Chapter 7

Summary and Conclusion

Malaria is a protozoan infection caused by the *Plasmodium* parasite and kills an estimated 655 000 people annually, with more than 90% of all deaths estimated to be in Africa and most are pregnant women and children under the age of 5 (WHO 2011:73). *P. falciparum* is the species that accounts for the highest percentage of case fatalities and predominates in Africa and Asia (Snow *et al*., 2005:2, White 2008:1201, WHO 2011:1).

The malaria parasite has the ability to acquire resistance against antimalarials, not necessarily as a result of treatment failure, but trough spontaneous chromosomal mutations (White 2004:1085, Le Bras *et al*., 2003:148). With this aggressive means of acquiring resistance, only a handful of drugs are still usable in all areas afflicted by this disease (Petersen *et al*., 2011:1559).

The artemisinin class of compounds are the basis of treatment favoured by the WHO for the treatment of uncomplicated *P. falciparum* (WHO 2010a:13). This important class of compounds have a lack of resistance/cross-resistance with other antimalarials, have the capability to induce a 10 000 fold reduction in parasitemia during the asexual stage, inhibits parasite metabolism and cytoadherence of infected RBCs to tissue and are active against the gametocyte forms of *P. falciparum* thereby preventing transmission to the mosquito (Posner *et al*., 2007:2516, Woodrow *et al*., 2005:72). Unfortunately, resistance has started to emerge against this class at both the Thai-Cambodian and Thai-Myanmar borders where a significantly longer *in vivo* parasite clearance time has been observed (Dondorp *et al.,* 2009:466, Phyo *et al*., 2012:6).

One method to overcome resistance and increase activity, is the coupling of two types of pharmacophores to one another forming a hybrid. A hybrid is defined as a chemical entity with more than one structural domain, each having its own biological function. Hybrids act as a double edged sword and has the advantage of having a dual mechanism of action, more predictable pharmacokinetic properties and if chosen correctly, the ability of one entity to convey favourable qualities onto the other (Meunier 2008:69).

Coupling the same pharmacophore to itself is another means of increasing the activity or decreasing the likelihood of acquired resistance. These dimer compounds have been showed in the past to show an increase in activity and have been known to overcome resistance (Galal *et al.,* 2009:746, Chaturvedi *et al*., 2010:1, Posner *et al*., 2007:2516).

Incorporating a moiety that has the capability of accumulating to a great extend at the site of action is another approach that can be used to overcome resistance, increase activity or decrease toxicity of a parent compound (Chadwick *et al*., 2010:2587). The natural occurring polyamine compounds are actively transported into malaria parasites by polyamine transport systems (Chadwick *et al*., 2010:2587). Coupling these polyamine compounds to artemisinin has proven in the past to increase the activity of the drugs, although only slightly (Chadwick *et al*., 2010:2589).

The polyamine polar head moiety as defined by Calas and co-workers has been shown to have an antagonistic effect on the transportation/absorption of naturally occurring polar head compounds that are crucial for the phospholipid metabolic pathways inside the parasite (Calas *et al*., 2007:6307, Ancelin *et al*., 1998:1426). The described pharmacophores were greatly influenced by the chain length separating the amino groups, the degree of substitution and the number of lipophillic moieties around the amines (Calas *et al*., 2000:509, Calas *et al*., 2007:6307). Forming a hybrid drug molecule between artemisinin and the amine moieties might result in an increase in activity against malaria.

As seen with chloroquine (CQ), amines have a tendency to concentrate inside the acidic food vacuole in the malaria parasite. In the normal *in vivo* environment amino groups with low pK_a values are unprotonated and can easily cross biological membranes of both the host and parasite. When these neutral amino groups enter an acidic environment, e.g. the food vacuole of the malaria parasite, they become protonated decreasing the amount of drug that can cross biological membranes, trapping the molecule inside the parasite's food vacuole (Egan 2003:118). Previous attempts to increase the concentration of artemisinin by ion trapping showed a modest increase in antimalarial activity (Hindley *et al*., 2002:1055, O'Niel *et al*., 1996:4513).

Chapter 3 (Article 1) described the synthesis of eleven 10-aminoethylether derivatives of artemisinin and their *in vitro* antimalarial activity against the chloroquine sensitive (D10) and resistant (Dd2) strains of *Plasmodium falciparum*. The aim of this article was to investigate the effect that the attached amine moieties would have on the antimalarial activity of these compounds, in comparison to existing artemisinin derivatives such artesunate and dihydroartemisinin (Cloete *et al*., 2012a:4701).

It was found that compound **8** had activity comparable to that of artesunate versus the CQS D10 strain, whilst **2**, **7** and **8** had activities comparable to the even more potent DHA versus the CQR Dd2 strain. None of the synthesised compounds showed any mentionable toxicity against CHO and had IC_{50} values statistically significantly above that of the reference drug, emetine (Cloete *et al*., 2012a:4707).

In contrast to Calas and co-workers, the amines coupled with a shorter chain length to the artemisinin pharmacophore displayed higher activity than those with longer chains. This observation is indicative that these derivatives do not share the same mechanism of action as those described by Calas and co-workers, having too great a difference in their pharmacophore (Cloete *et al*., 2012a:4702).

On the basis of their antimalarial activity against the D10 strain, compounds **2**, **4** and **11** could be arranged in the order **11** > **4** > **2**, from most to least active. Similarly the order of their resistance index (RI) and pK_a values were **11** > **4** > **2**, showing that the compound with the smallest pK_a value had the weakest activity and showed the least discrimination between the two strains. A lower pK_a value implies a weaker base, decreasing the likelihood that a compound will be trapped in the parasite's acidic food vacuole. This correlation might imply that ion trapping is involved in the mechanism by which these derivatives exert their antimalarial action. The difference in RI values also indicate that there might be a relationship between the difference in activity against the two strains, the pK_a values of the amine moieties and the mode by which the parasite acquires resistance (Cloete *et al*., 2012a:4708).

The short chain compounds with fewer amino groups had better activity than the long chain polyamine compounds on both strains. This may be as a result of an increase in polarity of the longer chain polyamino compounds, decreasing the amount of drug crossing the parasitic membranes. Intriguingly, the RI values of the long chain polyamino compounds are greater than for the short chain compounds. This might be due to the specific method by which the parasite acquires resistance warranting further investigation (Cloete *et al*., 2012a:4708).

Chapter 4 (Article 2) compared the same 10-aminoethylether derivatives of artemisinin discussed in chapter 3 with eight 10-*n*-alkyl/aryl/aroyl ester derivatives previously synthesised in our group. The *in vitro* antimalarial activity of these nineteen compounds was determined against both the chloroquine sensitive (3D7) and resistant (K1) strains of *Plasmodium falciparum*, whilst their cytotoxicity was determined against both human embryonic kidney cells (HEK 293) and hepatocellular carcinoma cells (Hep G2). The 10-*n*-

alkyl/aryl/aroyl ester and the 10-aminoethylether derivatives showed activity versus both strains, with no mentionable toxicity against either HEK 293 or Hep G2 cells (Cloete *et al*., 2012b).

The 10α-*n*-propyl and 10α-benzyl ester derivatives, **11** and **18** respectively, were the most active compounds against both strains, whilst the other ester derivatives also showed a slightly higher degree of activity than the aminoethers. Compound **29**, featuring an isobutylamine substituent, was the most active of all aminoethers (Cloete *et al*., 2012b).

Considering only the aminoethers, the short chain compounds with fewer amino groups had better activity than the long chain polyamine compounds against the CQS (3D7) and CQR (K1) strains, as was the case with compounds described in chapter 3 against the CQS (D10) and CQR (Dd2) strains. The increased polarity of the polyamino compounds decrease the amount of drug crossing the parasitic membranes, resulting in decreased activity (Cloete *et al*., 2012b).

As discussed in chapter 3, the aminoethers with higher pK_a values had a propensity for increased activity compared to the derivatives with lower pK_a values (Cloete *et al*., 2012a:4708). This observation was ascribed to the possibility of these aminoethers to undergo ion trapping. One would thus expect the aminoethers to be more active than the esters, which lack the amine(s) for ion trapping. However, the EC_{50} data showed that the opposite was true. Two facts should be kept in mind: firstly, although the attachment of the amine moieties to artemisinin had positive influences on the activity of the derivatives, other factors such as H-bonding, molecular shape, polarity, flexibility and charge/ionization also influence the accumulation of the drug at the site of action (Bigucci *et al*., 2008:168). Secondly, ester derivatives of artemisinin are known to be extremely labile in aqueous solution and mostly exert their antimalarial action *via* their active metabolite, DHA, whilst the ether derivatives are much more stable and exert their antimalarial action to a greater extend on their own (Krishna 2004:234, D'Hulst *et al*., 1996:277, Karbwang *et al.,* 1997:309, Woodrow *et al*., 2005:71). The active metabolite, DHA, has been proven in the past to be slightly more active than some of the synthesised aminoethers, explaining why the ester compounds were more active (Cloete *et al*., 2012a:4706).

Chapter 5 (Article 3) entailed the synthesis of seven artemisinin-triazine hybrids and the determination of their *in vitro* antimalarial activity against the chloroquine sensitive (3D7) and resistant (K1) strains of *Plasmodium falciparum*, whilst their cytotoxicity was determined against human embryonic kidney cells (HEK 293), hepatocellular carcinoma cells (Hep G2),

B-lymphocyte cells (Raji) and human fibroblast cells (BJ). The synthesised hybrids all showed activity against both strains and were found to be non-toxic to all mammalian cells.

Compound **17**, featuring *p*-anisidine and 2-(diisopropylamino)ethylamine substituents on the triazine ring, was the compound with the lowest EC_{50} value and had activity comparable to both AS and AM. The compound has a tertiary amine with a calculated pK_a value of 10.2, which is comparable to the calculated pK_a value of 10.4 for CQ. One of the suggested mechanisms of action for CQ is ion trapping, which relies on the action of its amino groups as weak bases (Egan 2003:118). This observation thus implies that ion trapping might play a role in this compound's activity as well.

Compounds **11** and **13** are homologous differing only by an extra oxygen atom in the *para* position of the substituents on the triazine moiety, resulting in a 7-fold difference in activity. This same phenomenon is also seen in compounds **12** and **14** with a 4-fold difference in activity. In both instances the compounds with the higher log P values were found to be less active. This observation is supported by Lipinski's rule of five, which states that a log P below 5 is ideal for crossing of biological membranes (Lipinski *et al*., 2001:9).

Compounds **11** – **16** were all slightly more active against the CQR versus the CQS strain and retained the same increasing order of activity against both strains *viz*. **17** > **13** > **14** > **16** > **11** > **12** > **15**.

Chapter 6 (Article 4) described the synthesis of six dimeric artemisinin triazine hybrids, the determination of their antimalarial activity and toxicity against the same strains of *P. falciparum* and mammalian cells as in chapter 5. All compounds showed activity against both strains and no noticeable toxicity towards any of the mammalian cell lines used.

Compared to both AS and AM, compound **15** with aniline and morpholine substituents on the triazine ring, showed a 3- and a 2-fold increase in activity, respectively, against the 3D7 strain. Compound **11** with *p*-anisidine and morpholine substituents on the triazine ring, is analogous to **15** differing only on the *para* position of the aniline ring with a methoxy group. This difference in structure, however, led to a 5-fold reduction in activity against the 3D7 strain. It's believed that the addition of an oxygen atom leads to an increase the polarity of the molecule, thereby decreasing the amount of compound crossing the biological membrane causing a reduction in activity.

Only compounds **11** and **15** had calculated log P values below 5, whereas that of all the other synthesised compounds in this article were above 5. Interestingly compounds **15** and **11** were the first and second most active compounds, with all the other compounds having

lower activity. Likewise, compounds **10** and **11** forms is a homologous pair that differs from one another only in the oxygen atom in the *para* position of the cyclic substituent on the triazine moiety, resulting a 3-fold difference in activity versus the 3D7 strain. Both these compounds have very similar pK_a values, but the calculated log P value of **10** is higher than that of **11**. As noted in chapter 5, we believe that a log P value above 5 leads to a decrease in activity.

Compounds **10**, **11**, **12** and **13** all had corresponding monomer equivalents that have been reported in chapter 5. Against both the CQS and CQR strains of *P. falciparum*, the dimer compounds were slightly more active than their monomer counterpart, except compound **13**.

To summarise, all of the synthesised compounds had activity not only against the CQS but also compared to the CQR strains of malaria, with no mentionable toxicity against mammalian cells. Thus, neither the amine moieties as described by Calas and co-workers nor the substituted triazine rings led to los of activity or to an increase in toxicity against mammalian- or Chinese hamster cells. A relationship between the calculated pK_a values and the antimalarial activity was observed, where a pK_a profile similar to that of CQ proved to mediate an increase in activity. It is hypothesised that this observed association might be a result of pH trapping. An increase in the amount of amine moieties in the parent compounds led to a reduction in activity, possibly as a result of increased polarity thereby decreasing the amount of drug able to cross the biological membranes and lowering the concentration at the site of action. The addition of the triazine moiety, consisting of several amino groups, did however not lead to a decrease in activity possibly as a result of its high lipophilicity. The calculated log P values also influenced the activity of these compounds, with higher values leading to a decrease in activity. It is postulated that a log P value above the preferred upper limit of Lipinski's rule of five leads to a decrease in the amount of drug able to cross the biological membrane lipid bilayer, reducing the activity.

Neither the amine moieties defined by Calas and co-workers, nor the coupled polyamine substituents intended to facilitate active transport lead to a massive increased activity. It is believed that the amine moieties however exerted their effect mainly through their influence on the pK_a and log P values and by their effects on the polarity of the compounds.

The attachment of the triazine moiety to the artemisinin pharmacophore, forming the hybrids, resulted in compounds that showed activity against both strains of malaria.

Coupling of a second artemisinin pharmacophore, forming artemisinin-triazine dimers, resulted in a minor increase in activity compared to their monomer counterparts, which may be the result of more artemisinin reaching the site of action.

Although the pK_a and log P values together with the difference in polarity seemed to influence the activity of the compounds, other factors such as H-bonding, molecular shape, flexibility and charge/ionization also play a role in the accumulation of the drug at the site of action.

Future experiment will be aimed at determining the experimental log P and pK_a values, to establish whether the same conclusions can be drawn as based on the calculated values. Additionally, the effects that these compounds elicit on both the folate- and phospholipid biosynthetic pathways needs to be investigated to determine whether they act on multiple targets.

This study has shown that there are still several ways and great opportunities in which to address the devastating disease of malaria by treating the condition with chemotherapy. A number of compounds synthesised in this study exhibits activity comparable to that of potent antimalarial drugs currently on the market, and they may constitute excellent compounds for further *in vivo* studies.