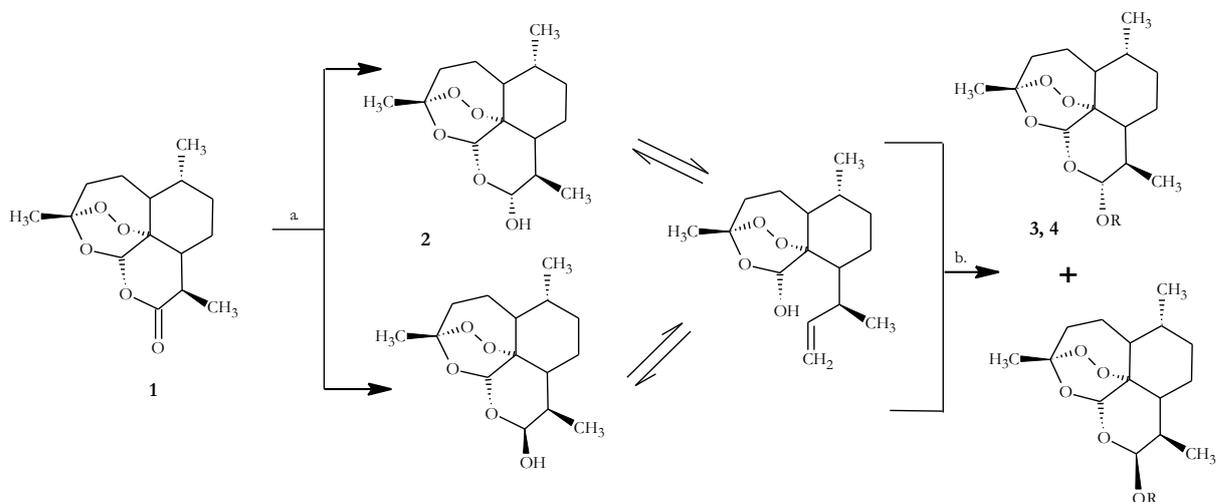

Figure 3 The preparation of dihydroartemisinin (**2**) from artemisinin (**1**), with the conversion into artemether (R=CH$_3$) and arteether (R=CH$_2$CH$_3$). The pure β-isomers of artemether (**3**) and arteether (**4**) are isolated by fractional crystallisation. a. NaBH$_4$, CH$_3$OH, 0 °C; b. CH$_3$OH or CH$_3$CH$_2$OH, Brønsted or Lewis acid (Ho, 2004).

Although the antimalarial activity of DHA is six times greater than that of the parent compound (Janse *et al.*, 1994), as a hemi-acetal, it is considerably less stable *in vivo* (Kuhn & Wang, 2008; Ho, 2004). The lactol ethers of DHA, artemether (**3**) and arteether (**4**) (Figure 4) (Kuhn & Wang, 2008), are oil-soluble derivatives, and therefore well absorbed intramuscularly (Muraleedharan & Avery, 2009). Through esterification, DHA was converted into the lactol hemi-ester, artesunate (**5**) (Figure 4). This water-soluble artemisinin derivative is used intravenously for the treatment of advanced cases of malaria (Kuhn & Wang, 2008). These analogues (**3**-**5**) are found to be highly effective and fast-acting agents against uncomplicated *P. falciparum* infections and severe malaria. However, as acid-labile acetals, they are rapidly metabolised to DHA, and therefore exhibit short biological half-lives (Baker, McChesney & Chi 1993). Artelinic acid (**6**) (Figure 4), a new water-soluble derivative of artemisinin, displayed a greater hydrolytic stability in aqueous solution in comparison with artemisinin and the above-mentioned derivatives (**2**-**5**). It also has the highest oral bioavailability and longest elimination half-life (t$_{1/2}$ = 2.6 h) (Li *et al.*, 1998), but is three times more toxic than artesunate (Li *et al.*, 2005). Artelinic acid was too toxic, and its use was discontinued.
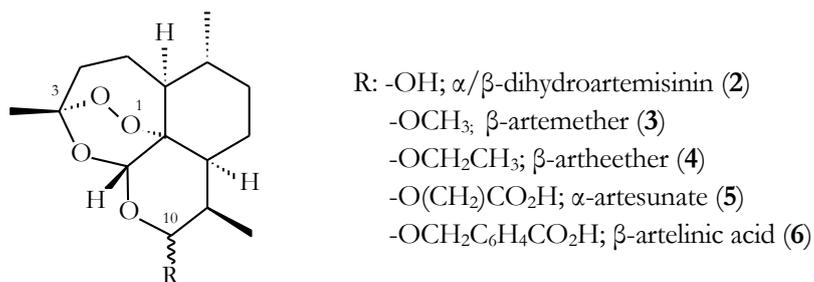
R: -OH; α/β-dihydroartemisinin (**2**)
-OCH$_3$; β-artemether (**3**)
-OCH$_2$CH$_3$; β-artheether (**4**)
-O(CH$_2$)CO$_2$H; α-artesunate (**5**)
-OCH$_2$C$_6$H$_4$CO$_2$H; β-artelinic acid (**6**)

Figure 4 The first-generation derivatives of artemisinin (**2** - **6**).

C-10 Acetal Analogues of Artemisinin

The new C-10 acetal derivatives were designed by introducing bulky groups in order to block or slow down the hydrolysis of the C-10 bond (Lin & Miller, 1995). The introduction of an aromatic group to artelinic acid (**6**) afforded new analogues (Figure 5A). Compounds with an electron-withdrawing functional group on their aromatic ring showed a substantial increase in antimalarial activity (Lin & Miller, 1995).

The 10-phenoxy derivatives (Figure 5B), designed by O'Neill *et al.* (2001a)*,* possessed an aryl instead of an alkyl group, and were expected to be more resistant to CYP-mediated oxidative dearylation to DHA (**2**) (Ploypradith, 2004). These analogues have a higher antiplasmodial activity compared with artemether (**3**) (O'Neill *et al.*, 2001a), but further work is required to determine their *in vivo* pharmacokinetics and hydrolytic stability.
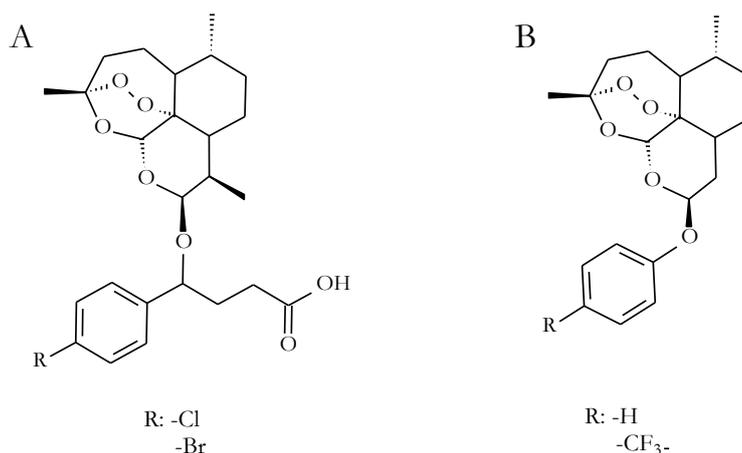

A

B

R: -Cl
-Br

R: -H
-CF$_3$-

Figure 5 The C-10 acetal (A) and -phenoxy (B) analogues of artemisinin.

