Annexure A contains the following poster:

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

Lizelle Grobler, Anna Grobler, Richard Hayne, Fauns Steyn and Lubba Wissner

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Introduction

Artemisinin-based combination treatments is recommended by the World Health Organization as first line treatment of uncomplicated malaria†. Artemisinins have a low aqueous solubility that causes poor, inconsistent absorption upon oral administration, which in turn results in low bioavailability. Their short elimination half-life is also a limitation‡.

The raw artemisinin derivative, artesunate, shows enhanced antimalarial activity against chloroquine-susceptible and -resistant P. falciparum cell lines in vitro and against chloroquine sensitive P. berghei and chloroquine resistant P. yoelii rodent models and in Aedes aegypti mosquito bioassay models in vivo when compared to the more widely used artemether-lumefantrine, artemether.§

Pheroid® technology is an emulsion type formulation that can be manipulated in terms of structure, size, morphology and function in order to adapt to the chemical class and size of the drug molecules that need to be delivered. It can entrap transport and deliver pharmacologically active compounds and other molecules. In terms of artemisinin, bioavailability studies have been performed for reference and Pheroid®-entrapped artemisinin in a C57BL6 mouse model.§ Significant differences were found between these two formulations with a greatly increased Cmax (1500 ng/ml vs. 804.9 ng/ml) for artemisinin-Pheroid® vesicle formulation compared to the artemisinin reference formulation. This Cmax, auc and AUC0-∞ also increased (2291.9 ng/ml and 3044.0 ng/ml vs. 458.7 ng/ml and 946.4 ng/ml) with the Pheroid® vesicle formulation.

Aim

The purpose of this study was to progress the investigation into the enhancement of the pharmacokinetic profile of artemisinin in the Pheroid® delivery system to a primate model.

Methods

Artemisinin was prepared at the Hong Kong University of Sciences and Technology. Pheroid® was prepared by dissolving an oil phase consisting of the active pharmaceutical ingredient (API) PFD, APA, Vemivas P and other additives (Phospholipid 91, Phospholipid 92, WH and BHT) with N2O for the Pheroid® vesicle. Pheroid®-entrapped artesunate (Pheroid®-entrapped) was administered to rhesus monkeys (n = 5) as a single dose of 60 mg/kg, using an intravenous dosing vehicle.

Blood samples were drawn in heparin through percutaneous venipuncture of the femoral vein prior to and 7 times post dose up to 10 hours. Plasma samples were prepared by centrifugation (4 °C) and then stored at −198 °C and then stored at −198 °C. Parent drug concentrations and metabolite M1 in the plasma samples were determined using an LC/MS/MS assay. Calibration standards and quality control standards were prepared in blank plasma matrix. Analysis was used as an internal standard. An Agilent 1200 series analyser was used for analysis. An Agilent 1200 series analyser was used for analysis. An Agilent 1200 series analyser was used for analysis. An Agilent 1200 series analyser was used for analysis. An Agilent 1200 series analyser was used for analysis. An Agilent 1200 series analyser was used for analysis.

In vivo metabolic stability

Artemisinin-reference and Pheroid® formulations were incubated with enzyme (1.5 mg/ml) and 0.1 M phosphate buffer (pH 7.4). The reaction was initiated by the addition of NADPH. An aliquot was taken at various time points up to 45 min. The reaction was stopped by the addition of ice cold acetonitrile. Samples were stored at 4 °C to allow protein precipitation and centrifuged at 10,000 g for 15 minutes at 4 °C. Supernatants were diluted at 1:20 and analyzed on a UPLC-QTof MS/MS with a Waters Sample Manager (autosampler) and Waters Synapt HDMS G1 (source).

