

CHAPTER 7

This chapter is the closing chapter. It provides a summary of all the preceding chapters and highlights areas for future research.

CHAPTER 7

SUMMARY AND FUTURE PROSPECTS

1 Introduction

The WHO guidelines recommend artemisinin-based combination treatments (ACTs) for the treatment of uncomplicated *P. falciparum* malaria (WHO, 2012), but there are limitations to artemisinins, such as their low aqueous solubility, low bioavailability and short half-lives (White, 2008). Furthermore, 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). In line with the emergence of resistance, studies have shown reduced susceptibility of *P. falciparum* ring stages to artemisinins in Western Cambodia (Witkowski *et al.*, 2013). An alternative approach to the costly and time consuming development of new drugs is the use of the drug delivery approach (Gardner, 1987; Speiser, 1998). The fatty acid-based Pheroid® delivery system is stable, safe and cheap to manufacture (Steyn, 2009; Grobler *et al.*, 2008) and Pheroid® based products have been used for alternative purposes in what may be termed a malaria 'environment' – Pheroid® containing products have been subjected to the formal thermal stress stability programmes required by the regulatory bodies for medications or bioagricultural products before they are marketed (Personal communication, Dr. R. Buitenbach, Nelesco 882 (Pty. LTD., 2012). In this thesis we examined the potential of the Pheroid® delivery system to enhance the therapeutic efficacy and bioavailability of the antimalarial drug artemisone.

2. Summary and future prospects

The bioavailability of artemisone (reference and Pheroid® test formulations) was evaluated in Vervet monkeys. Artemisone demonstrated rapid absorption and the Pheroid® delivery system improved the pharmacokinetic (PK) profile of artemisone by increasing the maximum artemisone concentration and the area under the curve while decreasing the time to reach the maximum concentration in a clinically significant manner.

During the development of any new medication, the *in vitro* metabolism of such medication in animals and humans, as well as its exposure in animals should be investigated prior to commencing human clinical trials. Metabolic and pharmacokinetic data from two rodent species and a non-rodent species are recommended by regulatory authorities (FDA, 1993). The results obtained from the current PK study indicate that the Vervet monkey is not a good model to predict human bioavailability of artemisone. The artemisone plasma levels are

much lower ($C_{\max}$ of 47 and 114 ng/ml for reference and Pheroid® test formulations) than expected for the dosages administered (60 mg/kg). In contrast, mice treated with artemisone reference or artemisone-Pheroid® at a dose of 50 mg/kg generated $C_{\max}$ values of 810 and 1550 ng/ml respectively (Steyn *et al.*, 2011). When artemisone was administered to humans at a dosage of ~1 mg/kg, the $C_{\max}$ was 140 ng/ml (Nagelschmitz *et al.*, 2008). When the rodent dosages are extrapolated to a human equivalent dose (HED) by using the body surface area normalization method of Reagan-Shaw *et al.* (2008), the 50 mg/kg bodyweight dosage given to the rodents is equivalent to a 4 mg/kg bodyweight human dose; the results correlate well with those of the human artemisone studies. Applying this extrapolation to the current Vervet monkey study, the dosage given is 20 times more than in the human clinical trials while the $C_{\max}$ is 3 fold lower and the AUC is 1.5 fold lower for the reference formulation.

Therefore, future artemisone-Pheroid® studies should be conducted with a different non-rodent model. The Vervet, Cynomolgus and Rhesus monkeys are old world monkeys. New world monkeys such as the marmoset, squirrel monkey or owl monkey could be used. In a study where squirrel monkeys received a single oral dose of 30 mg of artemisone, the $C_{\max}$ was 497 ng/ml. Using the extrapolation as above, this is equivalent to a dosage of ~5-10 mg/kg. Owl monkeys also received an oral dose of either 1, 3 or 10 mg/kg artemisone, which resulted in $C_{\max}$ values of 5, 44 and 276 ng/ml respectively. These concentrations are equivalent to dosages of 0.3 mg/kg, 1 mg/kg and 3 mg/kg. Therefore, although the concentrations used here are slightly lower, they compare well with those obtained in the human clinical trials. Thus, these old world monkeys are better models for future studies.