C-10 Carba Analogues of Artemisinin

These analogues were designed to be more chemically robust in acidic conditions such as those found in the stomach. The simplest derivative, deoxyartemisinin (**7**), was 10 times more stable than artemether (**3**) (Jung *et al.*, 2002). The C-10 naphthyl (**8**) and heteroaryl analogues (**9**) (Figure 6) all showed great *in vitro* activity in comparison with artemisinin, but similar to that of artemether (Sinishtaj, 2008).
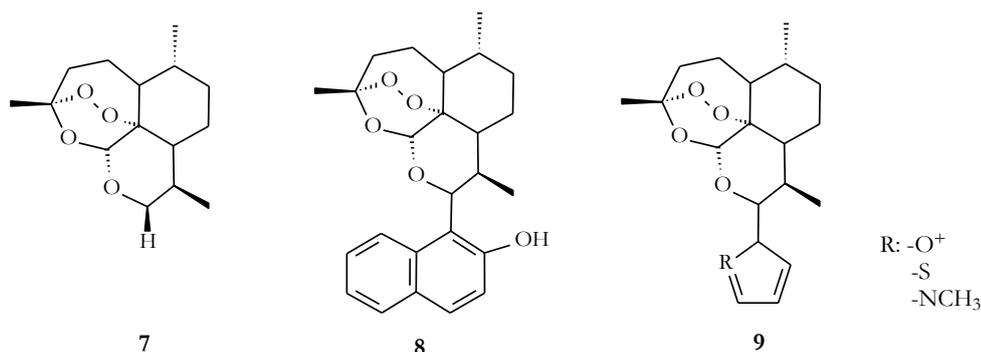

Figure 6 The C-10 carba analogues of artemisinin: deoxyartemisinin (**7**), C-10 napthyl (**8**) and -heteroaryl (**9**) derivatives.

Artemisinin Hybrids

Antimalarial hybrids are composed of two distinct antimalarial moieties joined covalently (Figure 7). In combination therapies, the risk of treatment failure is reduced and the partner drug may be protected from the spread of resistance. Fundamental design limitations, characteristic of a single targeting agent, can be addressed when in the form of a hybrid – especially with respect to lipophilicity, solubility, metabolic stability, bioavailability, potency, side effects, toxicity, etc. (Morphy, Kay & Rankovic, 2004). An added benefit of some antimalarial combinations is synergism (Angus, 2005), which will allow lower doses and a potentially wider safety margin.

Figure 7 Diagrams presenting the idea of a hybrid drug (Meunier, 2008).

At first, particular consideration should be given to: (i) the mechanism of action of the individual ligands, (ii) the nature of the linker unit employed, (iii) the distance between the individual components, and, (iv) the molecular geometry, if known, of the individual ligand-binding sites

(Buijsman *et al.*, 1999; Passarella *et al.*, 2008). The nature of the linker unit used to attach the individual components should be chosen carefully to allow the intact hybrid to dissociate into its individual components following administration (cleavable conjugates) or to remain fixed when metabolically resistant linker units are chosen. When constructing a hybrid molecule, the greatest design challenge is to ensure that there is an appropriate number of hydrogen-bond donor and acceptor sites, and the solubility characteristics and molecular weight to confer drug-like properties (Lipinski *et al.*, 1997).

Given the WHO's current preference for ACT for malaria first-line treatment (World Health Organisation, 2011), the hybrid drug would have to compare favourably not only with the known single agents but also with the combinations in use. Therefore, it is crucial to ask whether the hybrids offer an advantage over a 1:1 combination of the two constituent drugs. The hybrid may simply be less active than the combination of the individual constituents, for example, because the increased bulkiness of the molecule reduces penetration into the parasite cell.

Endoperoxide-and Quinoline-Based Hybrids

Walsh *et al.* (2007) synthesised an artemisinin–quinine hybrid (**10**) (Figure 8) by coupling dihydroartemisinin directly to the carboxylic acid derivative of quinine *via* an ester-linkage (Walsh *et al.*, 2007). The hybrid had potent *in vitro* activity against the 3D7-sensitive and FcB1-resistant strains of *P. falciparum*, superior to that of quinine alone, artemisinin alone and of the 1:1 combination of the two drugs, suggesting a tangible benefit in terms of activity from linking the two molecules covalently (Walsh & Bell, 2009).
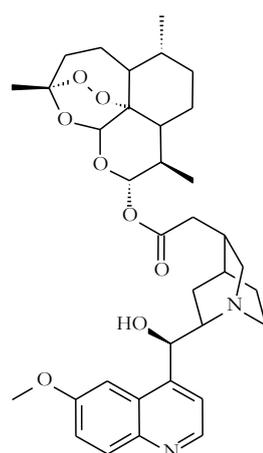

Figure 8 Artemisinin-quinine hybrid (**10**) as synthesised by Walsh *et al.* (2007)

Grellepois *et al.* combined trifluoromethylated artemisinin derivatives with mefloquine, using a cleavable diester and non-cleavable C–N linkage (Figure 9). The activity *in vitro* of the cleavable