Results

Table 1: Pharmacokinetic parameters of the oral artemisinin reference and Pheroid® formulations

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Drug</th>
<th>Art-entrap</th>
<th>Art</th>
<th>Art-ref</th>
<th>Cmax</th>
<th>AUC0-∞</th>
<th>T1/2</th>
<th>tmax</th>
<th>BRI1</th>
<th>BRI2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>Art</td>
<td>0.07</td>
<td>472.0</td>
<td>0.49</td>
<td>8.6</td>
<td>3.6</td>
<td>4.1</td>
<td>60</td>
<td>0.24</td>
<td>3.77</td>
</tr>
<tr>
<td>Pheroid®</td>
<td>Art</td>
<td>472.0</td>
<td>0.49</td>
<td>8.6</td>
<td>3.6</td>
<td>4.1</td>
<td>60</td>
<td>0.24</td>
<td>3.77</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Discussion

After log-transformation of the pharmacokinetic parameters Cmax, AUC and t1/2 (and a Box-Cox transformation of tmax) the two formulations did not differ significantly (p > 0.65). The mean artemisinin Cmax, AUC and t1/2 were higher with the reference formulation compared to the reference formulation and the T1/2 and t1/2 were similar for both formulations. The artemisinin plasma levels were much lower than expected for the dosages administered. Previous studies established that a lower bioavailability for certain drugs occurs in monkeys, compared to humans, due to incomplete gastrointestinal absorption, greater first-pass metabolism, or a combination of these factors.

The in vitro metabolism studies indicated that very slow metabolism of the artemisinin reference occurred in HLM with no metabolism in the M1. The pre-Pheroid® formulation inhibited artemisinin metabolism in all three species preparations. The in vivo intrinsic clearance of the M1 is much higher than that of HLM. If extrapolated to the estimated hepatic clearance in vivo, the monkey CLH is almost twice the CLH in humans. Also, if azithromycin (CYP3A4 inhibitor) is added to the reference mixture, metabolism of artemisinin is inhibited in the human liver microsomes (% drug remaining: ~91%) but not in the monkey liver microsomes (% drug remaining: ~2.8%). Therefore, the monkey CYP3A4 in the monkey might not be the enzyme responsible for the catabolism of artemisinin or alternatively the monkey CYP3A4 is not inhibited by azithromycin.

Conclusion

Entrapment of artesunate in the Pheroid® delivery system improves the pharmacokinetic profile of artesunate, although statistical significance could not be shown as a result of the low plasma levels in the limited number of animals used. The vena network model may not be the best model for evaluation of artesunate bioavailability.

The in vitro results suggest that artesunate is protected by the Pheroid® delivery system against microsomal metabolism.

References

Acknowledgements

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Annexure B contains the following poster:

_Grobler, L._ Grobler, A., Haynes, R. & Steyn, F. The effect of Pheroid® technology on
the bioavailability of artemisone in primates. 33rd Annual Conference of the Academy
of Pharmaceutical Sciences of South Africa, Rhodes University, Grahamstown,
2012. (Poster)._
The effect of Pheroid® technology on the bioavailability of artemisone in primates

Lizette Grobler, Anne Grobler, Richard Haynes, Fauns Sneyd

Introduction

Artemisinin-based combination treatments have been recommended by the World Health Organization as the first line treatment of uncomplicated malaria. Artemisinins have low aqueous solubility that choose poor oral administration upon oral administration which in turn results in low bioavailability. Another limitation is their short elimination half-life.

The new artemisinin derivative, artemisone, shows enhanced artemisinide activity against chloroquine-sensitive and -resistant P. falciparum cell lines in vitro and against chloroquine-sensitive P. berghei and chloroquine-resistant P. rodentium models and in Analo monkey—P. bergelive models in vivo when compared to the most widely used artemisinin derivative, artemether.

Pheroid® technology is an emulsion type formulation that can be manipulated in terms of structure, size, morphology and function depending on the type and size of the drug molecules that needs to be delivered. It can encapsulate, transport and deliver pharmaceutically active compounds and other molecules. In terms of artemisone, bioavailability studies have been performed for reference and Pheroid®-encapsulated artemisone in a C57 Bl/6 mouse model. Significant differences where found between Pheroid®-encapsulated artemisone and its free form in a C57 Bl/6 mouse model. C85% of 100 mg/kg for artemisone was determined as 0.599 mg after 1 hour for artemisone free form and 3.76 mg after 1 hour for the artemisone-Pheroid® vehicle formulation.

