

CHAPTER 1

This chapter is an introduction to the thesis and the problem statement and the aims of the study are discussed.

CHAPTER 1

PROBLEM STATEMENT AND AIM OF THE STUDY



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Malaria, AIDS and tuberculosis are the three main causes of mortality due to infectious diseases (Victoria *et al.*, 2009). Of the 216 million malaria cases reported in 2010, 91% were due to *P. falciparum*, resulting in an estimated 655,000 deaths (WHO, 2012).

Emergence of drug-resistant malaria has greatly diminished the effectiveness of previously widely-used antimalarial drugs, and has posed the greatest challenge to effective malaria chemotherapy (White, 2004; WHO, 2010a). The choice of treatment for malaria has consequently changed from chloroquine and sulfadoxine-pyrimethamine to artemisinin-based combination therapy (ACT). ACTs are a combination of artemisinin or one of its derivatives with an antimalarial or antimalarials with a longer half life of a different class (WHO, 2010b).

Artemisone is a new derivative of the natural product artemisinin and is described to be superior to other artemisinins, both in terms of activity and safety (Haynes, 2006). Compared to the most widely used artemisinin derivative, artesunate, it shows enhanced *in vitro* antimalarial activity against chloroquine-sensitive and -resistant *P. falciparum* parasite strains (Haynes *et al.*, 2006). In addition, three metabolites of artemisone showed *in vitro* antimalarial activity, with mean IC₅₀ values of 1.7 ± 0.5 ng/mL for M1, 25.7 ± 5.0 ng/mL for

M2, and 2.3 ± 0.2 ng/mL for M3 compared to the IC₅₀ value of 0.60 ± 0.15 ng/mL for artemisone against the K1 line (Nagelschmitz *et al.*, 2008). Artemisone displays enhanced *in vivo* activity against chloroquine sensitive *P. berghei* and chloroquine resistant *P. yoelii* rodent models. (Vivas *et al.*, 2007; Haynes *et al.*, 2006). In *Aotus* monkey-*P. falciparum* models artemisone also showed enhanced activity (Haynes *et al.*, 2006; Obaldia *et al.*, 2009). Artemisone elicits no neurotoxicity in standardized *in vitro* and *in vivo* assays, as compared to current artemisinins. The lack of neurotoxicity of artemisone is fundamental, given that artemisinins are neurotoxic in neuronal cell cultures *in vitro* (Fishwick *et al.*, 1995; Mclean & Ward, 1998; Wesche *et al.*, 1994; Schmuck *et al.*, 2002) and in animal models (Brewer *et al.*, 1994; Kamchonwongpaisan *et al.*, 1997; Nontprasert *et al.*, 1998). In Phase IIa trials, it was well tolerated and had a curative effect at dose levels of one third that of artesunate (Krudsood *et al.*, 2005; Nagelschmitz *et al.*, 2008).

Artemisinins have low aqueous solubility that results in poor and inconsistent absorption and low bioavailability upon oral administration (White, 2008). Another limitation is their short half-life values, which has required use of protracted treatment regimens. This, in turn has lead to non-compliance, which has resulted in recrudescence infections. Such protracted monotherapy is also proscribed by the WHO, given the development of resistance (Dondorp *et al.*, 2010; Gautam *et al.*, 2009). However, in the case of ACTs, there are now serious concerns about the emergence of artemisinin resistance. Prolonged *P. falciparum* parasite clearance times *in vivo* have been reported in Cambodia, Myanmar, Thailand and Vietnam (Noedl *et al.*, 2008; Dondorp *et al.*, 2009; Phyo *et al.*, 2012; WHO, 2012).

An alternative approach to the costly and time consuming development of new drugs is the use of the drug delivery approach to improve the bioavailability and patient compliance and to decrease side effects of an active compound (Gardner, 1987; Speiser, 1998). Although various drug delivery systems are available, they have several limitations i.e. stability, cost and safety issues (Cimato *et al.*, 2004; Hagar *et al.*, 1993; Muller & Keck, 2004). The Pheroid[®] delivery system is stable, safe and cheap to manufacture (Steyn, 2009, Grobler, 2009). This fatty acid-based system's size, charge, and the hydrophilic-lipophilic characteristics can be controlled, which is useful in enhancing drug absorption (Saunders *et al.*, 1999; Goodfield *et al.*, 2003; Tzaneva *et al.*, 2003)

In this thesis the focus is on the therapeutic efficacy and bioavailability of the antimalarial drug, artemisone, and to examine the potential of the novel formulation delivery system, the Pheroid[®]. The specific literature objectives of this study are to conduct relevant literature research on malaria, the antimalarial drug artemisone, and the pertinent aspects of the

Pheroid® drug delivery system. This literature review is presented in Chapter 2. The experimental results and data are presented in Chapters 3 -5 in article format and results of miscellaneous experiments that are not included in Chapters 3-5 are presented in Chapter 6.