hybrid (**11**) was comparable to that of artemether and trifluoromethyl artemisinin and superior to that of mefloquine. No comparison was made with a 1:1 mixture of the starting moieties. The non-cleavable hybrid (**12**) was less active, suggesting that the release of the individual components may be required for optimal activity. The relative proximity to each other of the individual units may also be an obstacle for optimum binding of the individual components to their respective targets, in the case of the non-cleavable hybrid (Grelepois *et al.*, 2005).
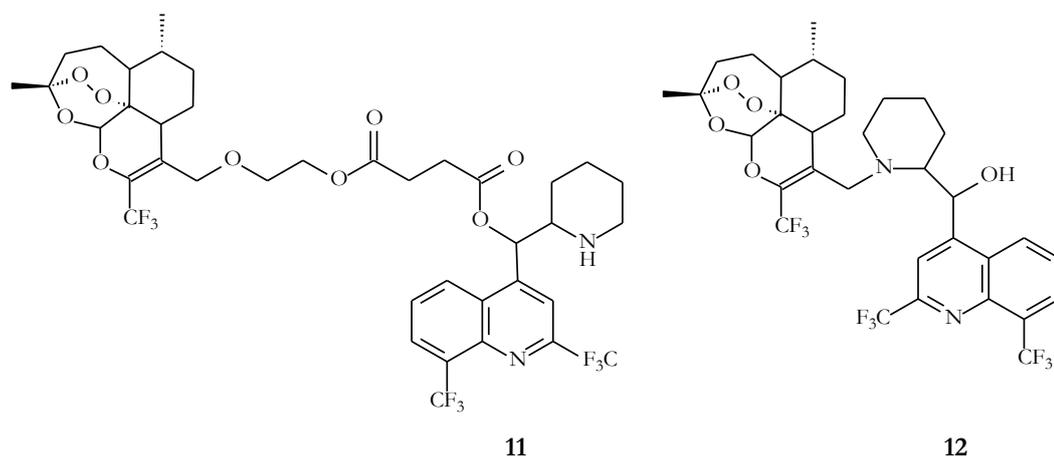

11   **12**

Figure 9 The cleavable (**11**) and non-cleavable (**12**) trifluoromethylated artemisinin-mefloquine hybrids.

The trioxaquine series (Figure 10), the most extensive and best characterised group of hybrid antimalarials, was originally designed by Meunier and co-workers. Synthetic peroxides (trioxanes or trioxolanes), with similar properties to artemisinins, were linked to aminoquinolines, in particular chloroquine. Invariably, the trioxanes have a cyclohexane ring fused to the trioxane, and in the case of the trioxolanes, a spiro carbon atom to promote the generation of an alkylating C-centred radical entity. These hybrids were in their citrate salt form (Meunier, 2008). The activities of the best trioxaquines against cultured parasites were generally superior to that of chloroquine and were maintained against chloroquine-resistant strains. However, activities were similar or slightly inferior to those of artemisinin and/or artesunate (Basco *et al.*, 2001; Dechy-Cabaret *et al.*, 2003; Dechy-Cabaret *et al.*, 2004; Benoit-Vical *et al.*, 2007; Cosledan *et al.*, 2008). A number of the trioxaquines have shown good activity against mouse malarial parasites (Dechy-Cabaret *et al.*, 2004; Benoit-Vical *et al.*, 2007; Cosledan *et al.*, 2008) and *P. falciparum* in mice (Cosledan *et al.*, 2008) when administered by oral or other routes. Trioxaquine PA1103-SAR116242 was in pre-clinical development for uncomplicated malaria by Palumed in collaboration with Sanofi-Aventis (Meunier, 2008).
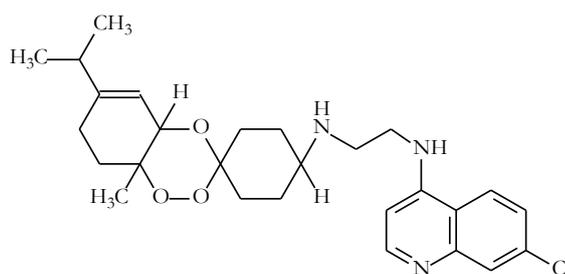
Figure 10 The structure of a trioxaquine

Tetraoxane moieties (Figure 11) were linked to aminoquinoline *via* an amide linkage, but required relatively high doses (>80 mg/kg/day) to cure *P. berghei* in mice, in spite of high activity on cultured *P. falciparum* (IC_{50} as low as 2 nM). Trioxaquines produced by Singh *et al.* (Singh, Malik & Puri, 2004), had limited activity at 96 mg/kg/day, inferior to that of artemisinin or chloroquine individually.

Figure 11 The structure of 1,2,4,5-tetraoxane

Artemisinin can also be modified on C-16 as was done by Paitayatat *et al.* (Paitayatat *et al.*, 1997) C-16-functionalized artemisinin derivatives (Figure 12) were obtained through a one-pot synthesis of artemisitene (**13**), followed by Michael addition with several nucleophiles. These derivatives inhibited a significant interaction with ferroprotoporphyrin IX.
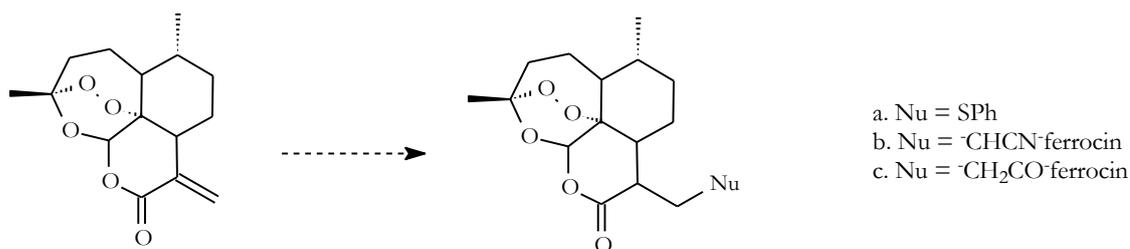

a. Nu = SPh
b. Nu = ⁻CHCN⁻ferrocin
c. Nu = ⁻CH₂CO⁻ferrocin

Figure 12 The synthesis of C-16 derivatives from artemistene (**13**).

Artemisinin Dimers

Investigations into artemisinin dimers, consisting of two structurally similar monomers, were prompted because of the metabolic instability of artemisinin and its derivatives'. The presence of a metabolically susceptible C-10 acetal linkage in these derivatives leads to rapid metabolism to DHA (**2**), and subsequent rapid clearance from the body. The replacement of the O-16 atom linked to C-10 with a C-atom produces dimers with a greater hydrolytic stability, resulting in a

prolonged half-life (O'Neill *et al.*, 1999; O'Neill *et al.*, 2001b). These dimers possess significant antimalarial and antiproliferative activities (Posner *et al.*, 1997; Jeyadevan *et al.*, 2004; Chadwick *et al.*, 2009; Galal *et al.*, 2009; Rosenthal *et al.*, 2009; Slade *et al.*, 2009; Stockwin *et al.*, 2009). However, as with the hybrids already discussed above, the importance of the linker is evident, as seen below.

A series of C-10 non-acetal dimer of 10β-(2-hydroxyethyl)deoxoartemisinin was prepared, which displayed low nanomolar antimalarial activities against K1 and HB3 strains of *P. falciparum*. Two of the phosphate ester dimers (**14**) (Figure 13) expressed growth-inhibitory (GI_{50}) values lower than 10 nM against a range of cancer cells in the National Cancer Institute (NCI) 60-cell line assay. The total growth inhibition (TGI) values were in the range of 1-10 μM against leukaemia, colon and melanoma cell lines. Interestingly, phosphate ester monomers were inactive as anticancer agents, even at concentrations in the millimolar region, emphasising the importance of two trioxane units for high antiproliferative activity. However, the two trioxane units alone are not sufficient; the separation between the two units is crucial. Dimers bearing longer linkers have poor antiproliferative activity. It was found that all cell lines sensitive to the peroxides overexpressed transferrin (an endogenous protein that transports iron from the circulation into cells) (Jeyadevan *et al.*, 2004).