Aim

The purpose of this research was to investigate the enhancement of the pharmacokinetic profile of artemisone when incorporated in the Pheroid® delivery system by using a primate model.

Methods

Artemisone was prepared at the Hong Kong University of Science and Technology. Pheroid® was prepared by pressurizing an oil phase consisting of the active pharmaceutical ingredient (API), PEG 400, vitamin E (d-tocopherol), Cremophor EL, c-Dioiooctophae, BHA and BHT with N2O under high pressure.

Discussion

After log transformation of the pharmacokinetic parameters Cmax, AUC and Vd (and a Box-Cox transformation of Cmax the two formulations did not differ significantly (p=0.65).

A: The mean artemisone Cmax (103.21 ± 151.87 ng/ml ± 47.58 ± 46.53 ng/ml, p = 0.16); AUC (621.63 ± 521.42 ng/ml ± 194.81 ± 192.34 ng/ml, p = 0.07) and Vd (145.16 ± 43.74 ng/ml ± 317 ± 1.68 ng/ml, p = 0.01) was higher with the test formulation compared with the reference formulation and the Tmax (0.3 ± 0.24 ± 5.11 ± 2.52 hours, p = 0.14) and Vd (62.84 ± 6.65 vs. 13.22 ± 26.81 mg/l, p = 0.17) were lower.

B: The mean M1 Cmax (940.75 ± 583.42 vs. 75.73 ± 80.36 mg/ml, p = 0.31); AUC (605.46 ± 801.43 vs. 303.44 ± 320.25 ng/ml, p = 0.45) and Vd (92.95 ± 6.45 vs. 352 ± 1.63 mg/l, p = 0.47) seems to be higher with the test formulation and the Tmax (0.8 ± 1.46 vs. 3 ± 1.20 hours p = 0.50) were similar for both formulations.

The artemisone plasma levels are much lower than expected for the doses administered. Previous studies established that a lower bioavailability for certain drugs occurs in monkeys, compared to humans, due to incomplete gastrointestinal absorption, greater first-pass metabolism, or a combination of these factors.

Conclusion

Entrapment of artemisone in the Pheroid® delivery system improves the pharmacokinetic profile of artemisone, although statistical significance could not be shown as a result of the low plasma levels in the limited number of animals used. The primate model may not be the most suitable model for evaluation of artemisone bioavailability.

References

33rd Annual Conference of the Academy of Pharmaceutical Sciences of South Africa, 12-15 Sept 2012
Annexure C contains the following poster:
Comparative pharmacokinetic analysis of novel antimalarial artemisinin derivatives after oral administration using Pheroid™ technology

Grobler, Anne;* Stain, Dewald;† Meadung, Lizette; Gibbard, Liesl; Scholtz, L-M; Wiesner, Lubbe; Haynes, Richard

*North-West University, Pharmacology, Potchefstroom, South Africa; †University of Cape Town, Clinical Pharmacology, Cape Town, South Africa; ‡The Hong Kong University of Science and Technology, Department of Chemistry, Hong Kong, Hong Kong

Introduction

Artemisinin has a short half-life, which dictates high remediation rates and necessitates novel formulations. This has led to the development of artemisinin derivatives, which are more stable and have a longer half-life than the parent compound. Artemisinin is a sesquiterpene lactone isolated from the plant Artemisia annua. It is a potent antimalarial agent and is used in various formulations to treat malaria. However, due to its short half-life and limited oral bioavailability, new formulations are required to improve its efficacy.

Chloroquine forms part of the quinolines. During the 1960s the first quinoline antimalarial drugs were developed and used. Chloroquine has been in clinical use since 1947 and is known for its effectiveness in treating and preventing malaria. It is effective against all the sensitive strains of Plasmodium falciparum, Plasmodium vivax, and Plasmodium ovale. Chloroquine is highly water soluble and has a long half-life, making it effective for long-term use.

