

CHAPTER 5

This chapter consists of a full length text article to be submitted to *The International Journal for Parasitology – Drugs and Drug Resistance*. In this chapter the author guidelines are given, followed by the article prepared according these guidelines. The aim of this chapter was to determine if artemisone acts as a P-gp substrate and if so, what the effect of the Pheroid® system will be. Also, the cytotoxic properties of the Pheroid® delivery system on Caco-2 cells were assessed.



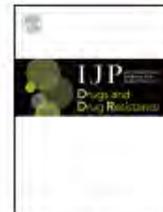
INTERNATIONAL JOURNAL FOR PARASITOLOGY: DRUGS AND DRUG RESISTANCE

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AUTHOR INFORMATION PACK

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DESCRIPTION

The International Journal for Parasitology – Drugs and Drug Resistance publishes the results of original research in the area of anti-parasite drug identification, development and evaluation, and parasite drug resistance. The journal also covers research into natural products as anti-parasitic agents, and bioactive parasite products. Studies can be aimed at unicellular or multicellular parasites of human or veterinary importance.

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Artemisone, a possible substrate of the multidrug transporter, P-glycoprotein?

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Abstract

P-glycoprotein and CYP3A4 have overlapping substrate specificities and since artemisone, a novel artemisinin derivative, is a CYP3A4 substrate, the objective of this study was to examine whether artemisone acts as a p-gp substrate. Using membranes overexpressing p-gp, the current study investigated the effects of artemisone and artemisone entrapped in the Pheroid® drug delivery system on p-gp ATPase activity. Cytotoxicity of the delivery system in Caco-2 cells was measured using the live/dead assay. Bidirectional *in vitro* transport assays using Caco-2 cells were carried out in the presence and absence of the p-gp inhibitor, verapamil. Samples were analysed for artemisone and verapamil using an UPLC-QTOF-MS. Artemisone stimulated the p-glycoprotein (p-gp)-ATPase activity in a concentration-dependent manner, while the Pheroid® drug delivery system inhibited drug stimulated p-gp-ATPase activity. The Pheroid® drug delivery system is not cytotoxic to Caco-2 cells at concentrations of $\leq 1.25\%$. Incubation of Caco-2 cells with artemisone alone and artemisone entrapped in Pheroid® vesicles showed moderate apical to basolateral ($2.01 \pm 0.11 \times 10^{-6}$ and $2.04 \pm 0.07 \times 10^{-6}$ cm/s respectively) and high basolateral to apical ($9.36 \pm 0.35 \times 10^{-6}$ and $12.40 \pm 0.12 \times 10^{-6}$ cm/s respectively) permeability (P_{app}). The P_{app} efflux ratios for artemisone and artemisone entrapped in Pheroid® vesicles were 4.65 ± 0.40 and 6.07 ± 0.22 , respectively, and decreased to 1.06 ± 0.04 when $50 \mu\text{M}$ verapamil was added. The data show that artemisone is a p-gp substrate.

Keywords: artemisone, Caco-2, permeability, p-glycoprotein, ATPase, Pheroid®

1. Introduction

The drug efflux pump P-glycoprotein (p-gp) belongs to the ATP binding cassette (ABC) transport superfamily. Human p-gp is encoded by the multidrug resistance protein 1 (MDR1) gene and is expressed in several human tissues such as the liver, kidney, gastrointestinal tract, and in the blood-brain barrier (Ayrton and Morgan, 2001). Intestinal expression of p-gp may have an effect on the oral bioavailability of p-gp substrates. Overexpression of p-gp in cancer cells also contributes to the mechanism of chemoresistance, also known as multidrug resistance (Chen et al., 1986, Gottesman and Pastan, 1993). Drug-drug interactions resulting from inhibition or induction of p-gp are regarded as clinically important (Englund et al., 2004; Balayssac et al., 2005; FDA, 2012). In early drug discovery it is, therefore, necessary to examine interactions between p-gp and drugs because this can affect the pharmacokinetics, efficacy and safety of drugs.

Artemisone, an artemisinin derivative, is classified as nonlipophilic as it has an aqueous solubility of 89 mg/liter at pH 7.2 and a log P (octanol–water partition coefficient) of 2.49 at pH 7.4. When compared to the most widely used artemisinin derivative, artesunate, artemisone possesses superior *in vitro* antimalarial activity against various chloroquine-sensitive and – resistant *P. falciparum* lines (see Chapter 4), as well as superior *in vivo* activity in the *Plasmodium berghei* infected rodent and *P. Falciparum* Aotus monkey models (Haynes, 2006). The regulation of p-gp and CYP3A4 is tightly linked and since they have similar substrate specificity and localization in the human body, synergistic actions of both CYP3A and p-gp may limit oral drug delivery (Lindell, et al., 2003, Zhang and Benet, 2001.). Artemisone, a novel artemisinin derivative is a CYP3A4 substrate (Haynes, 2006). In this study the aim was, therefore, to determine whether artemisone is a possible p-gp substrate.

Mouse Mdr1 and human MDR1 transfected cell lines are mainly used in p-gp studies although nonrodent species like monkeys are generally used as *in vivo* preclinical screening models (Xia et al., 2006.). The binding affinities of test compounds to rhesus monkey p-gp

were found to be close to those of human p-gp (Xia et al., 2006.). In order to extrapolate the pharmacokinetic behaviour of artemisone and artemisone entrapped in Pheroid® observed in monkeys (see Chapter 3) to those in humans, we investigated the species differences of p-gp activities. Takahashi *et al.* (2008) demonstrated that the mRNA levels of efflux transporters such as MDR1 were much higher in cynomolgus monkeys than in humans.

Several *in vitro* screening assays have been developed to identify p-gp substrates or inhibitors. These include direct assays using cell systems to monitor drug transport and indirect assays such as drug-stimulated ATPase activity or the Calcein-AM fluorescence assay (Polli et al., 2001). In this study the ATPase activity was determined by observing the liberation of inorganic phosphate by a colorimetric reaction. P-gp substrates stimulate the ATPase activity of human p-gp expressed in *Spodopterafrugiperda* Sf9 insect cell membranes (Sarkadi et al., 1992; Scarborough, 1995) and p-gp expressing mammalian cells (Senior et al., 1995; Doige et al., 1992; Shapiro and Ling, 1994). Because the ATPase assay cannot distinguish between substrates or inhibitors, drug transport was also studied in the well characterized Caco-2 cell monolayers. These intestinal adenocarcinoma cells are often used as the surrogate of the human intestine since they undergo spontaneous differentiation after 3 - 4 weeks into polarised enterocyte monolayers with highly expressed intercellular tight junctions when cultured on membranes (Wen-Loh et al., 2012). Due to their similarity to the small intestinal epithelium, Caco-2 cells are generally accepted as the *in vitro* model for assessing the transport of drugs or delivery systems across epithelial barriers (Ruiz-Garcia et al., 2007). By measuring trans-epithelial electrical resistance (TEER) and cell viability, Caco-2 cells can also be used to determine the possible cytotoxicity of drugs or delivery systems (Lin et al., 2011). In addition, Caco-2 monolayers are commonly used to measure efflux activity of p-gp by comparing basal-to-apical permeability of drugs to apical-to-basal permeability (FDA, 2012, Balimane et al., 2006). In the present study, we investigated the effect of the Pheroid® drug delivery system on the transport of artemisone across the Caco-2 cells.

The Pheroid® drug delivery system is a trademarked entity but for ease of reading may be referred to as Pheroid(s) for the remainder of this article. Pheroid technology is based on a stable, colloidal system, capable of entrapping, transporting and delivering pharmacologically active compounds and other molecules (Grobler et al., 2008, Steyn et al., 2011). By altering the ratios of components or the preparation process, the size and structure of the Pheroid carriers can be manipulated to form lipid-bilayer vesicles, microsponges or pro-Pheroid (Grobler, 2009). Unlike Pheroid vesicles and microsponges, the pro-Pheroid formulations do not contain a water phase, but consist only of an oil phase saturated with nitrous oxide gas. Upon addition of a water phase to the pro-Pheroid, Pheroid micro- and nano-particles form spontaneously, packaging the active pharmaceutical ingredient (API) present in the pro-Pheroid into the particles (Grobler, 2009).