Different drug delivery systems should be evaluated. Recently, polyethylene glycol (PEGylated) liposomes were shown to enhance the half-life of artemisinin 5-fold (Isacchi *et al.*, 2011). When *Plasmodium berghei* NK-65 infected mice were treated with artemisinin and artemisinin-curcumin loaded into conventional and PEGylated liposomes, the antimalarial activity was also enhanced (Isacchi *et al.*, 2012). These liposomal formulations very likely will prove useful in increasing the pharmacodynamic and pharmacokinetic properties of artemisone.

A description of the metabolism *in vitro* of artemisone (reference and Pheroid® test formulations) is also given in Chapter 3. The metabolism of artemisone was assessed *in vitro* by using human and monkey liver and intestinal microsomes, and recombinant CYP3A4 enzymes. The results indicate that microsomal metabolism of artemisone is inhibited by the

Pheroid® delivery system. The intrinsic clearance *in vitro* of the reference formulation with monkey liver microsomes is ~8 fold higher in the monkey liver microsomes compared to the human liver microsomes. The estimated hepatic clearance *in vivo* for the monkey is almost twice the CL_H in humans. Therefore, there is a species difference in artemisone metabolism, which may influence the *in vivo* PK results. . In retrospect, it would have been valuable to measure CYP levels in the individual monkeys before, during and after the PK study. Also, since very low levels of M1 metabolite were produced in the microsomes, it would have been worthwhile to include a positive control for CYP3A4 in the microsome studies.

Future studies should include inhibition studies to establish whether the Pheroid® inhibits CYP3A4. For this, use of the BD Gentest™ P450 high throughput inhibitor screening kits (CYP3A4/DBF, CYP3A4/BFC or CYP3A4/BQ) is recommended. It would also be worth testing if the Pheroid® inhibit all standard *in vitro* microsomal interactions at physiologically relevant doses. Also, release studies that assess the quantities of drug released in the microsomal incubations *in vitro* should be carried out. Such studies may establish if the drug is free to be metabolized in this system

The efficacy of artemisone (reference and Pheroid® test formulations), its major active metabolite M1 as well as standard antimalarial drugs were evaluated *in vitro* against *P. falciparum* W2, D6, 7G8, TM90-C2B, TM91-C235 and TM93-C1088 strains. Artemisone was the most active of the drugs assessed. Artemisone was approximately 2-fold more potent than either artesunate or DHA. The artemisone metabolite M1 was also highly active. The Pheroid® drug delivery system did not improve the *in vitro* efficacy of artemisone or DHA in these strains. However, results obtained in a different laboratory, showed that the Pheroid® improved the efficacy of artemisone against the *P. falciparum* 3D7 (Chapter 3) and RSA11 (Steyn, 2009) strains.

The composition of the Pheroid® used in the studies of Steyn (2009) and in this study differed and could have had an influence on the results. Steyn used Pheroid® vesicles (2% oil phase:98% water phase) while in this study pro-Pheroid® (100% oil phase) was used. In the assays performed, the final Pheroid® vesicle to water phase ratio in the study of Steyn was 1:2500 and, therefore, a final oil phase of 0.0008%. In this study, the pro-Pheroid to water phase ratio was 1:50000 and, therefore, a 0.002% oil phase solution. In addition to this difference, composition of the starting oil phase of the two formulations used in the *in vitro* efficacy experiments were different and are summarized in Table 1. The pro-Pheroid® contains PEG, BHA and BHT while the vesicles do not contain these components. Also, the ratio of vitamin F ethyl ester:Kollifor® EL:dl- α - tocopherol used in the study of Steyn was

70:25:10 while it is 66:27:1 for the pro-Pheroid®. Therefore, the ratio of vitamin F ethyl ester to Kollifor® EL is higher than with the pro-Pheroid® and the dl- α - tocopherol is tenfold higher in the formulation of Steyn.

Table 1: The composition of the starting oil phase of the two formulations used in the *in vitro* efficacy experiments are summarized in table 1.

Component/ Pheroid batch	V08036 (Steyn, 2009)	P12010 (this study)
Vitamin F ethyl ester	70%	65.57%
Kollifor EL	25%	27.32%
dl-α- tocopherol	10%	1.00%
PEG 400	-	4.90%
BHA	-	0.01%
BHT	-	0.01%

Another factor that might have had an influence on the results, was the process of entrapment of the active pharmaceutical, artemisone. Steyn used already prepared oil phase, added artemisone and then adjusted to volume with nitrous oxide water (NW) to achieve a 2% oil in water vesicle solution. In this study, artemisone was entrapped during the manufacturing of the oil phase.