A series of C-10 non-acetal dimers with varying linker lengths was prepared by Chadwick *et.al.* Anticancer activity was determined against human promyelocytic leukemia HL-60 cells, which are known to be particular sensitive to artemisinin derivatives. Activity decreased with reduced linker length. A methyl phosphate dimer (**15**) was the most potent (IC_{50} = 0.05 μM), whereas the amide-linked dimer (**16**) (Figure 13) displayed the best antiplasmodial activity against the CQ-sensitive (3D7) strain of *P. falciparum* (IC_{50} = 0.03 nM) (Chadwick *et al.*, 2009).
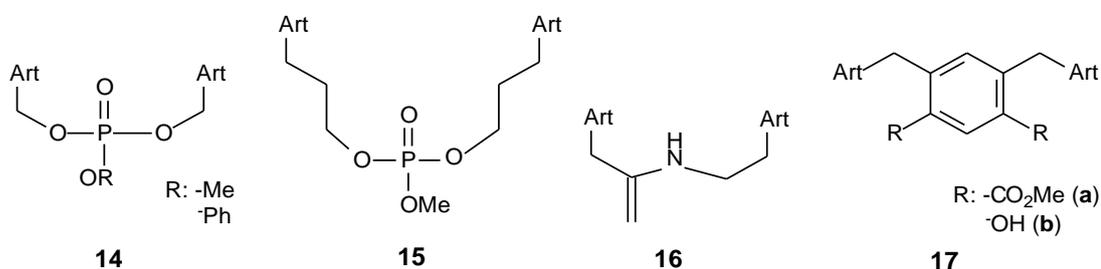

Figure 13 The phosphate ester (**14**), methyl phosphate ester (**15**), amide-linked (**16**) and bis-ester (**17a**) and -diol (**17b**) dimers of artemisinin (Art = artemisinin nucleus).

Single-dose treatment is one of the ultimate objectives of drug synthesis in the treatment of malaria, as that would ensure 100% patient compliance. Trioxane dimers, synthesized by Posner *et. al.*, prolonged mouse survival between 8.7 and 13.7 days with a single oral dose of 30 mg/kg. When given 3 x 30 mg/kg orally, malaria-infected mice were cured completely, whereas sodium artesunate prolonged average mouse survival to only 7.2 days with the same dose regimen (Posner *et al.*, 2008). A 5-carbon-linked trioxane dimerorthoester sulfone was administered orally to malaria-infected mice using a single dose of only 6 mg/kg body weight along with 18 mg/kg of mefloquine hydrochloride, and cured mice completely and safely. When artemether (6 mg/kg) plus lumefantrine (18 mg/kg) was given as a single dose, the average survival of mice was 24 days (Moon *et al.*, 2011).

Dimers synthesised by Paik *et al.* (2006), were administered as a single dose of 3, 10, or 30 mg/kg, either subcutaneously (sc) or orally (po). The bis-ester (**17a**) and diol (**17b**) dimer (Figure 13) displayed antimalarial sc ED_{50} values of 0.71 mg/kg and 0.06 mg/kg, respectively. The dimeric diol had a po ED_{50} value of 2.6 mg/kg, whereas the clinically used monomeric trioxane sodium artesunate had a sc ED_{50} of 2.2 mg/kg and po ED_{50} of 4.0 mg/kg. Thus, these two dimers were approximately 3-37 times more efficacious than artesunate administered sc, and the dimeric diol was approximately 1.5 times more efficacious than sodium artesunate administered po (Paik *et al.*, 2006).

New Derivatives

The need for new, fast-acting and effective antimalarial drugs has never been greater, especially with the emergence of artemisinin resistance, which threatens the conventional therapy. In conjunction with the Medicines for Malaria Venture (MMV), Charman and co-workers designed and optimised a complety synthetic ozonide antimalarial based upon the 1,2,4-trioxolane pharmacophore. Ozonide OZ439 (**18**) has successfully completed Phase I clinical trials, where it showed a maximum tolerated dose of 1600 mg in animal studies. It is currently undergoing Phase IIa trials in malaria patients (Charman *et al.*, 2011). OZ439 was a follow-up to OZ277 (**19**) (Figure 14), the first synthetic ozonide to be evaluated clinically. OZ277 was in Phase III clinical trials as a combination product with piperaquine phosphate (Vennerstrom *et al.*, 2004; Olliaro & Wells, 2009), but has recently been launched by Ranbaxy, an Indian pharmaceutical firm (Chemistry World, 2012). OZ277 exhibits antimalarial activity against all asexual blood stages of *P. falciparum* (Maerki *et al.*, 2006, Hofer *et al.*, 2008). In Phase I clinical trials, the half-life in healthy volunteers was about two- to three-fold longer than that of DHA (**2**) (1.4h *vs.* 0.5 for DHA)

(Orrell *et al.*, 2008). However, when administered to malaria patients as monotherapy (Valecha *et al.*, 2010), OZ277 displayed reduced plasma exposure compared with that in healthy volunteers (Olliaro & Wells, 2009). Therefore, the focus in designing the next generation of ozonides was to define the mechanistic basis for clearance and degradation of the first-generation ozonide, OZ277. The critical design criteria that led to the selection of OZ439 as a clinical candidate was the relationship between Fe(II)-mediated activation and antimalarial activity. The next-generation ozonides contained a *cis*-8'-phenyl substituent and were >50-fold more stable to Fe(II)-mediated degradation compared with first-generation ozonides, which contained a *cis*-8'-alkyl group. OZ439 displayed a significant reduction in clearance and overall improvement in bioavailability, increased volume of distribution as a result of an increase in Log D, and, together with reduced clearance, this contributed to a long half-life of greater than 20 h in rats following oral dosing. Therefore OZ439 completely cures *Plasmodium berghei*-infected mice with a single oral dose of 20 mg/kg and exhibits prophylactic activity superior to that of the benchmark chemoprophylactic agent, mefloquine. The outstanding efficacy and prolonged blood concentrations of OZ439 were the result of a design strategy that stabilises the intrinsically unstable pharmacophoric peroxide bond, thereby reducing clearance yet maintaining the necessary Fe-(II)-reactivity to elicit parasite death (Charman *et al.*, 2011).
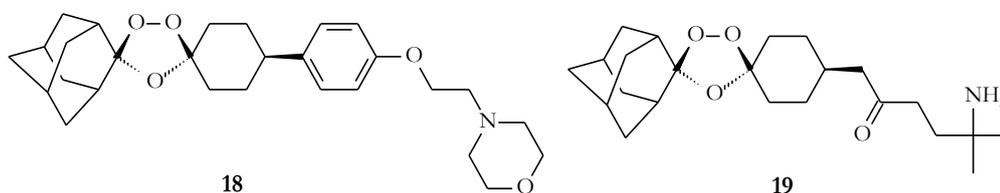

Figure 14 Structures of ozonides OZ439 (**18**) and OZ277 (**19**).