2. Materials and methods

2.1. Materials

Artemisone was prepared by Ho-Ning Wong and Wing-Chi Chan in the Department of Chemistry at the Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong. Nile red, verapamil, loperamide and vinblastine were obtained from Sigma-Aldrich (St. Louis, USA). Vitamin F ethyl ester was obtained from Chemimpo (South Africa) and Kolliphor® EL was obtained from BASF (South Africa). Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), polyethylene glycol (PEG 400) and dl- α -tocopherol were obtained from Chempure (South Africa). Purified water was obtained from SABAX (Johannesburg, South Africa). Hyclone® Dulbecco's modified Eagle medium (DMEM; with 4 mM glutamine, 4500 mg/L glucose and sodium pyruvate) and penicillin (10,000 units/mL)–streptomycin (10,000 mg/mL) solution were obtained from Thermo Scientific (Pittsburgh, PA, USA). Hank's balanced salt solution (HBSS with 0.35 g/L NaHCO₃, Ca²⁺ and Mg²⁺), N N-2-Hydroxyethylpiperazine-N' -2-ethane-sulfonate (Hepes), heat-inactivated fetal bovine serum (FBS) and amphotericin B (250 µg/ml) were obtained from Biochrom AG (Germany). Trypsin-Versene (EDTA) (1x), L-glutamine (200 mM) and nonessential amino acid solution

(NEAA, 100 x) were obtained from Lonza (Walkersville, USA). The ATPase kit, human MDR1 (p-gp) and cynomolgous monkey Mdr1-overexpressing membranes were purchased from Gentest Co. (Woburn, MA, USA). All other chemicals and reagents were of analytical grade and were used as received and instructed.

2.2. Preparation of solutions

2.2.1. ATPase assay

The 30 mM artemisone stock solution was prepared in acetonitrile. Pro-Pheroid containing artemisone (30 mM) and drug free pro-Pheroid were prepared. Drug free pro-Pheroid contained PEG 400, vitamin F ethyl ester, Kolliphor® EL, BHA, BHT and dl- α -tocopherol in a ratio of 4.9:66.41:27.67:1:0.01:0.01 w/w. The pro-Pheroid containing artemisone contained artemisone, PEG 400, vitamin F ethyl ester, Kolliphor® EL, BHA, BHT and dl- α -tocopherol in a ratio of 0.16:4.9:66.30:27.62:1:0.01:0.01 w/w. Artemisone was premixed into PEG400 for artemisone containing pro-Pheroid, the mixture was heated to 70 °C in a water bath, followed by sonication for 15 minutes in a digital ultrasonic cleaner (Jeken, China). Vitamin F ethyl ester, Kolliphor® EL, BHA and BHT were then added while the temperature was maintained at 70 °C, followed by sonication for 15 minutes with a digital ultrasonic cleaner (Jeken, China). Dl- α -tocopherol was then added and the mixture was gassed with nitrous oxide (N₂O) under pressure (200 kPA) for four days.

2.2.2. Caco-2 transport and cytotoxicity assays

For the Caco-2 transport study, HEPES buffered HBSS was prepared by adding 1M HEPES to HBSS (1:39 v/v) and adjusting the pH to 7.4. A 10mM artemisone stock solution was prepared in acetonitrile. Artemisone stock solutions (200 μ M) were prepared by spiking either HBSS or HEPES-HBSS with the 10mM artemisone stock solution and mixed by shaking on a GFL 3005-model shaker (Gesellschaft für Labortechnik, Germany) at 150 rpm for 17 hours before dilution with HBSS or HEPES-HBSS to a final artemisone concentration of

100 μ M. A 5mM verapamil stock solution was prepared in water and used to spike the 100 μ M artemisone solution to a final concentration of 50 μ M verapamil and 100 μ M artemisone.

For the cytotoxicity and transport studies, drug free Pheroid vesicles were prepared. The oil phase contained PEG 400, vitamin F ethyl ester, Kolliphor[®] EL, BHA, BHT and dl- α -tocopherol at a ratio of 0.196:2.530:1.054:0.2:0.01:0.01 w/w. Briefly, PEG 400 was heated to 70 °C, followed by sonication for 15 minutes. Vitamin F ethyl ester, Kolliphor[®] EL, BHA and BHT was then added, heated to 70 °C, followed by sonication for 15 minutes in a digital ultrasonic cleaner (Jeken, China). DI- α -tocopherol was then added and the oil phase was added to the N₂O saturated water phase (oil to water ratio, 4:96 % w/w) while homogenising with a Heidolph DiAx 600 homogeniser (Labotec, South Africa) at 13,500 rpm/min for 4 min. This solution was mixed by overnight shaking on a GFL 3005-model shaker at 150 rpm. For cytotoxicity studies, test samples for experiments were prepared by diluting the Pheroid vesicles with serum free medium. For transport studies, HBSS and HEPES-HBSS were spiked with Pheroid vesicles (1:0.002 v/v) to a Pheroid vesicles concentration of 0.2 % (v/v). These 0.2 % Pheroid solutions were spiked with 10mM artemisone to a final concentration of 200 μ M and mixed by shaking on a GFL 3005-model shaker at 150 rpm for 17 hours. The 200 μ M artemisone-Pheroid solutions were diluted with Pheroid free HBSS and HEPES-HBSS (1:1 v/v) to a final concentration of 100 μ M artemisone and 0.1% Pheroid. All concentrations of Pheroid vesicle solutions are presented in % (v/v).

2.3. Pheroid characterization

Pro-Pheroid was mixed with 0.1 N hydrochloric acid (1:100 v/v) to form Pheroid vesicles for characterization. The size and surface charge (zeta potential) of the Pheroid vesicles was measured using a Hydro Malvern Mastersizer 2000MU and a Malvern 2000 zetasizer (Malvern Instruments Ltd., Malvern, Worcestershire, UK). For particle size measurement,

samples were measured in duplicate by diluting samples with de-ionized water and injecting through the sample injection port. Span value were calculated as

$$\text{Span} = \frac{(d_{0.9} - d_{0.1})}{d_{0.5}} \quad (1)$$

Where $d_{0.9}$, $d_{0.5}$ and $d_{0.1}$ are the particle diameters determined at the 90th, 50th and 10th percentile of the particles respectively.

For zeta potential measurements, the experiments were performed in triplicate in de-ionized water. Confocal laser scanning microscopy (CLSM, Nikon D-eclipse C1 confocal laser scanning microscope) were used to characterize the morphology of the particles as described by Slabbert *et al.* (2011). Briefly, the Pheroid vesicles were stained with Nile Red (50:1 v/v) for 15 minutes in the dark and analysed, using a He/Ne laser.

2.4. Cultivation of Caco-2 cells

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and was grown in 25 and 75 cm² culture flasks in culture medium consisting of DMEM supplemented with 1% amphotericin B, 1% penstrep, 1% NEAA, 1% L-glutamine and 10% FBS. Cells were maintained at 37°C in an atmosphere of 5% CO₂ and 95% relative humidity.