The induction of dormant parasites was evaluated for artemisone (reference and Pheroid® test formulations) and metabolite M1 in the *P. falciparum* W2 strain. For both artemisone and the Pheroid® entrapped artemisone, the parasite growth was abruptly arrested and dormant ring stages were induced in a manner similar to that of DHA. Growing parasites were first detected in thick blood films on day 3 after drug treatment and reached the initial parasitaemia (>1%) by day 6. Following sorbitol-treatment that selectively kills all late-parasite stages except for ring forms, growing parasites were only detected on day 5 and initial parasitaemia of 1% was reached on day 9.

The future of artemisone, and indeed of artemisinins in general, lies in the identification of a suitable partner drug that can prevent the induction of parasite dormancy, or alternatively, induce dormant parasites to resume their normal growth programmes such that they will become susceptible to artemisone, or other artemisinin. This is a task of considerable urgency. It will require both a better understanding of how artemisinins exert their mode of

action, and the screening against dormant rings of libraries of compounds that have antimalarial activities,

Since P-glycoprotein (P-gp) and CYP3A4 have overlapping substrate specificities I evaluated the interaction of artemisone (reference and Pheroid® test formulations) with the P-gp efflux transporter. Artemisone stimulated P-gpATPase activity in a concentration-dependent manner. The ATPase activity stimulated by 100 µM artemisone was ~4 fold higher in human MDR1 expressed in insect cell membranes than with Cynomolgus monkey MDR1 expressed in insect cell membranes. These results illustrate the species differences between human and monkey P-gp ATPase activity. When artemisone was entrapped in pro-Pheroid®, the P-gp ATPase activity was not stimulated by artemisone, indicating complete inhibition of P-gp ATPase. Because the Pheroid® inhibited artemisone stimulated ATPase activity, the effects of the Pheroid® on other P-gp substrates such as verapamil, vinblastine and lopermide were evaluated. The Pheroid® inhibited the drug-stimulated ATPase activity of these drugs.

The apical-to-basolateral and basolateral-to-apical transport of artemisone (reference and Pheroid® test formulations) were determined across Caco-2 cell monolayers. Both artemisone alone and artemisone entrapped in Pheroid® vesicles showed moderate apical to basolateral and high basolateral to apical permeability (P_{app}). The P_{app} efflux ratio's for artemisone and artemisone entrapped in Pheroid® vesicles were >5, and decreased to ~1 when 50 µM verapamil was added. These data suggest that artemisone is a substrate for mammalian P-gp. Future studies can be carried out with Madin-Darby canine kidney (MDCK) epithelial cell line in order to establish if similar P_{app} values are obtained. Such studies should also include studies in wild-type and *mdr1a* knockout mice in order to assess the influence of P-gp efflux activity on the oral absorption of artemisone.

Chemoresistance typically occurs when access of P-gp substrates are limited by the overexpression of P-gp in cancer cells (Chen *et al.*, 1986, Gottesman & Pastan, 1993). Artemisone does show useful efficacies against different types of cancer cell lines (Gravett, *et al.*, 2010), suggesting that involvement of the P-gp efflux transporter is probably not important. However, more work is required to establish if artemisone, and other artemisinins, are adversely affected by mammalian P-gp in resistant cancer cells that overexpress this transporter. This study has to be repeated and expanded to other artemisinins and known to be a substrate for P-450, such as artemether, in order to validate the results.

The cytotoxic properties of the Pheroid® delivery system on Caco-2 cells were assessed by using a live-dead assay and fluorescent microscopy. The pro-Pheroid® is not cytotoxic to Caco-2 cells at concentrations of $\leq 1.25\%$. Previous studies on the toxicity of the Pheroid® included cytotoxicity analysed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay using MT-2 cells infected with an HIV-1 SW7-TCL strain (Botha, 2007), neurotoxicity using Nb2A cells and haemotoxicity (haemolysis and ROS assay) using red blood cells (Slabbert, 2011), the *in vitro* bacterial reverse mutation (Ames) test, as well as acute and subchronic *in vivo* studies in rats (personal communication; D. Elgar & A. Grobler). Future studies should include expansion of this study to a full toxicity screening of the Pheroid® drug delivery system using several different cell culture lines and different end points of toxicity detection. This could include panel tests to measure membrane integrity, mitochondrial function, immunotoxicity and apoptosis. Genetic toxicity studies such as *in vitro* mouse lymphoma thymidine kinase gene mutation assay, mammalian erythrocyte micronucleus test and *in vitro* mammalian chromosomal aberration test should also be conducted.