With emphasis placed on improving the systemic behaviour of artemisinin, artemisone (**20**) (Figure 15) was synthesised. Artemisone has shown that artemisinins which are structurally distinct from, but significantly more active than current artemisinins, can be prepared through embedding metabolically inert polar groups within alkylamino substituents attached directly to C-10 of the artemisinin nucleus. A different metabolic profile from the current artemisinins was displayed by artemisone, with favourable physicochemical properties and negligible neuro- and cytotoxicity. Inhibition of one of the possible targets, the parasite Ca^{2+}-pump, PfATP6, was much greater for artemisone than artemisinin (ED_{90} = 1.5 mg/kg sc for artemisone *vs.* ED_{90} = 12 mg/kg sc for artesunate (**5**)) (Haynes *et al.*, 2006). Artemisone has been through a Phase II trial for non-resistant malaria, where it displays faster parasite clearance times than any other artemisinin, and is curative

at one-third the dose of currently the most active artemisinin derivative, artesunate. If the resistant parasites found along the Thai-Cambodian border prove sensitive to it, it will be extremely useful (Enserink, 2010).
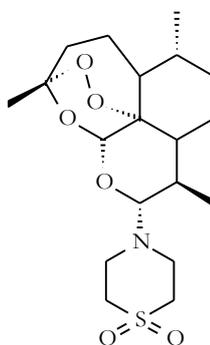

Figure 15 Structure of artemisone (**20**), an artemisinin derivative.

Antimalarial Activity and Mechanism of Action

Artemisinin, with its active derivative DHA (**2**), had become the first-line antimalarial treatment in areas of multi-drug resistance. However, monotherapy with artemisinin drugs results in high recrudescence rates, with the underlying mechanism being autoinduction of CYP-mediated metabolism (Liu *et al.*, 2011). Although a number of potential targets have been proposed, the actual mechanism of action remains ambiguous. Understanding the mechanism of action of this class of drugs will allow the prediction of potential resistance mechanisms and aid targeted design of future antimalarial agents (O'Neill, Barton & Ward, 2010).

Unlike quinoline-based antimalarials (e.g. chloroquine), which have only one well-documented mechanism of action, artemisinin kills malaria parasites by generating more than one type of cytotoxic intermediate. Although protein alkylation in *Plasmodia* is well established, a single molecular target having a direct role in cell death must still be identified (O'Neill, Barton & Ward, 2010).

The currently accepted mechanism of trioxane antimalarial action involves the generation of free radicals within or near susceptible sites, probably arising from the production of distonic radical anions (Drew *et al.*, 2006). This is believed to be triggered by ferrous iron to produce several different types of highly reactive intermediates (e.g. oxy radicals, carbon radicals, high-valent iron-oxo species) as well as several different kinds of longer-lived neutral electrophiles (e.g. epoxides, aldehydes, and dicarbonyl compounds (Posner & O'Neill, 2004)). Molecular calculations confirmed that the bioisosteric substitution of O3 most strongly influences the generation and

subsequent stability of both oxygen and carbon-centred radicals, which contributes to parasiticidal action (Drew *et al.*, 2007). An alternative mechanistic proposal involved the ionic scission of the peroxide group and consequent generation of a carbocation at C4. Drew *et al.* (2006) investigated the latter mechanism and the consequent decomposition pathways and hydrolysis sites. The preferred Lewis acid protonation sites for artemisinin and arteether were found to be O5>>O4≈ O3 > O2 > O1 and O4 ≥ O3 > O5>>O2>O1, respectively (see Figure 16 for numbering system). As protonation was unlikely to occur on the peroxide bond O1-O2 in either of the molecules, the alternative radical pathway remained the most likely explanation to account for antimalarial activity, they concluded (Drew *et al.*, 2006).
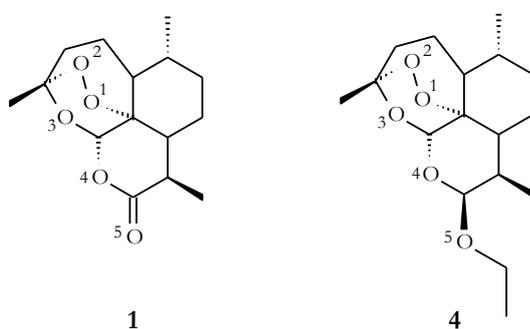

1 **4**

Figure 16 Numbering system used for the oxygen skeleton of artemisinin (**1**) and arteether (**4**), respectively (Drew *et al.*, 2006).

Artemisinin and its derivatives have also been investigated for their antiproliferative effects against a wide range of cancer cell lines (Krishna *et al.*, 2008). The monomeric forms of artemisinin have superior activity in the treatment of malaria, whereas the dimeric forms display enhanced anticancer activities (Posner *et al.*, 1997; Chadwick *et al.*, 2009; Arav-Boger *et al.*, 2010). The molecular targets of both artemisinin in *Plasmodia* and tumour cells are still under debate. However, in both cases, strong evidence suggests that the primary activator is an iron source, either in ferrous (Fe^{2+}) form, haem or both. This source mediates the release of reactive oxygen species (ROS) (Efferth 2006) and/or carbon-centred radicals (Mercer *et al.*, 2007), which may play an important role in inducing DNA damage, mitochondrial depolarisation and apoptosis induction (Stockwin *et al.*, 2009). It is important to consider the effect of artemisinin in cancer cells with respect to similarities and differences in their action in malaria chemotherapy, factors which may be important in elucidating potential common mechanisms of action (O'Neill, Barton & Ward, 2010).

What is apparent is the multi-faceted nature of cellular response to artemisinin and its derivatives in *Plasmodia* and tumour cells. Continuing responses may explain how this drug can be used against otherwise multi-drug resistant cells in both tumours and *Plasmodia*, to guide rational design of valuable, new, orally active antimalarial peroxides.