2.5. P-gp ATPase Assay

The effects of artemisone and the Pheroid drug delivery system on p-gp ATPase activity were evaluated using suspensions of p-gp overexpressing membranes. This is an indirect assay that measures the ability of test compounds to stimulate or inhibit p-gp ATPase activity in membranes prepared from recombinant baculovirus-infected insect cells. The p-gp ATPase activity assay was performed as per instructions by the manufacturer. Briefly, treatments were added to the reagent mixture consisting of p-gp overexpressing membranes and Mg²⁺ ATP solution and incubated at 37°C for 20 min for human p-gp membranes and 30

min for monkey p-gp membranes. Simultaneously, identical reaction mixtures containing 100 μ M sodium orthovanadate, which inhibits p-gp ATPase, was assayed. Therefore, ATPase activity measured in the presence of orthovanadate represents non-p-gp ATPase activity, which is subtracted from the total activity measured to generate p-gp ATPase activity. The reaction was stopped by the addition of 10% SDS. The detection reagent consists of ascorbic acid in ammonium molybdate solution and was added to all wells, followed by incubation at 37°C for 20 min. The liberation of inorganic phosphate was detected by measuring the colorimetric reaction at a wavelength of 630 nm using an ELX800 platereader (BioTek Instruments Inc., Winooski, USA).

2.6. Cytotoxicity of the Pheroid drug delivery system (Live/Dead assay)

Cells were seeded at a density of 10⁴ cells per well onto test surfaces contained within 96-well plates and cultured for 5 days. Medium was aspirated and replaced every other day. The Pheroid delivery system was added in triplicate and at different concentrations to the plated cells and incubated for 24 hr. Cells incubated in culture medium were used as the control. The study was repeated in three separate experiments using cells of different passages. The cytotoxic effects of the drug delivery system on Caco-2 cells were then measured by the Live/Dead assay for mammals (Invitrogen, Carlsbad, CA). The Live/Dead fluorescent labelling consists of calcein AM and ethidium homodimer-1 (EthD-1). Calcein AM penetrates viable cell membranes, producing green fluorescence in viable cells and EthD-1 penetrates damaged membranes and upon binding to nucleic acids, produces red fluorescence in dead cells. After the 24hr incubation, the cell culture plates were filled with serum free medium and then inverted for 10 minutes in order to remove poorly attached and detached Caco-2 cells from the surfaces. Thereafter, 100 μ L of combined Live/Dead assay reagent containing 1 μ M calcein AM and 2 μ M EthD-1 were added to each well and incubated at room temperature for 30 minutes. The cells were observed under a fluorescence microscope (Olympus IX70, Japan) at a 20 x magnification and images were acquired using an Olympus digital camera (Olympus IX-TVAD, Japan). For each well, five

images of live and dead cells were taken and counted using ImageJ software. The data represent the average number of live or dead cells over five images per wells in triplicate per experiment for each treatment. The total attached cells were calculated by the sum of live and dead cells per image. For each image, the total cells per well were calculated.

2.7. *Caco-2 transport studies*

Cells were plated at a density of 2.5×10^4 cells/well on 6-well Transwell® polycarbonate membranes (0.4 μm pore size). The apical and basolateral culture medium was replaced every other day. Confluent Caco-2 monolayers expressing p-gp were obtained 21 days postseeding. Prior to the experiments, the culture medium of Transwell®-grown Caco-2 cell monolayers was replaced with transport medium and equilibrated for 1 hour at 37°C before the integrity of the cell monolayers was determined by measuring the *trans*-epithelial electrical resistance (TEER) using an epithelial ohmmeter (Millicell®-ERS; Millipore Corporation, Billerica, MA). TEER ($\Omega \cdot \text{cm}^2$) was calculated by multiplying the measured resistance (Ω) with the filter surface area (4.67 cm^2). Only monolayers with a TEER of greater than 250 $\Omega \cdot \text{cm}^2$ were used. TEER values were also measured at the end of the experiment and recalculated as a percentage of initial TEER values.

Permeability studies were performed in triplicate in both transport directions, i.e. apical to basolateral (AP/BL) and basolateral to apical (BL/AP). All transport experiments were performed for 2 hours at 37°C, with time intervals of 20 minutes. Hanks Balanced salt solution (HBSS) was used as the transport buffer at the apical side and HBSS buffered with 1M HEPES (1:39 v/v) was used as the transport buffer on the basolateral side. The transport medium and transport medium containing the compounds were equilibrated at 37°C before the transport experiment. Triplicates of each compound were analyzed. In AP-to-BL transport studies, all wells received 2.5mL of transport medium. Transport medium (2.5mL) containing the relevant substance of which transport properties were to be determined, was then applied to the AP side. The cell culture plates were gently swirled to ensure complete

mixing before sampling at indicated time intervals. Samples (100µl) were then withdrawn carefully from the BL side and replaced with the same volume of fresh transport medium. In BL-to-AP transport studies, transport medium (2.5mL) containing the relevant substance of which transport properties were to be determined, was applied to the BL side and 2.5mL of transport medium was added to the AP side. Samples were withdrawn as described above, but from the AP side and replaced with the same volume fresh transport medium. The collected samples were diluted 1:2 (v/v) with acetonitrile before analyses using UPLC-QTOF-MS to determine artemisone and verapamil concentrations in each sample.

The qualitative analysis of artemisone and verapamil were performed on a Waters Ultra Performance HPLC system coupled to a G1 hybrid quadrupole time-of-flight mass spectrometer (UPLC-QTOF-MS, (Waters, Milford, MA, USA). Chromatographic separation was achieved on a Waters Acquity UPLC using an Acquity HSS C18 150 x 2.1 mm (1.7 µm) column maintained at 60°C. The mobile phase using the following gradient (mobile phase A 80% water containing 0.1% (v/v) formic acid and 20% acetonitrile; mobile phase B 10% water and 90% acetonitrile) was delivered at a flow rate of 400 µl/min. Mobile phase A was used as the initial mobile phase for 10 minutes, then changed to mobile phase B for 2 minutes and then returned to the initial conditions (mobile phase A) to equilibrate the column. The runtime was 15 minutes with an injection volume of 10µL. Calibration curves were designed to cover possible ranges of analyte concentrations in solutions. Ten dilutions ranging from 1–50 µM for artemisone and 0.5-25 µM for verapamil, respectively, were used. Linear regression analysis was used and the correlation coefficient in each calibration curve was higher than 0.998. Peak areas of analyte and calibration curves were determined using the Masslynx version 4.1 (SCN704) software (Waters, UK).

The apparent permeability coefficients (P_{app} , cm/s) were calculated based on the equation:

$$P_{app} = \frac{(dQ/dt)}{(A.C_0.60)} \quad (2)$$

where dQ/dt is the cumulative transport rate ($\mu\text{M}/\text{min}$) defined as the slope obtained by linear regression of cumulative transported amount after correcting for dilution as a function of time (min), A is the surface area (4.67 cm^2 in 6-well plates) of the monolayers, C_0 is the initial concentration of the compounds on the donor side ($100\mu\text{M}$ for artemisone and $50\mu\text{M}$ for verapamil) and 60 is the coefficient when minutes are converted to seconds.

The P_{app} efflux ratio was calculated based on the equation:

$$P_{\text{app}} \text{ efflux ratio} = \frac{P_{\text{app}}(\text{B-A})}{P_{\text{app}}(\text{A-B})} \quad \text{Equation 3}$$

$P_{\text{app}}(\text{B-A})$ is the apparent permeability coefficients in the basolateral to apical direction while $P_{\text{app}}(\text{A-B})$ is the apparent permeability coefficients in the apical to basolateral direction.

2.8. Statistical Analysis

Data are expressed as mean \pm standard deviation of triplicate treatments. Data were analysed with the univariate tests of significance, followed by the Levene's test for homogeneity of variance and subsequently the Dunnett's multiple comparison test. The data were considered statistically significant at $p < 0.05$.

3. Results

3.1. Pheroid characterization

The particle size and zeta-potential of the drug-free and artemisone entrapped pro-Pheroid formulation (P12006, P12010) for ATPase assay studies and drug-free Pheroid vesicle formulation (V13003) for cytotoxicity and transport studies are summarized in Table 1.