Previous *in vitro* efficacy studies with artemisone (reference and Pheroid® formulation) carried out at both the North-West University (NWU) and Swiss Tropical and Public Health Institute (STPHI) delivered inconsistent results. Results are discussed in Chapter 2, section 3. Briefly, Steyn (2010) from the NWU observed a 4.5 fold increase in the *in vitro* antimalarial efficacy of artemisone when entrapped into the Pheroid®. Jourdan (2011, from the STPHI) on the other hand observed either a decrease in efficacy or no change with the artemisone/Pheroid® formulation, although significant increases with other Pheroid® entrapped artemisinin-based compounds such as artesunate and artemether were found in the same study.

Therefore, the aim of the *in vitro* studies in this thesis is twofold:

- (i) to determine the *in vitro* efficacy of artemisone and its major active metabolite M1 on *P. falciparum* strains and also to evaluate the potential of the Pheroid® system to enhance the activity these drugs. Efficacy was established by using the [³H]-hypoxanthine incorporation assay in various sensitive and multidrug resistant *P. falciparum* strains W2, D6, 7G8, TM90-C2B, TM91-C235 and TM93-C1088. The drug concentrations that led to a 50% and 90% inhibition of parasite growth (IC₅₀ and IC₉₀) were calculated.
- (ii) to determine if artemisone (reference and Pheroid® test formulations) and metabolite M1 induce dormant parasites in the *P. falciparum* W2 strain. The drugs were added to the malaria cultures and washed out after a 6 hour incubation period. The formation of dormant rings and their recovery was observed by means of Giemsa slides and light microscopy.

Steyn *et al.* (2011) performed artemisone *in vivo* bioavailability studies using C57 BL/6 mice. They observed a 4.6 fold increased exposure of artemisone *in vivo* when entrapped in the Pheroid®. They observed a significant improvement in the C_{max} and T_{1/2} of artemisone and an increase in T_{max}, which effectively translates to a scenario where the drug concentration could be significantly decreased and still achieve therapeutic drug plasma concentrations (Steyn *et al.*, 2011).

While rodents are typically used as experimental animal models in preclinical studies, variation between rodents compared to humans in many physiological and biochemical

functions exist (Sharer *et al.*, 1995; Tang, 2007). This makes it difficult to extrapolate rodent data to humans and, therefore, non-rodent species, such as monkeys and dogs, are used as additional models by the pharmaceutical industry in preclinical metabolism and toxicology studies of new drugs (Stevens *et al.*, 1993; Sharer *et al.*, 1995). According to Jolivet and Ward (2005) extrapolation of monkey pharmacokinetic data is the most accurate method for predicting human clearance. The Cynomolgus (*Macaca fascicularis*) and Rhesus (*Macaca mulatta*) monkeys have been widely used in preclinical pharmacokinetic studies, but these species are in short supply and, therefore, studies on these species are expensive (Ward *et al.*, 2008; Ward *et al.*, 2009). When compared to Cynomolgus or Rhesus monkeys, the Vervet monkey (*Chlorocebus aethiops*) was shown by these authors to be a similar predictor of human oral bioavailability for a number of active pharmaceutical ingredients (Ward *et al.*, 2009).

Therefore, the third aim of the thesis was to investigate the bioavailability of artemisone entrapped in Pheroid® (Pheroid® test formulation) and artemisone only (reference formulation) in a non-human primate model. Vervet monkeys received a single dosage of 60 mg/kg using a crossover study design. Plasma samples were taken at specific time intervals and analysed using a validated LC/MS/MS method.

Teja-Isavadharm *et al.* (2004) described a bioassay for the measurement of the concentration of artemisinin derivatives in plasma and serum. By using a modified *in vitro* drug susceptibility test, it was possible to relate antimalarial activity in plasma or serum at an unknown concentration of drug to the known concentrations of dihydroartemisinin (DHA) required for parasite growth inhibition.