Pharmacokinetics and Pharmacodynamics

Artemisinin (**1**), the parent compound, exhibits remarkable dose- and time-dependent pharmacokinetics and a saturable first-pass metabolism (Ashton *et al.*, 1998). Studies conducted on escalating doses of artemisinin revealed higher concentrations towards the end of the treatment period (Gordi *et al.*, 2002), whereas repeated oral administration over five days was associated with an increase in oral clearance as result of artemisinin's pronounced capacity for autoinduction (Gupta, Svensson & Ashton, 2001; Asimus & Gordi, 2007). Artemisinin and its derivatives act much more rapidly than the quinoline antimalarials, but are chemically less stable and have a higher first-pass metabolism, which is reflected in their relatively low bioavailability. Oral bioavailability in animals ranges between 19 and 35%. Due to their high liphophilicity, the artemisinins tend to cross the blood-brain barrier, causing neurotoxicity in animal models (Gautam *et al.*, 2009). The combination of the artemisinins with other antimalarials does not influence their pharmacokinetics. Interactions of the artemisinins with cytochrome P450 inhibitors are documented, but these affect neither their efficacy nor their toxicity (Giao & de Vries, 2001; Medhi *et al.*, 2009).

The rapid *in vivo* elimination of artemisinin and its derivatives and their unusual pharmacokinetic properties requires a sensitive, robust and accurate bio-analytical method for quantification. This has traditionally been problematic since these compounds do not have UV or fluorescent chromophores. Sensitive and specific liquid chromatography tandem mass spectrometric (LC-MS/MS) methods were developed and validated for the quantification of artemisinin and DHA in human heparinised plasma and rat plasma, respectively. The limit of detection was 0.257 ng/mL for artemisinin and the calibration range was 1.03–762 ng/mL using 50 μL plasma (Lindegardh *et al.*, 2009). For β-dihydroartemisinin, the method had a lower limit of quantification (LLOQ) of 0.2 ng/mL in 100 μL of plasma (Xing *et al.*, 2007).

Furthermore, the semi-synthetic derivatives of artemisinin are metabolised to its major metabolite DHA or the so-called artenimol, which possesses greater antimalarial potency than the parent drug. It is therefore important to monitor DHA in addition to the parent drug for pharmacokinetic studies (Xing *et al.*, 2007).

DHA has a high binding capacity with both rat and human plasma proteins (76–82%). The total concentration was two-fold higher in rat brain than in plasma and three- to four-fold higher in red blood cells (RBC) than in the plasma. This confirms that the powerful antimalarial potency of DHA in the treatment of blood stage malaria may relate to the high RBC binding (Xie *et al.*, 2009). The mean values for C_{max}, t_{max}, AUC_{0-t} and CL/F of β-DHA in rat plasma were 142.2 ± 21.1 ng/mL, 0.8 ± 0.1 h, 145.8 ± 33.6 ng h/mL and 1.19 ±0.25 L/min/kg, respectively (Xing *et al.*, 2007). The $t_{1/2}$ of ip-administered DHA (Figure 17) in malaria-infected and healthy mice (19–25 min) was approximately two- to three-fold shorter than in humans (40–70 min), whereas CL/F and V/F were substantially larger than in humans (Batty *et al.*, 2008). The mean terminal half-lives of rat plasma and blood radioactivity (75.57-122.13 h) were significantly prolonged compared with that of unchanged DHA (1.03 h) (Xie *et al.*, 2009).
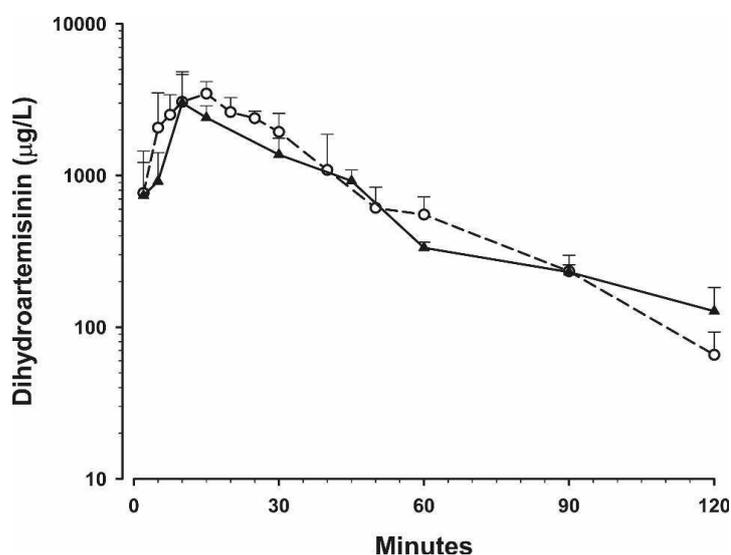

Figure 17 A plasma concentration–time profile for DHA in
P. berghei malaria-infected (▲) and uninfected (o) Swiss mice.
Data are means ± SD for 3–6 mice (Batty *et al.*, 2008).

Metabolism

Artemisinin and its derivatives are converted primarily, but to different extents, to the bioactive metabolite artenimol. The rate of conversion is the lowest for artelinic acid (**6**), which was designed to protect the molecule against metabolism, and highest for the water-soluble artesunate (**5**) (Gautam *et al.*, 2009; Navaratnam *et al.*, 2000). Such conversion occurs largely in the liver by CYP-enzymes (Gautam *et al.*, 2009). However, monotherapy with artemisinin drugs results in comparatively high recrudescence rates. The proposed mechanism underlying this is the saturable first-pass metabolism of artemisinin (Gordi *et al.*, 2002), which influences its

CYP-mediated autoinduction metabolism (Ashton *et al.*, 1998), resulting in a reduced exposure of the drug.

Although pathways of artemisinin elimination are poorly understood, available data suggest hepatic metabolism as the main route of elimination. Because of drug re-absorption in the intestines (enterohepatic circulation), the majority of eliminated DHA is through urinary excretion (Xie *et al.*, 2009). Four metabolites, deoxyartemisinin, dihydrodeoxyartemisinin, deoxydihydroartemisinin, and the so-called "crystal-7" (all lacking the endoperoxide bridge), have been identified in urine after oral administration of artemisinin to humans (Ashton *et al.* 1998; Lee & Hufford 1990). Artemether and arteether are metabolised to release methanol and ethanol *in vivo*, respectively (Drew *et al.*, 2007).

The intake of artemisinin antimalarials affects the activities of several principal human drug-metabolising CYP450 enzymes including CYP3A and CYP2C19, which are increased, and CYP1A2 and CYP2D6, which are decreased by artemisinin (Asimus *et al.,* 2007). Artemisinin is also an inducer of CYP2A6 and glucuronidation (Asimus *et al.*, 2008).