Table 1: The particle size, span and zeta potential of the different Pheroid formulations

| Formulation | Particle size (μm) | Span | Zeta-potential (mV) |
|-------------|---------------------------------|-----------------|---------------------|
| P12006 | 12.38 ± 0.97 | 2.75 ± 0.15 | 1 ± 0.28 |
| P12010 | 0.20 ± 0.00 | 0.69 ± 0.00 | 3.05 ± 1.91 |
| P13003 | 3.20 ± 0.09 | 2.63 ± 0.00 | -15.45 ± 0.07 |

The Pheroid vesicles display a negative zeta potential while the pro-Pheroid formulations exhibit a near neutral zeta potential. The near neutral zeta potential may be due to the presence of PEG at the surface. According to the Gouy–Chapmann theory, the surface PEG chains' hydrodynamic drag moves the hydrodynamic plane of shear away from the charge-bearing plane, and causes the electrophoretic mobilities to decrease. The confocal laser scanning electron microscopy (CLSM) images depict successful formation of spherical particles (Figure 1).

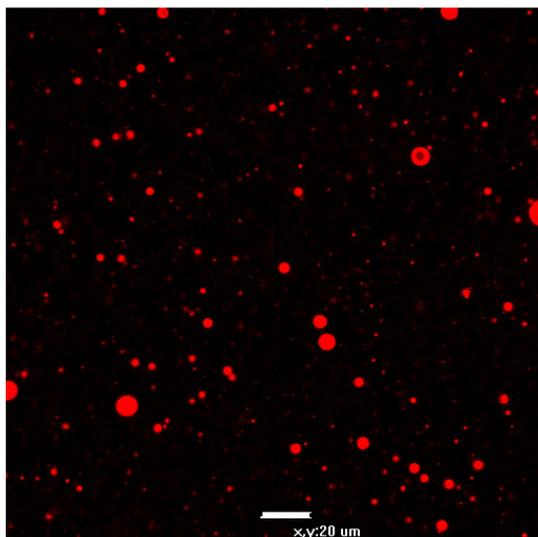


Figure 1: CLSM micrograph of the Pheroid vesicle (P12006) formulations.

3.2. Effects of artemisone and Pheroid on p-gp-ATPase and p-gp drug interaction

Artemisone stimulates the ATPase activity of human p-gp expressed in Sf9 insect cell membranes. The ATPase activity increased in a concentration dependent manner, but did not reach a steady state at the highest concentration (300 μM) tested and when artemisone

was entrapped in pro-Pheroid, the p-gp ATPase activity was not stimulated by artemisone (Figure 2).

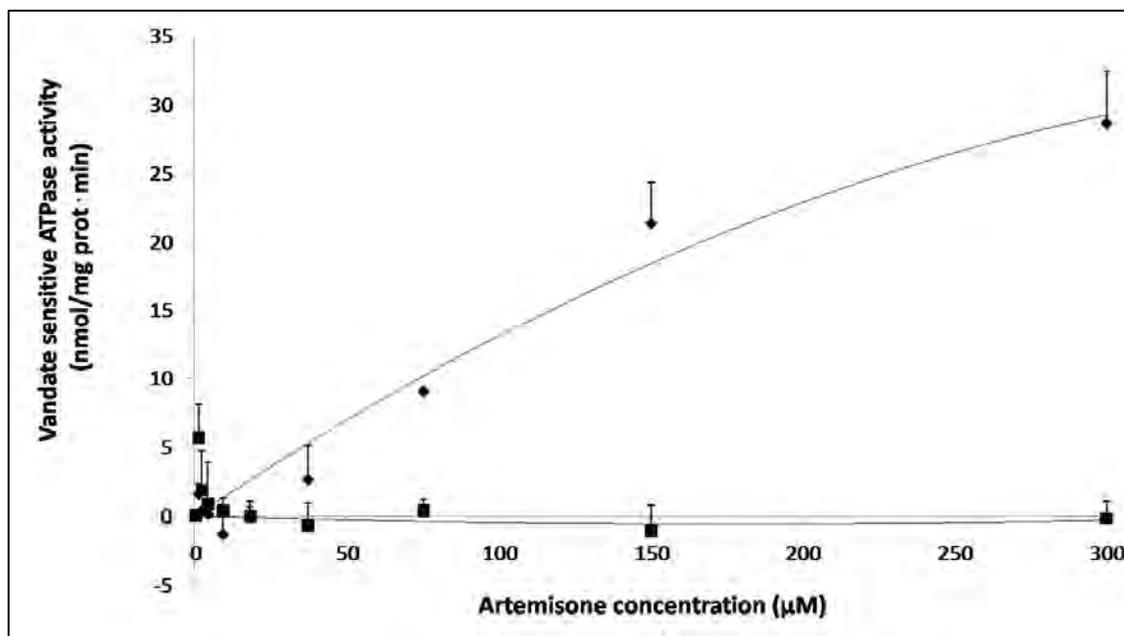


Figure 2: The effect of artemisone (diamonds) and artemisone-pro-Pheroid (squares) on the p-gp ATPase activity. Each point depicts the mean of triplicate experiments \pm SD.

When artemisone and verapamil were used in combination, the ATPase activity was less than the sum of their separate ATPase activities (Figure 3). This suggests that verapamil and artemisone might have the same modulating sites on p-gp, thereby supporting a competitive mechanism. Since drug transport by p-gp is related to its ATPase activity (Gottesman and Pastan, 1993), a corresponding competitive effect of artemisone and verapamil can be expected *in vivo*. When artemisone was entrapped in pro-Pheroid, the p-gp ATPase activity was not stimulated by artemisone, indicating complete inhibition of ATPase. The ATPase activity stimulated by both 20µM verapamil 100µM artemisone was ~ 4 fold higher in human MDR1 expressed insect cell membranes than with cynomolgus monkey Mdr1 expressed insect cell membranes. The ATPase activity stimulated by 20µM artemisone was equal to and 100µM artemisone was ~ 3 fold higher in human MDR1

expressed insect cell membranes than with cynomolgus monkey Mdr1 expressed insect cell membranes. These results illustrate the species differences between human and monkey p-gp ATPase activity.