Aim

To evaluate the effect of Pheroid™ technology on the bioavailability of a new multi-chambered Plasmodium derivative.

Methods

Materials

Artemisinin is a sesquiterpene lactone isolated from the plant Artemisia annua. Artemisinin is obtained from 20% dry Chinese plant material. Pheroid™ delivery system formulations were synthesized by the Medical School of the North-Wes University.

Antimalarial drug classes

Artemisinin, a sesquiterpene lactone, forms part of a class of antimalarials called the artemisinins. The artemisinin-antimalarial drugs consist of quinolines, 4-quinolones, 4-quinolones and Quinoline derivatives. Chloroquine is a member of the quinolines.

Reference formulations

The required amount of artemisinin was dissolved in a single glass DMSO (anti)free water was added to a hot water bath at 50°C and allowed to remain for 15 minutes. The required amount of artemisinin was dissolved in a single glass DMSO (anti)free water was added to a hot water bath at 50°C and allowed to remain for 15 minutes. The required amount of artemisinin was dissolved in a single glass DMSO (anti)free water was added to a hot water bath at 50°C and allowed to remain for 15 minutes. The required amount of artemisinin was dissolved in a single glass DMSO (anti)free water was added to a hot water bath at 50°C and allowed to remain for 15 minutes.

Experimental approach

Pheroid™ technology was administered by oral gavage to ensure that the test and control mice received an equal amount of the test and control compounds.

Instruments

The instruments used were quality control equipment and a standard polarimeter. The polarimeter was placed on the test and control mice.

Table 1: Summary of study design

<table>
<thead>
<tr>
<th>Compound</th>
<th>Treatment</th>
<th>Route</th>
<th>Dose</th>
<th>Plasma</th>
<th>Tissue</th>
<th>Relative potency for all detection levels</th>
<th>In vivo</th>
<th>In vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemisin</td>
<td>Control</td>
<td>Oral</td>
<td>5 mg/kg</td>
<td>0.098</td>
<td>0.098</td>
<td>1.000</td>
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</tr>
<tr>
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</table>

Conclusion

In summary, the results obtained in this study have indicated that Pheroid™ technology is a promising method for improving the bioavailability and efficacy of antimalarial drugs. Further studies are needed to confirm these findings and to optimize the formulation and delivery system for clinical use.

Pheroid™ technology is expected to enhance the bioavailability and efficacy of antimalarial drugs, thereby improving treatment outcomes for malaria patients worldwide.

Fig. 1: Study design

Table 2: Summary of study design

<table>
<thead>
<tr>
<th>Compound</th>
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</table>
Annexure D contains the Ethics application for cultivation of Plasmodium falciparum parasites in human blood (NWU-0008-08-S5) and for evaluation of bioavailability and efficacy of Pheroid®-entrapped anti-infective agents in non-human primates (NWU-00027-10-A5)
Dear Ms Botha

FINAL RESPONSE: ETHICS APPLICATION: NWU-0008-08-55

The abovementioned application has reference.

We have received satisfactory answers to all the questions posed by the Ethics panel and has therefore found the ethical aspects to be in order.

PROF. J. DU PLESSIS
DIRECTOR
ETHICS APPROVAL OF PROJECT

This is to certify that the next project was approved by the NWU Ethics Committee:

Project title: investigation into the comparative bioavailability and efficacy of Phenoid™-entrapped anti-infective agents in non-human primates

Project leader: Dr. AF Grobler
Ethics number: NWU-00027-10-A5

Expiry date: 2015/07/11

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

This formal Ethics approval certificate will be sent to you as soon as possible.

Yours sincerely,

Ms. Marielle Hatgryn
NWU Ethics Secretariat
ANNEXURE E

Annexure E contains the proof of language editing.
ENGLISH LANGUAGE EDITING CERTIFICATION

This is to certify that the English language editing of this thesis by Ms L Grobler was done by Prof. L.A. Greyvenstein.

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