The antimalarial activities 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 was evaluated. Antibody-mediated growth inhibition of blood stage *P. falciparum* occurred with Vervet monkey plasma. This may mean that these monkeys are protected against malaria infection. Monkey plasma, either drug-free or containing artemisone, was heat inactivated or treated with protein A gel to suppress growth-inhibitory activity. This proved useful in the elimination of the growth-inhibitory activity of the drug-free plasma, but the plasma samples containing the drug and collected from healthy monkeys could not be analysed by the *ex-vivo* bioassay method, since this method lacked the necessary sensitivity for detecting the artemisone, and was not reproducible. It is recommended that this study be repeated using a different assay method, such as the ^3H -hypoxanthine or the HRP2 ELISA method. Also, it is of interest to isolate IgGs from Vervet monkey plasma in order to assess their growth inhibitory effects against various *P. falciparum* parasites.

As for the ROS assay, dual labelling with SYTO 61 and CM-H₂DCFDA proved useful to distinguish between uninfected and *P. falciparum* infected red blood cells. The DCF signal increased as the parasite progressed to the trophozoite stage, which is consistent with increased metabolic activity and ROS production. However, the positive control, H₂O₂, as well as the various artemisinin drugs, did not increase ROS production in the malaria

parasite. Several new fluorescent probes have been developed by a number of researchers in order to address the weakness in the methods that rely on use of DCF fluorescence. Future studies may be conducted by using the membrane permeable Peroxy Green 1 (PG1) H₂O₂ probe (Rothe & Valet, 1990) or other appropriate ROS probes as suitable partners (with SYTO 61) for labelling the malaria parasite and allowing improved ROS detection.

3. References

- Botha, M.M. 2007. Pre-clinical evaluation of the possible enhancement of the efficacy of antiretroviral drugs by Pheroid™ technology. Potchefstroom: NWU. (Dissertation – M.Sc).
- Chen, H., Zhou, H. & Fang, X. 2003. Inhibition of human cancer cell line growth and human umbilical vein endothelial cell angiogenesis by artemisinin derivatives in vitro. *Pharmacological research: The official journal of the italian pharmacological society*, 48(3):231-236.
- Dondorp, A.M., Nosten, F. & White, N.J. 2009. Artemisinin resistance in plasmodium falciparum malaria. *New england journal of medicine*, 361(18):1807-1808.
- Dondorp, A.M., Nosten, F., Yi, P., Das, D., Phyto, A.P., Tarning, J., Lwin, K.M., Arie, F., Hanpithakpong, W., Lee, S.J., Ringwald, P., Silamut, K., Imwong, M., Chotivanich, K., Lim, P., Herdman, T., An, S.S., Yeung, S., Singhasivanon, P., Day, N.P.J., Lindegardh, N., Socheat, D. & White, N.J. 2009. Artemisinin resistance in plasmodium falciparum malaria. *The new england journal of medicine*, 361(5):455-467.
- FDA. 1993. Draft Redbook II: Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food, Chapter VB: Metabolism and Pharmacokinetic Studies
www.fda.gov/downloads/Food/GuidanceRegulation/UCM078741.pdf Date of access: 1 November 2013.
- GARDNER, C.R. 1987. Drug delivery - where now? (In Johnson, P. & Lloyd-Jones, J.G., eds. Drug delivery systems: Fundamentals and techniques. 1st ed. Ellis Horwood LTD, England. p. 11-31.).
- Gottesman, M.M. & Pastan, I. 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annual review of biochemistry*, 62:385-427.
- Gravett, A.M., Liu, W.M., Krishna, S., Chan, W., Haynes, R.K., Wilson, N.L. & Dalgleish, A.G. 2011. In vitro study of the anti-cancer effects of artemisone alone or in combination with other chemotherapeutic agents. *Cancer chemotherapy and pharmacology*, 67(3):569-577.

