The effect of Pheroid® technology on the bioavailability of artemisone in primates

L Grobler

13065513

BSc, M.Sc. (Biochemistry)

Thesis submitted for the degree Philosophiae Doctor in Pharmaceutics at the Potchefstroom Campus of the North-West University

Promoter: Prof. A.F. Grobler

Co-promoter: Prof. R.K. Haynes

May 2014

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to God for giving me this opportunity.

This thesis could not have been written without the encouragement and generous assistance of countless individuals.

Rick Grobler, my husband, thank you for all the love and patience. Thank you for being there through times of tears and smiles and all of the sacrifices that you had to make. Your spiritual support and encouragement throughout this journey really meant the world to me.

J.P and Elmarie Meeding, my parents, thank you for your financial and emotional support. I could not have asked for better parents and am proud to be your daughter. Thank you for giving me this opportunity and never giving up on me.

Hans and Denise Grobler, my parents-in-law, thank you for your love, understanding and encouragement. Thank you for accepting me as your own daughter and supporting me in a way that you do with your own children.

Prof. Anne Grobler, my promoter, thank you for the opportunity to do my PhD in Pharmaceutics. Thank you for all your advice and for your inputs throughout the process of my research. Thank you for the funding and enabling me to visit other laboratories and meet amazing people.

Prof Richard Haynes, my co-promoter, thank you for your enthusiasm for my work. Thank you for always having an open door. I appreciate the way you shared your vast knowledge on malaria and your assistance in the writing of this thesis.

Mike, Marina and Kerryn, my colleagues from the army malaria institute in Australia, thank you for making my visit there a memorable and enjoyable one. I really learned so much from you and was truly blessed by the way I was received in your laboratories. Mike and Maria, thank you for opening your home for me, for all the beautiful places you showed me and the delicious food.

Liezl Gibhard, my PhD buddy, as we walked this road together, I thank God that I could share all those times in the laboratory with you. Whether it was early mornings, late nights or labelling tubes until the break of dawn, we got through it together. I will always remember

and treasure the verse that God gave us for our PhD's. Rom 5:3-5 "And not only *this*, but we glory in afflictions also, knowing that afflictions work out patience, and patience *works out* experience, and experience *works out* hope."

I would also like to thank my friends and colleagues for your encouragement, prayers and support.

Lizette Grobler

This thesis is submitted in fulfilment of the requirements of a Doctor of Philosophy in Pharmaceutics. This study forms part of the project T90020: “An investigation into drug resistance reversal of antimalarials using Pheroid™ technology” and was financially supported by the South African Technology Innovation Agency (TIA), the Swiss South African Joint Research Project Initiative and the North-West University. This thesis is submitted in a manuscript format in accordance with the General Academic Rules (A.7.5.7.4) of the North-West University. Each chapter is written in accordance with specific guidelines as stipulated by the journals intended for publication. Manuscript 1 has been submitted to *Expert Opinion on Drug Metabolism and Toxicology*, manuscript 2 will be submitted to *Antimicrobial Agents and Chemotherapy* and manuscript 3 will be submitted to *The International Journal for Parasitology – Drugs and Drug Resistance*

The contribution of each author is as follows:

L. Grobler (Candidate)

- Planning and design of the studies.
- Experimental work.
- Interpretation of results.
- Writing of the thesis and manuscripts.
- Corresponding author of manuscripts.

Prof. A.F. Grobler (Promotor)

- Supervision of the planning and design of the studies.
- Assistance in the interpretation of results.
- Supervision and critical review of the writing of the thesis and manuscripts.
- Corresponding author of manuscripts.

Prof. R.K. Haynes (Co-promotor)

- Supervision of the planning and design of the studies.
- Assistance in the interpretation of results.
- Supervision and critical review of the writing of the thesis and manuscripts.
- Supplied artemisone and metabolite M1.

Prof. P.A. Steenkamp

- Critical review of the writing of the manuscripts 1 and 3.
- Performed the UPLC-QTOF-MS analysis.
- Assistance in the interpretation of metabolism and p-gp results.

Prof. C. Masimirembwa

- Critical review of the writing of the manuscript 1.
- Supervision of the planning and design of the metabolism study.
- Assistance in the interpretation of the metabolism results.

Mrs. R. Thelingwani

- Experimental work and assistance in the interpretation of the metabolism results.

Prof. H.S. Steyn

- Performed the statistical analysis.
- Critical review of the writing of the manuscripts.

Dr M. Chavchich

- Critical review of the writing of manuscript 2.
- Supplied *P. falciparum* isolates.
- Supervision of the planning and design of the *in vitro* efficacy and dormancy study.
- Experimental work and assistance in the interpretation of the *in vitro* efficacy and dormancy study.

Prof. M. D. Edstein

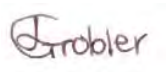
- Critical review of the writing of the manuscript 2.
- Supplied *P. falciparum* isolates.
- Supervision of the planning and design of the *in vitro* efficacy and dormancy study.
- Assistance in the interpretation of the *in vitro* efficacy and dormancy study.