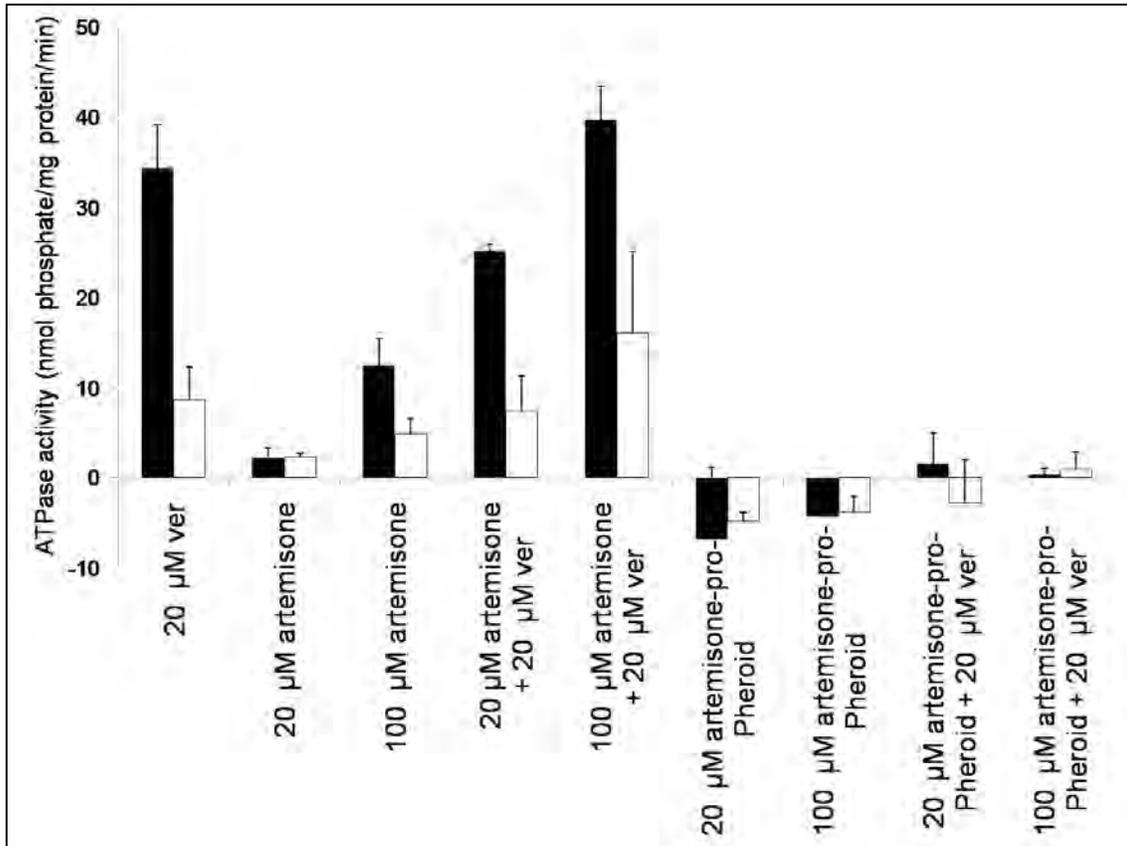


Figure 3: The effect of verapamil (ver), artemisone and artemisone-pro-Pheroid on the p-gp ATPase activity in human MDR1 (black bars) and cynomolgus monkey Mdr1 (white bars) expressed insect cell membranes. Each point depicts the mean of triplicate experiments \pm SD.

Because the Pheroid technology inhibited artemisone stimulated ATPase activity, the p-gp substrates, verapamil, vinblastine and lopermide were used to evaluate the effects of this technology on p-gp-drug specific interactions. These substrates are known to increase p-gp ATPase activity (Polli et al., 2001) and, therefore, alterations in p-gp ATPase activity caused by the Pheroid in the presence of the substrates, should reflect changes in p-gp interaction

induced by the Pheroid. As shown in Figures 3 and Table 2, the Pheroid inhibited the drug-stimulated ATPase activity. Therefore, we propose that pro-Pheroid, like Pluronic P85, impacts the fluidity of the lipid bilayers by adhering to and incorporating into the cell membranes, thereby significantly decreasing p-gp ATPase activity (Batrakova et al., 2004). The pro-Pheroid is composed of excipients that have an effect on transporter mediated absorption. Kolliphor® EL, vitamin F and PEG 400 demonstrated inhibitory effects on p-gp efflux activity (Rege et al., 2002., Hugger et al., 2002, Shen et al., 2006, Johnson et al., 2002, Jacarcz et al., 2005, Baracos et al., 2004).

The artemisone stimulated ATPase activity was completely inhibited (Table 2), while verapamil, vinblastine and loperamide stimulated ATPase activity was not completely inhibited. The reasons for this are two-fold: (i) The concentration of pro-Pheroid used in the artemisone studies was very high (1%) while it was much lower with the other substrates; (ii) Artemisone was entrapped into the pro-Pheroid during manufacturing and the other substrates were only added to pro-Pheroid just before the experiment commenced, implying that not all of the substrates were entrapped by the Pheroid when the ATPase experiments were conducted.

Table 2: The effect of pro-Pheroid on the p-gp ATPase activity of the substrates: verapamil, loperamide and vinblastine. L-PEG is a pro-Pheroid formulation containing 4.9% PEG and H-PEG is a pro-Pheroid formulation containing 20% PEG. Each value depicts the mean (expressed as percentages of values normalised to the activity in the control with no Pheroid added to the respective substrate) of triplicate experiments \pm SD.

| Substrate | Verapamil | | Loperamide | | Vinblastine | |
|----------------------------|------------------|--------------|-------------------|--------------|--------------------|--------------|
| Formulation | L-PEG (%) | H-PEG (%) | L-PEG (%) | H-PEG (%) | L-PEG (%) | H-PEG (%) |
| pro-Pheroid free control | 100 | 100 \pm 0 | 100 \pm 0 | 100 \pm 0 | 100 \pm 0 | 100 \pm 0 |
| 0.004 pro-Pheroid (L-PEG) | 32 \pm 6 | 43 \pm 20 | 63 \pm 8 | 43 \pm 8 | 31 \pm 17 | 32 \pm 7 |
| 0.008 pro-Pheroid (L-PEG) | 44 \pm 5 | 28 \pm 66 | 45 \pm 6 | 60 \pm 5 | 12 \pm 4 | 52 \pm 12 |
| 0.0167 pro-Pheroid (L-PEG) | 37 \pm 9 | 40 \pm 5 | 56 \pm 5 | 47 \pm 3 | 5 \pm 4 | 45 \pm 11 |
| 0.033 pro-Pheroid (L-PEG) | 20 \pm 7 | 72 \pm 32 | 33 \pm 7 | 43 \pm 4 | 33 \pm 7 | 41 \pm 13 |
| 0.067 pro-Pheroid (L-PEG) | 18 \pm 4 | 31 \pm 11 | 31 \pm 0 | 84 \pm 3 | 50 \pm 23 | 72 \pm 11 |

3.3. Cytotoxic effects of the Pheroid particles on Caco-2 cells

Before the live/dead assay was initiated, the 96-well plates were viewed using a Nikon eclipse TS100 microscope. All wells were confluent, but after the inversion step, poorly attached cells from wells treated with high concentration Pheroid, were removed. Figure 5 shows that the detachment of Caco-2 epithelial cells from culture surfaces only occur upon incubation with high concentrations of the Pheroid drug delivery system. Pheroid concentrations $<$ 1.67 % did not show significant cell death. Apoptotic epithelial cells are known to detach from growth substrates as well as neighbouring cells (Wylie *et al.*, 1980). The detachment of Caco-2 cells from cell culture substrates is, therefore, possibly due to the onset of apoptosis. The concentration of Pheroid used in the transport study, therefore, did not have a cytotoxic effect on the Caco-2 cell line during permeability studies.