- GROBLER, A., KOTZÉ, A.F., DU PLESSIS, J. 2008. The design of a skin-friendly carrier for cosmetic compounds using Pheroid™ technology. (In: Wiechers, J. W. eds. *Science and Applications of Skin Delivery Systems*. Allured Publishing Corporation, Wheaton, IL, USA. p. 283-311
- Isacchi, B., Bergonzi, M.C., Grazioso, M., Righeschi, C., Pietretti, A., Severini, C. & Bilia, A.R. 2012. Artemisinin and artemisinin plus curcumin liposomal formulations: Enhanced antimalarial efficacy against plasmodium berghei-infected mice. *European journal of pharmaceutics and biopharmaceutics*, 80(3):528-534.
- Isacchi, B., Arrigucci, S., Marca, G.I., Bergonzi, M.C., Vannucchi, M.G., Novelli, A. & Bilia, A.R. 2011. Conventional and long-circulating liposomes of artemisinin: Preparation, characterization, and pharmacokinetic profile in mice. *Journal of liposome research*, 21(3):237-244.
- Kotecka, B. M., Edstein, M.D., Rieckmann, K.H., Kyle, D.E., Obaldia, N., Addison R. S. & Dickinson, R.G. 2005. Final report artemisone (bay44-9585): Evaluation of the in vitro and ex vivo antimalarial activities of Artemisone (BAY44-9585) against Plasmodium falciparum isolates and in vivo antimalarial activity of Artemisone in Saimiri and Aotus monkeys. 152 p.
- Nagelschmitz, J., Voith, B., Wensing, G., Roemer, A., Fugmann, B., Haynes, R.K., Kotecka, B.M., Rieckmann, K.H. & Edstein, M.D. 2008. First assessment in humans of the safety, tolerability, pharmacokinetics, and ex vivo pharmacodynamic antimalarial activity of the new artemisinin derivative artemisone. *Antimicrobial agents and chemotherapy*, 52(9):3085-3091.
- Noedl, H., Se, Y., Schaecher, K., Smith, B.L., Socheat, D. & Fukuda, M.M. 2008. Evidence of artemisinin-resistant malaria in western cambodia. *The new england journal of medicine*, 359(24):2619-2620.
- Phyo, A.P., Nkhoma, S., Stepniewska, K., Ashley, E.A., Nair, S., McGready, R., ler Moo, C., Al-Saai, S., Dondorp, A.M., Lwin, K.M., Singhasivanon, P., Day, N.P.J., White, N.J., Anderson, T.J.C. & Nosten, F. 2012. Emergence of artemisinin-resistant malaria on the western border of thailand: A longitudinal study. *Lancet*, 379(9830):1960-1966.
- Reagan-Shaw, S., Nihal, M. & Ahmad, N. 2008. Dose translation from animal to human studies revisited. *FASEB journal: Official publication of the federation of american societies for experimental biology*, 22(3):659-661.
- Rothe, G. & Valet, G. 1990. Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescein. *Journal of leukocyte biology*, 47(5):440-448.

- Slabbert, C. 2011. Formulation, characterization and cellular toxicity of lipid based drug delivery systems for mefloquine. Potchefstroom: NWU. (Thesis – PhD).
- SPEISER, P.P. 1998. Poorly soluble drugs, a challenge in drug delivery. (In Müller, R.H., Benita, S. & Böhm, B.H.L., eds. Emulsions and nanosuspensions for the formulation of poorly soluble drugs. 1st ed. Stuttgart: Medpharm. p. 15-28.).
- STEYN, J.D. 2009. Novel artemisinin derivatives with pheroid™ technology for malaria treatment. Potchefstroom: NWU. (Thesis – PhD).
- Steyn, J.D., Wiesner, L., du Plessis, L.H., Grobler, A.F., Smith, P.J., Chan, W., Haynes, R.K. & Kotzé, A.F. 2011. Absorption of the novel artemisinin derivatives artemisone and artemiside: Potential application of pheroid™ technology. *International journal of pharmaceutics*, 414(1):260-266.
- White, N.J. 2008. Qinghaosu (artemisinin): The price of success. *Science*, 320(5874):330-334.
- WHO (WORLD HEALTH ORGANIZATION). 2012. World Malaria Report 2010. 195p http://www.who.int/malaria/publications/world_malaria_report_2012/report/en/index.html
Date of access: 18 August 2013.
- Witkowski, B., Khim, N., Chim, P., Kim, S., Ke, S., Kloeung, N., Chy, S., Duong, S., Leang, R., Ringwald, P., Dondorp, A.M., Tripura, R., Benoit-Vical, F., Berry, A., Gorgette, O., Arieu, F., de Ric, Barale, J., Mercereau-Puijalon, O. & Menard, D. 2013. Reduced artemisinin susceptibility of plasmodium falciparum ring stages in western Cambodia. *Antimicrobial agents and chemotherapy*, 57(2):2 914-923.