Prof. H. J. Viljoen

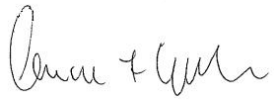
- Set up of the mathematical model of the caco-2 study and assistance in the interpretation thereof.

Declarations

I hereby declare that I have approved the manuscripts/thesis and that my role in the study as indicated above is representative of my actual contribution. I give permission as author or co-author for submission of the manuscripts/thesis for degree purposes.



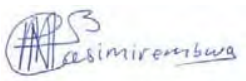
L. Grobler



Prof. A.F. Grobler

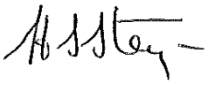
Prof. R.K. Haynes

Prof. P.A. Steenkamp



Prof. C. Masimirembwa

Mrs. R. Thelingwani



Prof. F. Steyn

Dr M. Chavchich

Prof. M. D. Edstein

Prof. H. J. Viljoen

ABSTRACT

Malaria is one the world's most devastating diseases. Several classes of drugs are used to treat malaria. Artemisinin combination therapy is the first line treatment of uncomplicated malaria. The artemisinin derivative, artemisone in conjunction with the Pheroid® drug delivery system, is the focus of this thesis.

The impact of the Pheroid® on the bioavailability of artemisone was evaluated in vervet monkeys. The resulting artemisone plasma levels were much lower (C_{max} of 47 and 114 ng/mL for reference and Pheroid® test formulations respectively) than expected for the dosages administered (60 mg/kg). The Pheroid® improved the pharmacokinetic profile of artemisone in a clinically significant manner. The metabolism of artemisone was assessed *in vitro* by using human and monkey liver and intestinal microsomes, and recombinant CYP3A4 enzymes. The Pheroid® inhibits the microsomal metabolism of artemisone. In addition, there is a species difference in artemisone metabolism between man and monkey since the *in vitro* intrinsic clearance of the reference formulation with monkey liver microsomes is ~8 fold higher in the monkey liver microsomes compared to the human liver microsomes and the estimated *in vivo* hepatic clearance for the monkey is almost twofold higher than in humans.

Artemisone has potent antimalarial activity. Its *in vitro* efficacy was approximately twofold higher than that of either artesunate or dihydroartemisinin when evaluated against *P. falciparum* W2, D6, 7G8, TM90-C2B, TM91-C235 and TM93-C1088 parasite strains. The Pheroid® drug delivery system did not improve or inhibit the *in vitro* efficacy of artemisone or DHA. Artemisone (reference and Pheroid® test formulations) and metabolite M1 abruptly arrested the growth of *P. falciparum* W2 parasites and induced the formation of dormant ring stages in a manner similar to that of DHA.

Interaction of artemisone with the p-glycoprotein (p-gp) efflux transporter was investigated. Artemisone stimulates ATPase activity in a concentration-dependent manner, whereas the Pheroid® inhibited this p-gp ATPase activity. P-gp ATPase activity stimulation was fourfold greater in human than cynomolgus monkey MDR1 expressed insect cell membranes. Artemisone alone and artemisone entrapped in Pheroid® vesicles showed moderate apical to basolateral and high basolateral to apical permeability (P_{app}) across Caco-2 cells. The P_{app} efflux ratio of artemisone and artemisone entrapped in Pheroid® vesicles were both >5, and decreased to ~1 when the p-gp inhibitor, verapamil, was added. Therefore, artemisone is a substrate for mammalian p-gp. The cytotoxic properties of Pheroid® on Caco-2 cells were assessed and the pro-Pheroid® seems to be non-toxic at concentrations of ≤1.25%.

Vervet monkey plasma caused antibody-mediated growth inhibition of *P. falciparum*. Heat inactivated or protein A treatment proved useful in the elimination of the growth-inhibitory activity of the drug-free plasma. Plasma samples containing artemisone could not be analysed by the *ex-vivo* bioassay method. The dual labelling ROS assay did not prove to be useful in the evaluation of ROS production by artemisone and the Pheroid® delivery system.

In conclusion, entrapment of artemisone in the Pheroid® delivery system improves the pharmacokinetic properties of artemisone, but does not improve or inhibit its antimalarial efficacy *in vitro*. The Pheroid® inhibited both the microsomal metabolism of artemisone and P-gp ATPase activity and was shown to be non-toxic at clinically usable concentrations.

Keywords: artemisone, bioavailability, clearance, drug delivery, efficacy, malaria, metabolism, monkey, Pheroid®.

UITTREKSEL

Malaria is een van die wêreld se mees verwoestende siektes. Verskeie geneesmiddelklasse word gebruik om malaria te behandel. Artemisinin gebaseerde kombinasie terapie is die eerste linie van behandeling van ongekompliseerde malaria. Die artemisinin derivaat, artemisoon, in kombinasie met die Pheroid® geneesmiddel afleweringsisteem, is die fokus van hierdie proefskrif.