α-DHA-β-glucuronide has been identified as the major metabolite, and UGT1A9 (UDP-glucuronosyltransferases) and UGT2B7 as the predominant isoforms involved in the clearance of DHA from humans (Ilett *et al.*, 2002). Other metabolites are desoxy-DHA and its glucuronide, 3-hydroxydesoxy-DHA glucuronide, and the glucuronide of a ring-contracted tetrahydrofuran acetate isomer of DHA. These metabolites are products of reductive cleavage and rearrangement of the endoperoxide bridge, a process known to generate reactive radical intermediates and for abolishing antimalarial activity (Maggs *et al.*, 1997).

The biotransformation of artemisinin and its active derivative DHA was evaluated *in vitro* and *in vivo*, using the LTQ-Orbitrap hybrid mass spectrometer in conjunction with online hydrogen/deuterium exchange high-resolution (HR)-LC/MS (mass spectrometry) for rapid structural characterisation. Thirteen Phase I metabolites of artemisinin were identified in liver microsomal incubates, rat urine, bile and plasma, including six deoxyhydroxylated, five hydroxylated and one dihydroxylated metabolite and deoxyartemisinin. Twelve Phase II metabolites were detected in rat bile, urine and plasma. DHA underwent similar metabolic pathways. Thirteen Phase I metabolites and three phase II metabolites were detected. Artemisinin drugs mainly undergo hydroxylation and loss of oxygen (deoxyl products) for their Phase I metabolic pathways and are subsequently subjected to Phase II glucuronidation processes (Figure 18) (Liu *et al.*, 2011).
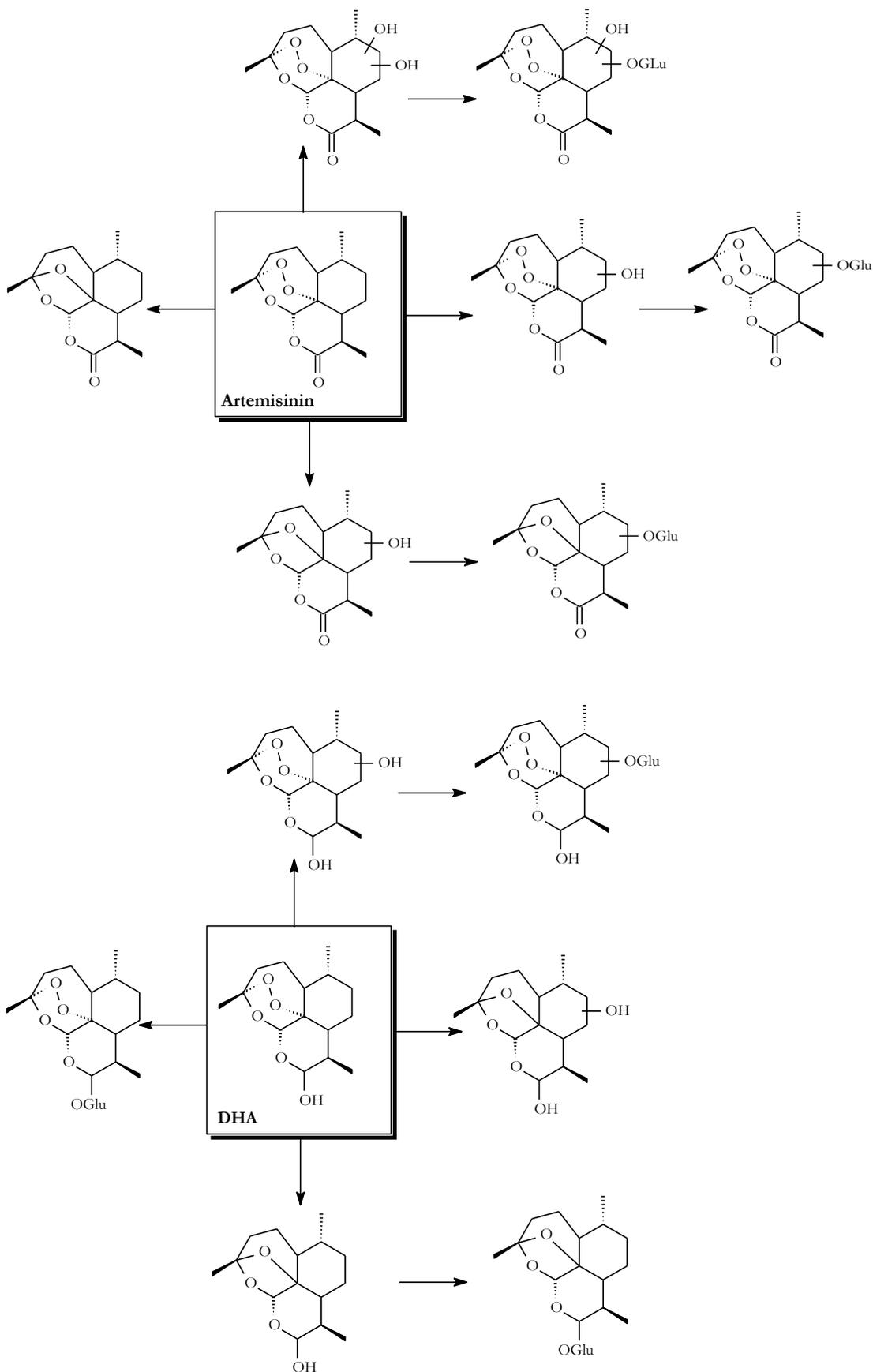
Figure 18 The proposed metabolic pathways for artemisinin (**1**) and DHA (**2**) *in vitro* and *in vivo* (Liu *et al.*, 2011).

Artemisinin Combination Therapy

The major limitation of artemisinin derivatives is their short half-lives, resulting in rapid elimination, which requires frequent administration, leading to noncompliance and recrudescence. Therefore, the WHO recommends their use in combination with long-acting antimalarial drugs to manage drug resistance, recrudescence, and non-compliance (Gautam *et al.,* 2009). Thus, all uncomplicated *P. falciparum* infections should be treated with an artemisinin-based combination therapy, and *P. vivax* with chloroquine and primaquine. When resistant, an ACT and primaquine should be given. The four ACTs currently recommended by the WHO are: artemether/lumefantrine; artesunate/amodiaquine; artesunate/mefloquine and artesunate/sulfadoxine-pyrimethamine (World Health Organization, 2011).

Since artemisinin and its derivatives are rapidly eliminated, ACTs are now recommended to delay parasite resistance mechanisms (O'Neill, Barton & Ward, 2010). The ACT regimes may offer complete and rapid eradication of the parasite load in symptomatic patients, and thus reduce the chance of survival of resistant strains. As higher numbers of gene mutations are required to express complete resistance against each drug, this may retard further development of resistance against the combination (Giao & de Vries, 2001).