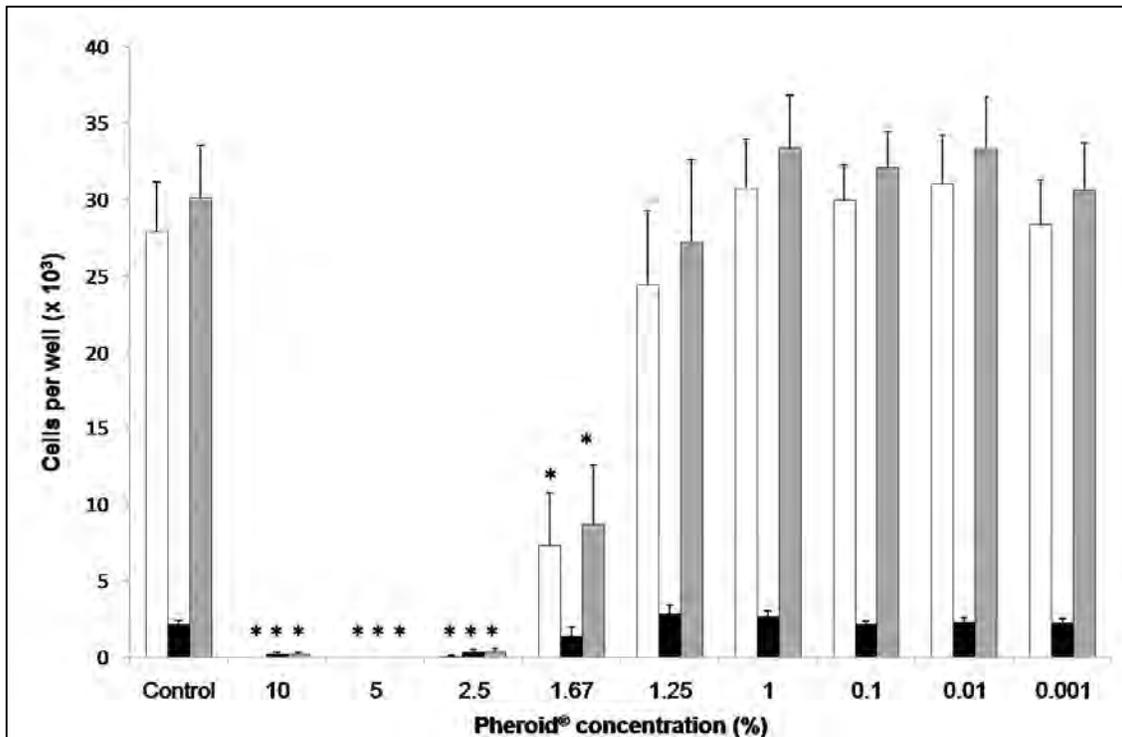


Figure 5: The toxicity of the Pheroid on Caco-2 cells was analysed by Caco-2 cell attachment (live cells as white, dead cells as black and total attached cells as grey bars). Data represent the mean \pm SE of three separate experiments, in triplicate, using cells of different passages. Statistically significant differences in attached live, dead and total cell number between the control and the Pheroid treated cells are indicated by an asterisk (*) ($p < 0.05$).

3.4. Transport study

The integrity of Caco-2 cell monolayers was determined by measuring the TEER of the cells. TEER measurements across each well both before and after transport experiments displayed similar values and confirmed the integrity of the monolayers during all of the experiments. Caco-2 P_{app} values of artemisone alone, artemisone incubated with Pheroid vesicles and artemisone incubated with verapamil, a p-gp inhibitor, were determined for both the AP-BL and BL-AP directions.

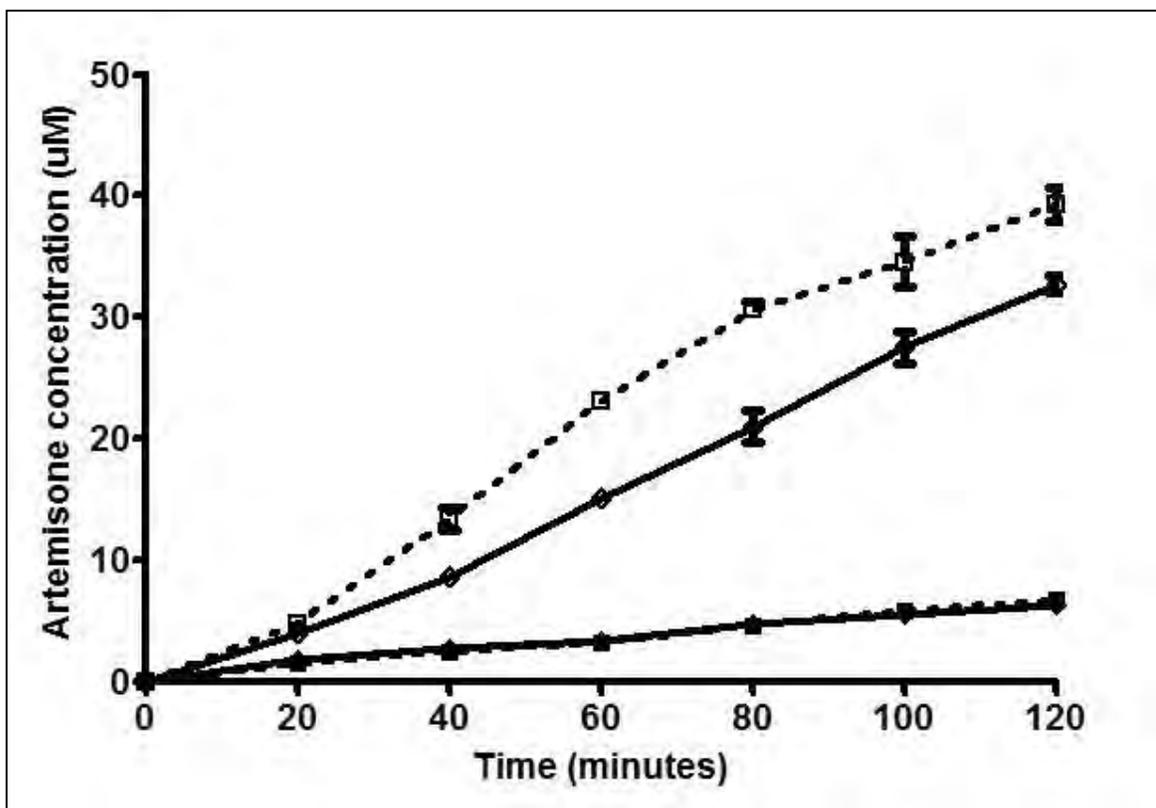


Figure 6: Artemisone (diamonds) and artemisone-Pheroid (squares) transport kinetics. The transport of artemisone across Caco-2 cell monolayers are depicted against time and data are presented as mean value \pm standard deviation ($n=3$). For artemisone treatment, open diamonds connected by solid lines represent transport of artemisone in the apical-to-basolateral (AB) direction and closed diamonds in the basolateral-to-apical (BA) direction. For artemisone-Pheroid treatment, open squares connected by dashed lines represent transport of artemisone in the AB direction and closed diamonds in the BA direction.

The P_{app} values of $< 1 \times 10^{-6}$, $1-10 \times 10^{-6}$, $>10 \times 10^{-6}$ cm/s were considered as low, moderate and high, respectively (Yee, 1997). Artemisone alone and artemisone entrapped in Pheroid vesicles showed high apical to basolateral permeability ($2.01 \pm 0.11 \times 10^{-6}$ and $2.04 \pm 0.07 \times 10^{-6}$ cm/s respectively, Figure 6a and Table 3).

The basolateral to apical permeability of artemisone and artemisone entrapped in Pheroid vesicles was even higher ($9.36 \pm 0.35 \times 10^{-6}$ and $12.40 \pm 0.12 \times 10^{-6}$ cm/s respectively, Figure 7a and Table 3). The transport across Caco-2 monolayers from BL to AP was higher than that from AP to BL with P_{app} efflux ratio's for artemisone and artemisone entrapped in Pheroid vesicles of greater than two (4.65 ± 0.40 and 6.07 ± 0.22 , respectively). This suggests that artemisone could be actively transported from the intestinal epithelial cells back into the gut lumen.

Furthermore, the P_{app} efflux ratios for artemisone and artemisone entrapped in Pheroid vesicles differed significantly, showing possible enhancement of artemisone efflux by the Pheroid technology. The preferred method for identifying p-gp substrates is the comparison of efflux ratios generated in the presence and absence of a p-gp inhibitor (FDA, 2012, Balimane et al., 2006). The FDA decision tree was used to determine whether artemisone is a substrate for p-gp (FDA, 2012, Balimane et al., 2006).

Potential inhibition of artemisone efflux was screened at a high concentration (50 μ M) of verapamil, a drug established as a p-gp inhibitor. The addition of verapamil resulted in significantly increased P_{app} values in the AP to BA direction and a ~4 fold lower efflux ratio (Table 3, Figure 7, $p < 0.001$). This observation confirms that the p-gp efflux protein is responsible for artemisone efflux. The Caco-2 transport data for artemisone are consistent with the ATPase data, illustrating that artemisone is a substrate of human p-gp. In contrast to the ATPase results, the Pheroid vesicles did not inhibit p-gp, but enhanced BA/AP transport of artemisone.

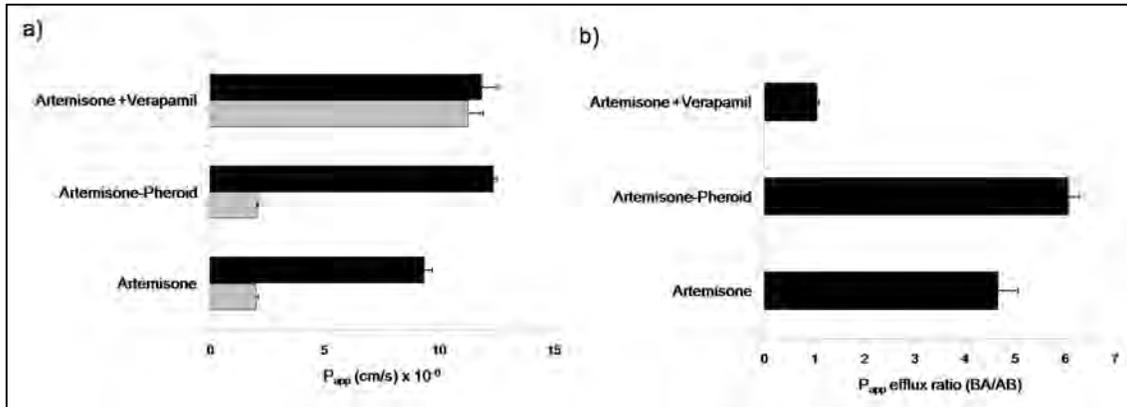


Figure 7: a) The apparent permeabilities (P_{app}) in apical-to-basolateral (AB) direction depicted as grey bars and basolateral-to-apical (BA) direction as black bars; and b) the ratio between P_{app} in BA and AB directions [P_{app} ratio(BA/AB)]. Data are presented as mean value \pm standard deviation (n=3).

Table 3: The apparent permeabilities (P_{app}) in Caco-2 cell assay from apical-to-basolateral (AB) and basolateral-to-apical (BA) side without and with inhibition of p-glycoprotein (p-gp) efflux pump by Verapamil. Data are depicted as mean value \pm standard deviation (n=3). Statistical difference between the artemisone control group and treatment groups are indicated as the p-value in brackets.

| Parameters | Artemisone | Artemisone-Pheroid | Artemisone + Verapamil | |
|--|---------------------------------------|--|--|--|
| | | | Artemisone | Verapamil |
| AP-BL P_{app} (cm/s) | 2.01 \pm 0.11 x 10 ⁻⁶ | 2.04 \pm 0.07 x 10 ⁻⁶ (>0.05) | 11.27 \pm 0.65 x 10 ⁻⁶ (<0.001) | 11.09 \pm 0.34 x 10 ⁻⁶ (<0.001) |
| BL-AP P_{app} (cm/s) | 9.36 \pm 0.35 x 10 ⁻⁶ | 12.40 \pm 0.12 x 10 ⁻⁶ (<0.001) | 11.89 \pm 0.72 x 10 ⁻⁶ (<0.001) | 12.33 \pm 0.55 x 10 ⁻⁶ (<0.001) |
| P_{app} ratio | 4.66 \pm 0.40 | 6.07 \pm 0.22 (<0.001) | 1.06 \pm 0.04 (<0.001) | 1.11 \pm 0.02 (<0.001) |

3.5. Mathematical model of the caco-2 study

3.5.1. The mathematical model

The experimental set-up described above is a closed system, i.e. no flow through occurs. The volumes of the apical and basal lateral sides are denoted as V_a and V_b . For the purpose of the model it is assumed that all the cells in the monolayer behave in similar fashion, so there is no reason to distinguish between them. Therefore, a volume V_c can be assigned to the monolayer. The drug concentration is $C_{a,b,c}$ where the subscript denotes the apical, basolateral and cell domains respectively. The interface between the apical side and the monolayer has a surface area of A_{ac} and the area between the cell layer and the basal lateral side is denoted by A_{bc} .

The passive transport parameter is the permeability, denoted by P and if the cell-to-apical or cell-to-basolateral have different permeability coefficients, then the distinction can be easily accommodated by the model. For the time being, the same parameter value for both sides was used and there was no need to distinguish between them. Drug efflux is affected by p-gp located in the cell membranes. Assume for now that p-gp are only present at the apical side. The third assumption of the model is that the active transport follows Michaelis-Menten kinetics.



Note that the product is C_{as} and it has, like P_{gp} , units that are expressed as a surface concentration. Although drug concentrations are usually expressed in μM , P_{gp} and C_{as} have units of $\mu mole/cm^2$. The rate constant k_1 has units of $(\mu M sec)^{-1}$, and the forward and reverse rates from the complex have units of sec^{-1} . The rate at which the drug is actively transported into the apical volume is;

$$\frac{dC_{as}}{dt} = q = k_2 C_c \cdot P_{gp} \quad (4)$$

It is known that cells express more P_{gp} under pressure from potential substrates that could bind to the p-glycoproteins, but for the cell line studied here, that is not the case. (Again, constancy of P_{gp} is not a constraint and can be easily included in the model.) For this case, we can write,

$$P_{gptot} = P_{gp} + C_c \cdot P_{gp} \quad (5)$$

Therefore the flux (2) can be written as;

$$q = k_2 [P_{gptot} - P_{gp}] \quad (6)$$

P_{gp} need to be tracked as a function of time in order to calculate q .

$$\frac{dP_{gp}}{dt} = [k_{-1} + k_2] [P_{gptot} - P_{gp}] - k_1 [C_c] [P_{gp}] \quad (7)$$

The balancing equations for the drug at the three sites are:

$$V_a \frac{dC_a}{dt} = A_a k_2 [P_{gptot} - P_{gp}] - P A_a [C_a - C_c] \quad (8)$$

$$V_c \frac{dC_c}{dt} = -A_a k_2 [P_{gptot} - P_{gp}] + P A_a [C_a - C_c] + P A_b [C_b - C_c] \quad (9)$$

$$V_b \frac{dC_b}{dt} = -P A_b [C_b - C_c] \quad (10)$$

In summary, the mathematical model is given by equations (7-10). Together with the initial conditions, which are determined by experiments, the model constitutes a closed system, implying unique solutions. In the following section some of the parameters of the model are discussed. Since $V_{a,b,c}$, $A_{a,c}$ and P are reported in terms of cm^3 , cm^2 and cm/s respectively, it is best to use for bulk concentrations $C_{a,b,c}$ units of $\mu mole/cm^3$, instead of μM . ($\mu mole/cm^3 \equiv nano\ molar$).

3.5.2. Parameters of the model

The physical parameters are $A_{a,b}$, $V_{a,b,c}$; therefore, the average cell size and the area of the support membrane need to be known. The physical transport parameter is P and the rate constants are k_{-1} , k_1 and k_2 . The remaining parameter is P_{gptot} .

Let the membrane surface area be A_{mem} . According to Hellinger *et al.* (2012), the height of Caco-2 cells varies between 8 – 15 μm . Approximate surface area is estimated as 160 μm^2 per cell. The number of cells in the monolayer is therefore;

$$N_c = A_{mem} \times 10^8 / 160$$

Since villi are absent at the basolateral side, $A_b = A_{mem}$. Based on scanning electron microscopy, there are approximately 25 villi/ μm^2 on the apical side, each one has a surface of 0.6 μm^2 . The apical side has considerable more surface area than the basolateral side, $A_a = 15A_b$.

3.5.3. Results

Table 4: The parameter values that reproduced the experimental values (figure 6) well are:

| | V_a | V_b | V_c | A_a | A_b | k_1 | k_{m1} | k_2 | P_{gptot} | P |
|------------------------|-------|-------|--------|-------|-------|--------|----------|-----------|-------------|--------|
| Artemisone | 2.5 | 2.5 | 0.0056 | 70 | 4.67 | 0.0014 | 0.01 | 10^{-5} | 6 | 0.002 |
| Artemisone- Pheroid | 2.5 | 2.5 | 0.0056 | 70 | 4.67 | 0.0014 | 0.01 | 10^{-5} | 6 | 0.0021 |

The parameter values that reproduced the experimental data (figure 6) well are summarized in Table 4. When the experiments with Pheroid were fitted, there was a slight increase in P from 0.002 cm/s (artemisone reference) to 0.0021 cm/s (artemisone-Pheroid).

The number of cells on the membrane is estimated to be $4.67 \times 10^8 / 160 \approx 3$ million cells.

The total number of P_{gp} 's is:

$$P_{gptot} \times A_a = 4.2 \times 10^{-4} \text{ mole}$$

Therefore, it can be calculated that there are $1.4 \times 10^{-10} \times 6.023 \times 10^{23} \approx 8 \times 10^{13}$ P_{gp} 's/ cell, due to the large surface area on the apical side.

4. Discussion

Based on these findings, artemisone appears to differ from artemisinin and other artemisinin derivatives. Unlike artemisone, artemisinins are not substrates for human p-gp (Crowe et al., 2006). Artemisinin activates CAR (constitutive androstane receptor) and PXR (pregnane X receptor), which induce *MDR1* expression in primary human hepatocytes and intestinal LS174T cells (Burk et al., 2005). Artemisinins also inhibit p-gp activity to a degree as demonstrated by the inhibition of p-gp-mediated digoxin efflux in Caco-2 cells and inhibition of verapamil stimulated ATPase activity of p-gp (Augustijns et al., 1996, Burk et al., 2005).

Oral drugs are absorbed by the GI tract and efflux transporters such as p-gp present in intestinal epithelia may reduce the absorption of p-gp substrates. This in turn, may limit systemic availability of the drug and consequently influence the pharmacokinetics and pharmacodynamics. However, the moderate absorptive permeability of artemisone ($2.01 \pm 0.11 \times 10^{-6}$ cm/s) suggests that, for artemisone, p-gp mediated transport on membrane passage may be expected and that poor absorption could contribute to its poor bioavailability [5 to 25% in rats dosed with 3 to 30 mg/kg bodyweight (bw) and 6 to 16% in dogs dosed with 3 to 10 mg/kg bw respectively (Fischer, 2004, Feser-Zügner, 2005.)].

In addition to its high expression in the GI tract, p-gp is also highly expressed in the blood-brain barrier. Passive membrane permeability (P_{app} (AP-BL) and p-gp can limit blood-brain barrier penetration (Polli et al., 1999; Schinkel, 1999). If a drug has a molecular weight > 400 g/mol, has passive permeability lower than 5×10^{-6} cm/s and has an efflux ratio >5, central nervous system exposure would be diminished, which also implies inadequate blood-brain barrier penetration (Mahah Doan et al., 2002). Artemisone has a molecular weight of 401.5 g/mol, a passive P_{app} of $<5 \times 10^{-6}$ cm/s and an efflux ratio close to 5. This may indicate that artemisone might not be able to reach adequate central nervous system exposure and penetrate the blood-brain barrier.

In multidrug resistant (MDR) *Plasmodium falciparum* strains, the *pfmdr-1* gene and *pgh-1* has approximately 54% homology to *mdr-1* gene of MDR cancer cells (Karcz and Cowman, 1991, Foote et al., 1989, Hasson et al., 1992, Reed et al., 2000). Artemisone may prove to be more useful in treatment of multidrug-resistant *P. falciparum* malaria. It was 10 times more potent than artesunate *in vitro* against a panel of drug-sensitive and chloroquine-, pyrimethamine – and cycloguanil-resistant *P. falciparum* strains. Artemisone was 4 to 10 times more potent than artesunate in rodent models against drug-susceptible and primaquine- or sulfadoxine/pyrimethamine-resistant *Plasmodium berghei* lines and chloroquine- or artemisinin-resistant lines of *Plasmodium yoelii* (Vivas et al., 2007).

Artemisinin combination therapy (ACT) is widely promoted in many parts of Africa for the treatment of malaria (Baird, 2005). If artemisone is to be used as an ACT for malaria, these findings provide new insights into potential drug-drug interactions (DDI). Specifically, antimalarial combination partners such as the p-gp substrates mefloquine or chloroquine and p-gp inhibitors such as primaquine, quinine, lumefantrine or amodiaquine may cause clinically relevant DDIs to occur (Kerb et al., 2009; Oga et al., 2012). Another area of concern may be the treatment of patients co-infected with HIV or TB, because many anti-HIV and anti-TB drugs are substrates and inhibitors of MDR1. For example, the HIV protease inhibitors amprenavir and nelfinavir are p-gp inducers, and rifampicin, an important first line anti-TB drug, induces p-gp (Huang et al., 2001, Chen and Raymond, 2006).

Overexpression of p-gp in cancer cells limits tumour cell access of p-gp substrates and, therefore, chemoresistance occurs (Chen et al., 1986, Gottesman and Pastan, 1993). In addition to artemisinins being effective antimalarial drugs, the artemisinins also have anti-cancer properties in *in vitro* and in *ex vivo* animal models (Woerdenbag et al., 1993, Efferth et al., 2001, Chen et al., 2003, Nakase et al., 2008, Li et al., 2007, Chen et al., 2009). Anti-cancer activity has also been seen in humans (Berger et al., 2005, Singh and Panwar, 2006, Zhang et al., 2008). Recently, artemisone has also shown anti-tumour efficacy.

Furthermore, artemisone are more potent against cancer than other artemisinins (Gravett et al., 2010). Reungpatthanaphon and Mankhetkorn (2002) concluded that at the artemisinin concentration range used in cancer treatment, they did not decrease the function of P-glycoprotein, suggesting a mechanism by which the drugs did not reverse MDR phenomenon at the P-glycoprotein level but at the mitochondrial level. This demonstrates that artemisone overcomes p-gp efflux in cancer cells, probably due to its high permeability or by modulate mitochondrial function (Reungpatthanaphon and Mankhetkorn, 2002).

With the ATPase assay, the Pheroid vesicles inhibited the stimulation of p-gp ATPase. However, the Pheroid did not enhance the AP/BA transport of artemisone, but BA/AP transport of artemisone was enhanced in caco-2 cells. This indicates stimulation of p-gp activity. The final Pheroid concentration used in the transport study was 0.05% (v/v), which is much lower than the concentrations used in the ATPase assay. Also, pro-Pheroid were used instead of Pheroid vesicles. Pheroid vesicles contained a 4% oil phase and a 96% nitrous oxide water phase while pro-Pheroid contains only an oil phase. Therefore, the lowest concentration tested (0.1% oil phase) in the ATPase assay is twofold higher than the concentration used in the transport study (0.05% oil phase).

The possibility exists that, in the ATPase assay, artemisone is not released by the Pheroid and, therefore, it is not able to stimulate ATPase activity. Artemisone was entrapped in the pro-Pheroid during manufacturing. The other substrates (i.e. verapamil, loperamide and vinblastine) were only added to pro-Pheroid just before the experiment commenced. Therefore, all of the substrate might have not been entrapped by the Pheroid when the ATPase experiments were conducted and, therefore, the free substrate could still stimulate the ATPase to a certain degree.

5. Conclusion

Our data demonstrate that the Pheroid vesicles are not cytotoxic to confluent Caco-2 cells at concentrations of $\leq 1.25\%$. Artemisone behave as a moderately permeable substance in Caco-2 cells. Artemisone is a p-gp substrate and Pheroid vesicles increased efflux of artemisone from intestinal epithelial cells. In order to evaluate the influence of p-gp efflux activity on the oral absorption of artemisone, *in vivo* studies in wild-type and mdr1a knockout mice should be considered.

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