All three metabolites of artemisone possess antimalarial activities, with M1 being the most potent. Although the metabolites are not as active as the parent drug, high concentrations of the metabolites are present in the plasma after oral artemisone exposure (Nagelschmitz *et al.*, 2008). This also proved true in the monkey pharmacokinetic study and, therefore, the metabolites would make an additional contribution to the overall parasitocidal effect of artemisone. The owl (*Aotus sp.*) monkeys are some of the few nonhuman primates that are susceptible to infection with the human malaria parasite (Singh *et al.*, 2006). However, these monkeys can be expensive and hard to find even in primate breeding centres. *In vivo* antimalarial efficacy could consequently not be evaluated.

Therefore, the fourth aim of this thesis was to determine the antimalarial activity of artemisone and its metabolites in plasma samples (*ex vivo* activity) after oral administration

of artemisone and artemisone entrapped in the Pheroid[®] to Vervet monkeys. The composite antimalarial activity of plasma samples collected from the monkey subjects after drug treatment was assessed against the *P. falciparum* 3D7 strain by using the SYBR green assay. Furthermore, this study may also explain whether the low bioavailability of artemisone may have resulted from first-pass metabolism and production of active metabolites.

Because of low artemisone plasma levels found with the monkey model, we hypothesized that first pass metabolism or incomplete gastrointestinal absorption might be involved. Indeed, lower bioavailability in monkeys may be due to incomplete gastrointestinal absorption, greater first-pass metabolism, or a combination of these factors (Chiou & Buehler, 2002; Emotoa *et al.*, 2011; Akabane *et al.*, 2010). Akabane *et al.* (2010) stated that lower bioavailability may be due to intestinal metabolism rather than poor absorption or liver metabolism. Therefore, the fifth aim was to determine the *in vitro* metabolism of artemisone (reference and Pheroid[®] test formulations). This was carried out by measuring the hepatic and intestinal metabolism of artemisone by using human and monkey liver and intestinal microsomes, as well as recombinant CYP3A4 enzymes.

Since P-glycoprotein (P-gp) and CYP3A4 have overlapping substrate specificities and artemisone is a CYP3A4 substrate, the possibility that artemisone acts as a P-gp substrate was examined. This was carried out by using two assays using the ATPase assay kit and Caco-2 cells. Artemisone (reference and Pheroid[®] test formulations) was tested for interaction with the p-gp ATPase of human and monkey p-gp membranes with the ATPase assay kit. The apical-to-basolateral and basolateral-to-apical transport of artemisone (reference and Pheroid[®] test formulations) were determined across Caco-2 cell monolayers. Also, the cytotoxic properties of the Pheroid[®] delivery system on Caco-2 cells were assessed by using a live-dead assay and fluorescent microscopy.

During the intra-erythrocytic stage of the malaria parasite, the parasite consumes host hemoglobin and produces reactive oxygen species (ROS). Thioredoxin- and glutathione-based redox systems protect *P. falciparum* against oxidative damage (Becker *et al.*, 2004; Haynes *et al.*, 2010, Haynes *et al.*, 2011, Haynes *et al.*, 2012). Fu *et al.* (2010) previously showed that the signal due to 2',7'-dichlorofluorescein (DCF), a ROS probe, increases as the intra-erythrocytic parasite matures from the ring to the trophozoite stage, when hemoglobin digestion is high (Fu *et al.*, 2010). Previous studies indicated that artemisinins induced ROS production in parasite membranes (Hartwig *et al.*, 2009.; Krungkrai & Yuthavong, 1987.; Scott *et al.*, 1989) and recently Klonis *et al.* (2011) showed that artemisinin treatment

increased the DCF signal indicating ROS formation. Therefore, the final aim of this thesis was to determine the effect on the generation of ROS formation within the malaria parasite by treatment with artemisone and the Pheroid® formulation. This was carried out by dual labeling of drug treated *P. falciparum* samples with the fluorescent dye nucleic acid-binding dye SYTO 61 to distinguish between uninfected and infected erythrocytes and fluorescent reactive oxygen species reporter 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester to measure oxidative stress in different stages of live *P. falciparum* parasites.

By addressing the specific aims described above and correlating the *in vivo* bioavailability results with the *in vitro* efficacy, dormancy, *ex vivo* and ROS assays, we will be able to evaluate the impact of Pheroid® delivery on artemisone's antimalarial activity. The *in vivo* pharmacokinetic, metabolism, Pgp ATPase and Caco-2 transport studies should provide information as to the effect of the Pheroid® on the pharmacokinetics of artemisone.

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