Artemether/lumefantrine (Coartem®) is a highly effective and well-tolerated antimalarial combination that achieves cure rates of up to 95%, even in areas of multi-drug resistance. It is the only pre-qualified fixed-dose ACT. Various other drug combinations, including dihydroartemisinin/ piperaquine (Artekin™); artesunate/ amodiaquine; artesunate/ mefloquine and artesunate/ pyronaridine have been developed (Kuhn & Wang, 2008). Sodium artesunate, in combination with other antimalarial drugs, is rapidly becoming the drug of choice in most third-world cases of malaria (Posner & O'Neill, 2004).

The possibility of drug-drug metabolic interactions during combination regimens is expected, since artemisinin exhibits such an unusual autoinductive effect on drug metabolism (Ashton *et al.*, 1998). Artemisinin has demonstrated a capacity to increase the metabolism of other drugs mediated by several different CYP enzymes (Mihara *et al.*, 1999), and thus the advantages of combination therapy should be balanced against the increased chance of drug interactions (Giao & de Vries, 2001).

Toxicity

Artemisinins are generally regarded to be of low toxicity. Two problems currently associated with the artemisinins are neurotoxicity and reproductive toxicity (Medhi *et al.*, 2009). Artemisinin analogues are rapidly metabolised to DHA, which is known to induce neurotoxicity in animal models (Brewer *et al.*, 1994a; Brewer *et al.*, 1994b), although such toxicity has not yet been observed in humans (Gordi & Lepist, 2004). The extent of this neurotoxicity is dependent on: (i) the nature of the compound; (ii) the route of administration; and, (iii) its formulation. The use of artemisinins in the first trimester of pregnancy is contraindicated (Medhi *et al.*, 2009).

Resistance

ACT, the current recommended first-line therapy for uncomplicated *P. falciparum*, followed a period of increasing failure rates with chloroquine, and later, sulphadoxine-pyrimethamine treatment, which arose from the development of resistant *P. falciparum* strains. Artemisinin's rapid onset of action and short half-life minimises the period available for the selection of resistant strains, known as the selective window, which implies that resistance to artemisinin can occur only by inadequate treatment (Stepniewska & White, 2008). However, when artemisinin is used as monotherapy, drug pressure is increased and resistance can emerge. Therefore, the WHO recommended artemisinin combination therapy, which mutually protects both compounds and minimises the risk of resistant parasites emerging and spreading (White, 2004).

First reports of decreased cure rates of *P. falciparum* after treatment with ACTs emerged from observational data collected in Cambodia since 2001. Cure rates decreased in Pailin from 90.1% with 28 follow-up days to 79.3% with 42 follow-up days, whereas the cure rates remained at 100% at the two other sites in Cambodia (Denis *et al.*, 2006). Conclusive evidence came from a recent study comparing the therapeutic responses to artesunate in patients with uncomplicated malaria in Pailin, western Cambodia, and Wang Pha, in western Thailand (Figure 19) (Dondorp *et al.*, 2009). The clearance rates were much slower in Pailin (median parasite clearance time was 84 h in Pailin, compared with 48 h in Wang Pha). The difference in clearance rates was not explained by genetic polymorphisms in the *P. falciparum* genes, although heritability studies suggested that the observed artemisinin-resistant phenotype of the parasites has a genetic basis, and is therefore expected to spread. To date, the molecular basis for the resistance mechanism remains unknown (Dondorp *et al.*, 2010).

Figure 19 Pailin (western Cambodia), near the Thailand border, where the first decreased artemisinin sensitivity was detected (Dondorp *et al.*, 2010).

Several factors may have contributed to the emergence of artemisinin resistance in Cambodia, as it was one of the first countries to implement ACTs as first-line treatment in 2001. Unregulated artemisinin or artesunate monotherapy has been available since the mid-1970s. The current availability of ACTs as separate tablets results in the continued use of artemisinin or artesunate as monotherapy. In 2001-2002, co-blister packs of artesunate and mefloquine were used, whereas drugs were individually given in 2003 and 2004 at a total dose of 12 mg/kg of artesunate and 25 mg/kg of mefloquine over three days. Thus, the co-blister packs led to under-dosed regimens (Denis *et al.*, 2006). The different pharmacokinetic properties of artemisinins in subgroups of the population could also result in under-dosing. Migrants have been attracted to work in the area, and because they are a highly mobile and susceptible population, it is a matter of great concern that this population may carry and spread artemisinin-resistant strains to other countries (Dondorp *et al.*, 2010).

A multifaceted programme was launched for the containment of partial artemisinin resistance in western Cambodia and eastern Thailand, which included early diagnosis and appropriate treatment of malaria, drug pressure decrease, optimisation of vector control, targeting of the mobile population, strengthening of management and surveillance systems and operational research. The sale of artemisinin as monotherapy has recently been banned in Cambodia, and there are ongoing efforts to strengthen the monitoring of drug quality. Recently, Cambodia's ministry of Health has also changed the first-line treatment of uncomplicated *P. falciparum* malaria to dihydroartemisinin–piperaquine in the affected regions in western Cambodia (Dondorp *et al.* 2010).

However, ACTs are still effective in western Cambodia, as resistance is not complete and the parasites are still killed by artemisinins, albeit at much lower rates. Continuous monitoring of the spread of the artemisinin-resistant phenol type through the region is of utmost importance (Dondorp *et al.*, 2010).

Vaccine

The RTS,S vaccine, targeting the circumsporozoite protein is given with an adjuvant system (AS01 or AS02). It has consistently shown protection against *Plasmodium falciparum* malaria in children and infants in Phase 2 trials (Kester *et al.*, 2009; Sacarlal *et al.*, 2009; The RTS,S Clincal Trails Partnership, 2011). Phase 3 study of the efficacy, safety and immunogenicity of the candidate malaria vaccine RTS,S/AS01 is being conducted in seven African countries (The RTS,S Clincal Trails Partnership, 2011).

Evaluation of the proportionality of the hazard assumption showed that the efficacy was not constant over time. The efficacy was higher at the beginning than at the end of the follow-up period. At least one episode of severe malaria that met the primary case definition occurred in 57 of 2830 children (2.0%) in the RTS,S/AS01 group and in 56 of 1466 children (3.8%) in the control group, resulting in a vaccine efficacy of 47.3% (The RTS,S Clincal Trails Partnership, 2011).

Despite its relatively high efficacy against severe malaria, a reduction in the rate of death from malaria in the RTS,S/AS01 group was not observed (The RTS,S Clincal Trails Partnership, 2011). Thus, RTS,S/AS01 did not meet the requirements of a successful vaccine.

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