Die impak van die Pheroid® op die biobeskikbaarheid van artemisoon is geëvalueer in blouape. Die artemisoon plasma vlakke was laer (C_{max} van 47 en 114 ng/mL vir verwysing en Pheroid® toets formulerings onderskeidelik) as wat verwag is vir die dosis wat toegedien is (60 mg/kg). Inkorporering van artemisoon in die Pheroid® afleweringsisteem het die farmakokinetiese profiel van artemisoon op 'n klinies beduidende wyse verbeter. Die metabolisme van artemisoon is beoordeel *in vitro* deur die gebruik van 5 toetsisteme: menslike lewer, aaplewer, menslike en aap intestinale mikrosome, en die rekombinante ensiem CYP3A4. Die Pheroid® inhibeer die mikrosomale metabolisme van artemisoon. Daarbenewens is daar is 'n spesie verskil in artemisoon metabolisme tussen mens en aap aangesien die *in vitro* intrinsieke opruiming van die verwysing formulering gemeet in aap lewer mikrosome ~8 keer hoër is in die aap lewer mikrosome in vergelyking met die menslike lewer mikrosome. Die beraamde *in vivo* hepatiese opruiming vir die aap is byna twee keer hoër as in die mens.

Artemisoon toon goeie anti-malaria effektiwiteit *in vitro*. Die effektiwiteit was ongeveer twee keer hoër as dié van óf artesunaat óf dihydroartemisinin teen *P. falciparum* W2 , D6 , 7G8 , TM90 - C2B , TM91 - C235 en TM93 - C1088 parasiet isolate in *in vitro* effektiewiteits toetse. Die Pheroid® het nie die *in vitro* effektiwiteit van artemisoon of DHA verbeter of geinhibeer nie. Artemisoon (verwysing- en Pheroid® formulerings) en metaboliet M1 het ‘n vinnige staking in die groei van *P. falciparum* W2 parasiete veroorsaak en het ook gelei tot die vorming van dormant ring stadia op 'n wyse soortgelyk aan dié van DHA .

Interaksie van artemisoon met p-glikoproteïen (p-gp) effluks sisteem se ATPase is ondersoek. Artemisoon stimuleer ATPase aktiwiteit op 'n konsentrasie-afhanklike wyse, terwyl die Pheroid® hierdie aktiwiteit inhibeer. Stimulasie van p-gp ATPase aktiwiteit was vier keer hoër in menslike MDR1 uitgedrukte insek selmembrane as in dié van die cynomolgus aap. Artemisoon alleen en artemisoon in die Pheroid® vesikels het matige apikale tot basolaterale en hoë basolaterale tot apikale deurlaatbaarheid (P_{app}) deur Caco-2 selle getoon. Die P_{app} uitvloei verhouding van artemisoon en artemisoon vasgevang in Pheroid® vesikels was albei >5, en het tot ~1 gedaal wanneer die p-gp inhibitor, verapamil,

bygevoeg is. Artemisoon is dus 'n substraat vir soogdier p-gp. Die sitotoksiese eienskappe van Pheroid® op Caco-2 selle is bepaal en die pro-Pheroid® blyk nie-toksies te wees by konsentrasies van ≤1,25% nie.

Blouaap plasma veroorsaak teenliggaam-bemiddelde inhibisie van die vermeerdering van *P. falciparum* parasiete. Inaktivering van plasma deur verhitting of protein A behandeling is gebruik vir die vernietiging van die groei- inhiberende effek van die geneesmiddel-vrye plasma. Artemisoon bevattende plasma monsters kon nie ontleed word deur die *ex-vivo* bioassay metode nie. Die dubbele merking ROS metode was nie bruikbaar vir die evaluering van ROS produksie deur artemisoon en die Pheroid® nie.

Ten slotte, toevoeging van artemisoon tot die Pheroid® verbeter die farmakokinetiese eienskappe van artemisoon, maar daar is nie ‘n verbetering of inhibisie van die *in vitro* anti-malaria effektiwiteit nie. Die Pheroid® inhibeer beide die mikrosomale metabolisme van artemisoon en P-gp ATPase aktiwiteit en bleik nie-toksies te wees by klinies toepaslike konsentrasies.

Sleutelwoorde: aap, artemisoon, biobeskikbaarheid, effektiwiteit geneesmiddel aflewering, , malaria, metabolisme, opruiming, Pheroid®.

TABLE OF CONTENTS

LIST OF FIGURES (SHORT LIST)

CHAPTER 2:

CHAPTER 3:

CHAPTER 4:

CHAPTER 5:

CHAPTER 6:

LIST OF TABLES (SHORT LIST)

CHAPTER 2:

CHAPTER 3:

CHAPTER 4:

CHAPTER 5:

CHAPTER 6:

CHAPTER 7: