HIGH IN SITU RAT INTESTINAL PERMEABILITY OF ARTEMISININ UNAFFECTED BY MULTIPLE DOSING AND WITH NO EVIDENCE OF P-GLYCOPROTEIN INVOLVEMENT

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ABSTRACT:
The objective of this study was to investigate whether the decrease in artemisinin bioavailability after repeated oral dosing in humans can be a result of increased efflux of artemisinin by P-glycoprotein or decreased membrane transport at the intestinal barrier. The effective jejunal permeability (P_{eff}) of artemisinin was investigated using an in situ rat perfusion model. Fifty-four rats were randomized to one of three treatment arms: no pretreatment, pretreatment with artemisinin emulsion for 5 days (60 mg/kg/day, p.o.), or pretreatment with emulsion vehicle for 5 days. The rats in each treatment arm were randomized further to be jejunally perfused with either low (500 ng/ml) or high (5000 ng/ml) artemisinin concentration or low artemisinin concentration plus the P-glycoprotein inhibitor R,S-verapamil (400 μg/ml). Perfusate samples were assayed for content of artemisinin, R,S-verapamil, and perfusion viability markers. Artemisinin P_{eff} was 1.44 ± 0.38, 1.17 ± 0.32, and 1.71 ± 0.29 (10^{-4} cm/s) in rats receiving no pretreatment and perfused with low, high, or low artemisinin concentration plus verapamil, respectively. Multiple oral dosing of artemisinin did not affect the jejunal permeability of artemisinin. R,S-verapamil P_{eff} was similar in artemisinin-pretreated rats (1.09 ± 0.54 · 10^{-4} cm/s) and rats pretreated with only vehicle (1.07 ± 0.37 · 10^{-4} cm/s). The decrease in artemisinin bioavailability after multiple oral dosing in human is probably not a result of changes in P-glycoprotein expression or general intestinal transport. It seems more likely attributed to increased hepatocellular activity. Furthermore, artemisinin exhibits high jejunal permeability and is neither a substrate nor inducer of P-glycoprotein.

Artemisinin represents a new class of antimalarials that is effective against drug-resistant Plasmodium falciparum strains (Hassan Alin et al., 1992). It is a sesquiterpene lactone with an internal peroxide bridge (Fig. 1) necessary for its antiparasitic effect (Klayman, 1985). Artemisinin pharmacokinetics in humans are characterized by a short half-life of about 2 to 3 h, a high and variable oral clearance of 200 to 400 liters/h after a single dose, and an extraordinary time dependence (Ashton et al., 1996, 1998a,b; Hassan Alin et al., 1996; Sidhu et al., 1998; Svensson et al., 1998). In several multiple oral dose studies in both patients and healthy subjects, artemisinin plasma concentrations decreased by the fifth day of administration to about 20% compared with those on the first day. Because there was no concomitant change in half-life with decreasing area under concentration-time curve, the time dependence was thought to be caused by a decrease in bioavailability (Ashton et al., 1996, 1998a,b; Hassan Alin et al., 1996; Sidhu et al., 1998; Svensson et al., 1998). However, the mechanism behind the pronounced time-dependent pharmacokinetics still remains unidentified.

The possibility of induction of membrane proteins that may cause an increased efflux out from cell membranes and thereby reduce the bioavailability of artemisinin has not yet been investigated. P-glycoproteins, located in the apical membrane of the enterocytes, may serve as a barrier to protect cells from cytotoxic agents (Borst et al., 1993), causing decreased absorption and/or increased metabolism by apical recycling. This may contribute to low oral bioavailability of hydrophobic drugs (Lown et al., 1997; Sparreboom et al., 1997; Kim et al., 1998). Transient exposure of drugs may transcriptionally increase the level of expression of mdr1, one of the encoding genes of the multidrug-resistant (MDR) P-glycoprotein (Silverman and Thorgeirsson, 1995).

P-glycoprotein in MDR tumor cells appears to exhibit relatively

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FIG. 1. Chemical structure of artemisinin (mol. wt. = 282).

1 Abbreviations used are: P_{eff, rat}, effective permeability in rat; P_{eff, man}, effective permeability in humans; MDR, multidrug resistant; CYP, cytochrome P-450; Q_{pw}, perfusion flow rate; NWF, net water flux.
broad substrate specificity (Ford and Hait, 1990). The substrates are generally amphiphilic, have partition coefficients (octanol/water) greater than 2, and are protonated at physiological pH (Germann et al., 1993). Many drugs metabolized by human cytochrome P-450 (CYP) 3A4 are also transported by P-glycoprotein (Wacher et al., 1995). Artemisinin has an octanol–water partition coefficient of 160 (Augustijns et al., 1996) and is partly metabolized by CYP3A4 in human liver microsomes (U.S.H.S. and M.A., submitted). Verapamil, a P-glycoprotein substrate, is a competitive inhibitor of intestinal P-glycoprotein in the rat (Saitoh and Angus, 1995).

The aims of this study were to elucidate whether the decrease in artemisinin bioavailability after repeated oral dosing in human can be a result of an increased efflux of artemisinin by P-glycoprotein or a decreased membrane passage at the intestinal barrier. We also wanted to investigate whether or not artemisinin is a substrate for P-glycoprotein.

Materials and Methods

Design. The study involved three treatment arms: no pretreatment, pretreatment with artemisinin in soya bean oil emulsion for 5 days (54 mg/kg/day p.o.), and pretreatment with the emulsion vehicle for 5 days (10 ml/kg/day p.o.). Each treatment arm was divided further into three groups jejunally perfused with either 500 ng/ml artemisinin, 5000 ng/ml artemisinin, or 500 ng/ml artemisinin along with 400 µg/ml of the P-glycoprotein inhibitor R,S-verapamil. Fifty-four rats were randomized to one of the nine experimental groups.

Chemicals. Artemisinin was a gift from the Institute of Malariology, Parasitology and Entomology (Hanoi, Vietnam). Pluronic F-68 was obtained from Fluka Chemie AG and glycerol was obtained from Merck. Antipyrine and verapamil. Fifty-four rats were randomized to one of the nine experimental groups.

Jejunum Perfusion Experiments. Rats weighing 205 to 341 g were fasted overnight before the perfusion experiment with access to tap water only. Anesthesia was induced with an i.p. injection of Inactin-Byk (thiobutabarbital sodium, 150 mg/kg), and the rats were placed on a heating pad to maintain a body temperature of 37°C. Breathing was facilitated by inserting a plastic tube into trachea. By a midline longitudinal incision, the abdomen was opened and a 10-cm jejunal segment was isolated and cannulated at both ends with plastic tubing. The segment was rinsed with saline to clear the segment, and approximately 10 cm of the inlet tubing was placed inside the abdominal cavity to achieve an inlet perfusion solution temperature of 37°C. Drips of saline were added onto the surgical area, which then was covered with a plastic sheet to avoid loss of fluid. The experiment was initiated by filling the segment with a 4-ml bolus of the perfusion solution and time set to zero with the immediate start of the perfusion. The perfusion rate was 0.2 ml/min (Harvard Apparatus Syringe Infusion Pump model 22; B&K, Sollentuna, Sweden). The perfusate samples were collected on ice in intervals after 45, 60, 75, 90, and 105 min. The length of the segment was measured after 45 min. At the end of the perfusion, the segment was flushed with approximately 20 ml of saline to recover remaining perfusion solution. A sample was taken from the inlet perfusion solution for analysis. All perfusion syringes and sample cups were weighted before and after the perfusion. Samples were frozen immediately and stored at −20°C. A blood sample was taken by heart puncture from pretreated rats at the end of the perfusion. Blood samples were centrifuged immediately (10,000g, 10 min) and plasma was frozen at −20°C.

Collection and Preparation of Rat Small Intestinal Fluid and Jejunum Homogenate. In three male overnight-fasted rats, a 10-cm long jejunal segment was isolated as described above. The segment was perfused with saline and the first 2.5 ml of intestinal fluid was collected from the outlet tube. Jejunal homogenate was prepared from a 35-cm segment of the upper small intestine excised beyond the 10-cm perfused section. The segment was flushed with ice-cold saline and the mucosa was scrapped off with a razor. Perfusion buffer was added to the mucosa (5 ml/g mucosa) and the mixture was homogenized. The intestinal fluid and homogenate were stored at −80°C until use.

Incubation of Artemisinin with Rat Small Intestinal Fluid and Rat Jejunum Homogenate. Incubations were performed to test the chemical and metabolic stability of artemisinin in rat intestinal juice and rat jejunal homogenate. The collected rat intestinal juice was diluted with perfusion buffer (1:1) and preincubated at 37°C for 6 min in a shaking water bath (Haake SWB 20). Artemisinin (5000 ng/ml, 1% methanol in final concentration) was added to the incubation, and samples (100 µl) were taken out at 0, 30, 90, and 105 min. The samples were mixed with 200 µl H2O and boiled for 3 min, chilled on ice, and centrifuged at 10,000g for 10 min. The supernatant was harvested and 60 µl injected directly onto the high-performance liquid chromatography column for quantitation of artemisinin. The incubation volume was 1 ml. Experiments with artemisinin incubated with 4, 9 and 17% rat jejunal homogenate in perfusate buffer were performed in the same way.

Analytical Analysis. Artemisinin concentrations were determined by high-performance liquid chromatography postcolumn on-line derivatization and UV detection at 289 nm (Edlund et al., 1984). Perfusate samples and incubation samples were analyzed by direct injection onto the column. Plasma samples (100 µl) were extracted and analyzed as previously described (Ashton et al., 1996). Standard curve and quality control samples were prepared by spiking rat plasma or perfusate buffer with artemisinin. Detection was linear in the range of the standard samples (40–5200 ng/ml). Six quality control samples, two of each concentration (80, 2000, and 4500 ng/ml), were run on each occasion with the standard curve and experimental samples. The run was rejected if two quality-control samples of the same concentration or more than two quality-control samples deviated more than 20% from their nominal values. The intra- and interday precisions were 4%, 2%, and 4% and 5, 2%, and 3%, respectively for the low, intermediate, and high quality-control concentrations. The limit of quantification of artemisinin in plasma was 40 ng/ml. Antipyrine in the perfusion solution and the perfusate leaving the intestinal segment was analyzed by high-performance liquid chromatography (Lenner- näs et al., 1992). Peak areas of coeluted R,S-verapamil were compared for any change in the effective permeability of R,S-verapamil.
The fraction absorbed in humans (fa, man) can be predicted from rat for highly permeability (Peff, cm/s) was calculated according to a parallel tube model concentrations achieved after 45 min. The steady-state intestinal effective (5500 vapor pressure osmometer; Wescor Inc., Logan, UT).

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The radioactivity of 14C-labeled PEG 4000 and D-[^3H]glucose in the perfusate leaving the intestinal segment was determined during steady state. The perfusion flow rate (0.2 ml/min) and A is the mass transfer

\[ \text{PEG}_{\text{in}} / \text{PEG}_{\text{out}} \]

where \( \text{PEG}_{\text{in}} \) and \( \text{PEG}_{\text{out}} \) are the inlet and outlet concentrations of the nonabsorbable, water flux marker 14C-labeled PEG 4000. A negative net water flux indicates loss of fluid from the mucosal side (lumen) to the serosal side (blood). A positive net water flux indicates secretion of fluid into the segment.

The recovery of PEG 4000 was estimated from eq. 3:

\[ \text{PEG}_{\text{rec}} = \Sigma \text{PEG}_{\text{in}} / \Sigma \text{PEG}_{\text{out}} \]

where \( \Sigma \text{PEG}_{\text{in}} \) and \( \Sigma \text{PEG}_{\text{out}} \) are the accumulated amounts of 14C-labeled PEG 4000 entering and leaving the segment, respectively. PEG_{rec, ss} is the PEG 4000 recovered during steady state.

In vivo drug intestinal permeability in humans (P_{eff, man}) can be predicted from rat P_{eff} values according to (Fagerholm et al., 1996):

\[ P_{\text{eff, man}} = 3.6 \cdot P_{\text{eff, rat}} + 0.03 \cdot 10^{-4} \]

The fraction absorbed in humans (fa_{man}) can be predicted from rat for highly soluble, stable, and passively and carrier-mediated absorbed drugs by (Fagerholm et al., 1996):

\[ fa_{\text{man}} = 1 - e^{-2 \cdot P_{\text{eff, rat}} \cdot L / R^{2.8}} \]

where \( t_{res} \) and \( r \) are the average small intestine transit time and radius in humans, assumed to be 3 h and 1.75 cm, respectively.

The possible effect of pretreatment with artemisinin on the estimated parameters and the involvement of P-glycoprotein in the absorption of artemisinin were tested by using two-way analysis of variance, Tukey HSD test (Statistica; StatSoft, Tulsa, OK). The possible effect of artemisinin pretreatment on verapamil effective permeability compared with vehicle pretreatment was tested with Student’s unpaired t test (Statview Student; Abacus Concepts, Inc., Berkeley, CA). The significance level was set to 0.05. All parameters are presented as average ± S.D.

### Results

The effective jejunal permeability (P_{eff}) of artemisinin at the low artemisinin concentration (500 ng/ml) was 1.44 ± 0.38, 1.59 ± 0.50, and 1.50 ± 0.33 \cdot 10^{-4} cm/s in untreated, artemisinin-pretreated, and vehicle-pretreated rats, respectively (Table 1). Artemisinin P_{eff} values obtained in rats receiving no pretreatment did not differ for low (1.44 ± 0.38 \cdot 10^{-4} cm/s), high (1.17 ± 0.32 \cdot 10^{-4} cm/s), and low artemisinin concentration plus R,S-verapamil (1.71 ± 0.29 \cdot 10^{-4} cm/s) (Table 1). Artemisinin P_{eff} values in rats pretreated with artemisinin did not differ significantly from values obtained in vehicle-pretreated rats (Table 1). Using rat intestinal permeability values obtained in untreated rats at the low and high artemisinin perfusion concentration resulted in corresponding predictions of in vivo jejunal effective permeability estimates in humans of 5.21 \cdot 10^{-4} cm/s and 4.24 \cdot 10^{-4} cm/s, respectively. Thus, the fraction absorbed (fa) of dissolved artemisinin from the gastrointestinal tract was predicted to be 100% in humans.

P_{eff} of the P-glycoprotein inhibitor R,S-verapamil was 1.09 ± 0.54 \cdot 10^{-4} cm/s in rats receiving an artemisinin emulsion orally for 5 days. This was not significantly different from 1.07 ± 0.37 \cdot 10^{-4} cm/s obtained in rats pretreated with only emulsion vehicle.

The results for the different viability markers, P_{eff, antipyrine}, P_{eff, D-GLUCOSE}, NWF, and PEG_{rec, ss} are presented in Table 2. In rats receiving no pretreatment, P_{eff, antipyrine} was similar between the three different perfusate compositions, 0.56 ± 0.31, 0.49 ± 0.21, and 0.87 ± 0.18 \cdot 10^{-4} cm/s at low, high, and low artemisinin concentration plus R,S-verapamil, respectively. No difference in P_{eff, D-GLUCOSE} values were observed at low (1.05 ± 0.07 \cdot 10^{-4} cm/s), high (0.94 ± 0.16 \cdot 10^{-4} cm/s), and low artemisinin concentration plus R,S-verapamil (1.04 ± 0.24 \cdot 10^{-4} cm/s), respectively. Similar results of P_{eff, antipyrine} and P_{eff, D-GLUCOSE} were obtained in artemisinin- and vehicle-pretreated rats. The average negative NWF of −0.08 ± 0.05 ml/h/cm indicated some absorption of fluid from the mucosal (lumen) to the serosal side (blood). There was a significantly (p < .001) higher net water absorption at the high compared with the low artemisinin concentration. The overall average PEG_{rec, ss} for all nine groups was 103 ± 7%.

### Discussion

Artemisinin pharmacokinetics exhibit a remarkable time dependence in both malaria patients and healthy subjects with a decrease in bioavailability after repeated oral administration (Ashton et al., 1996,
1998a,b; Hassan Alin et al., 1996; Sidhu et al., 1998; Svensson et al., 1998). The present study examined the hypothesis of induction of enteroctyotical P-glycoprotein expression or decrease jejunal membrane passage after multiple oral administration resulting in reduced bioavailability of artemisinin. We also wanted to investigate whether P-glycoprotein is involved in the jejunal absorption of artemisinin.

Induction of P-glycoprotein or a general decrease in jejunal transport are not likely explanations for the reduced bioavailability of artemisinin in humans seen after multiple administration because there was no difference in artemisinin effective permeability (P_{eff}) in rats pretreated with artemisinin compared with emulsion vehicle. Also, adding the P-glycoprotein inhibitor R,S-verapamil to the perfusate did not change artemisinin P_{eff} in rats pretreated with artemisinin (Table 1). There was no evidence of artemisinin being an inducer of P-glycoprotein expression in rat jejunum, because R,S-verapamil P_{eff} was not different in the artemisinin-pretreated rats compared with the vehicle-pretreated rats.

The present study demonstrated that artemisinin was not a substrate for rat jejunal P-glycoprotein because artemisinin P_{eff} was not increased by coperfusion of the P-glycoprotein inhibitor R,S-verapamil. There also was no indication of a saturable carrier-mediated transport of artemisinin (Table 1). In another study using Caco-2 epithelial cells, no evidence of carrier-mediated transport of artemisinin was found (Augustijns et al., 1996). Cell culture have a significant lower functional expression of carrier-mediated processes (Artursson et al., 1996). Cell culture have a significant lower functional expression of carrier-mediated processes (Artursson et al., 1996). Cell culture have a significant lower functional expression of carrier-mediated processes (Artursson et al., 1996).

Whereas compounds with P_{eff} < 0.03 \cdot 10^{-4} \text{cm/s} in the rat small intestine are classified as poorly absorbed whereas compounds with P_{eff} > 0.2 \cdot 10^{-4} \text{cm/s} are completely absorbed (Fagerholm et al., 1996). The same classification of in vivo absorption may be defined in humans; poorly absorbed compounds have P_{eff} < 0.1 \cdot 10^{-4} \text{cm/s} whereas

\begin{table}[h]
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\begin{tabular}{|c|c|c|c|}
\hline
Parameter & Perfusate & No Pretreatment & Pretreatment with ART\(^a\) & Pretreatment with Vehicle\(^b\) \\
\hline
P\(_{\text{eff, antipyrine}}\) (10\(^{-4}\), cm/s) & 500 ng/ml ART & 0.56 ± 0.31 & 0.93 ± 0.17\(^c\) & 0.57 ± 0.30 \\
& 5000 ng/ml ART & 0.49 ± 0.21 & 0.68 ± 0.38 & 0.69 ± 0.29 \\
& 500 ng/ml ART + inhibitor\(^d\) & 0.87 ± 0.18 & 0.52 ± 0.18 & 0.81 ± 0.24 \\
& 5000 ng/ml ART & 1.05 ± 0.07 & 1.20 ± 0.19\(^e\) & 0.95 ± 0.16 \\
& 500 ng/ml ART & 0.94 ± 0.16 & 0.90 ± 0.24 & 0.96 ± 0.26 \\
& 5000 ng/ml ART + inhibitor\(^d\) & 1.04 ± 0.24 & 0.96 ± 0.20 & 0.97 ± 0.23 \\
\hline
P\(_{\text{eff, c-glucose}}\) (10\(^{-4}\), cm/s) & 500 ng/ml ART & -0.09 ± 0.03 & -0.13 ± 0.05\(^f\) & -0.12 ± 0.05 \\
& 5000 ng/ml ART & -0.02 ± 0.02 & -0.04 ± 0.05 & -0.05 ± 0.06 \\
& 500 ng/ml ART + inhibitor\(^d\) & -0.05 ± 0.03 & -0.11 ± 0.03 & -0.12 ± 0.05 \\
& 5000 ng/ml ART & 102 ± 3 & 110 ± 6\(^g\) & 106 ± 2 \\
& PEG\(_{\text{res, ss}}\) (%) & 102 ± 3 & 110 ± 6\(^g\) & 106 ± 2 \\
\hline
\end{tabular}
\caption{Mean ± SD (n = 6/group) P_{eff, NWF, and PEG_{res, ss}} in the in situ perfused rat jejunum}
\end{table}
A, increased efflux of artemisinin into the lumen due to induction of carrier-mediated transport proteins, for instance, P-glycoprotein (1). Decreased passive diffusion of artemisinin through the intestinal cell membrane due to, e.g., membrane atrophy (2). Increased gut wall metabolism due to induction of, e.g., CYP3A (3). B. decreased protein binding, C. increased hepatocellular capacity due to induction of enzymes via increased transcription (1), mRNA stabilization (2), increased translation (3), or protein stabilization/activation (4).

completely absorbed compounds have $P_{eff} > 0.7 \cdot 10^{-4}$ cm/s. The observed artemisinin $P_{eff, rat}$ of $1.44 \cdot 10^{-4}$ cm/s, yielding a predicted $P_{eff}$ of $5.21 \cdot 10^{-4}$ cm/s in humans, clearly classifies artemisinin as being completely absorbed. Intestinal effective permeability estimated from in situ perfused rat intestine has been shown to correlate well with the extent of in vivo absorption in humans after oral administration of highly soluble, stable, and passively and carrier-mediated absorbed drugs (Amidon et al., 1988). Our prediction of the fraction artemisinin absorbed in humans of $100\%$ assumes that the drug is completely dissolved in the gastrointestinal fluids. Artemisinin is sparingly soluble in water (Trigg, 1989), possibly limiting its availability after oral administration. Because artemisinin has a high jejunal effective permeability and is not a substrate for P-glycoprotein, it can be concluded that the assumed low oral bioavailability in humans after single-dose administration (Ashton et al., 1996, 1998a,b; Hassan Alin et al., 1996; Sidhu et al., 1998; Svensson et al., 1998) is a result of either solubility limitations and/or extensive first-pass metabolism rather than absorption problems.

The experimental viability of the segments was good. The obtained $P_{eff, antipyrine}$ and $P_{eff, d-glucose}$ indicate that artemisinin within the concentration span studied had no effect on passive or carrier-mediated transport. A decreased water absorption was observed at the high artemisinin concentration although the decrease was relatively low compared with the perfusate flow of 0.2 ml/min during 105 min. In the calculation of $P_{eff}$ values, differences in NWF were corrected for.

Effective permeability values estimated from drug disappearance in the in situ perfusion rat model is well validated (Fagerholm et al., 1996). However, if the compound studied is susceptible to chemical or enzymatic instability in the lumen and/or brush-border membrane, $P_{eff}$ values will be overestimated. The intestinal segment was flushed before the start of the perfusion; therefore, only membrane-bound enzymes could interact with artemisinin. Because the $P_{eff}$ is estimated from drug disappearance from the lumen, any putative gut metabolism of artemisinin by, for instance, CYP3A, the major intestinal CYP enzyme (de Waziers et al., 1990), should not affect the estimated permeability. Furthermore, artemisinin was metabolically stable in incubations with jejunum homogenate without NADPH, indicating low activity of intestinal enzymes other than CYP. Therefore, disappearance of artemisinin from the perfusate should give good estimates of the jejunal effective permeability of artemisinin. A jejunal segment was chosen because artemisinin is available as an immediate release product for oral administration. P-glycoprotein transport also has been studied previously in the rat jejunum with the in situ perfusion technique (Sandström et al., 1998).

Any decrease in artemisinin plasma concentrations after multiple oral administration could not be demonstrated in this study because the artemisinin plasma concentrations were below the detection limit for the assay.

In conclusion, artemisinin is not a substrate for P-glycoprotein, nor does artemisinin appear to be an inducer of P-glycoprotein. Artemisinin therefore is not susceptible to efflux-type drug resistance. Intestinal enzymes apart from CYP3A probably do not contribute to the overall elimination of artemisinin. The low oral bioavailability seen in humans is probably a result of high hepatic first-pass metabolism and/or solubility problems in the gastrointestinal tract, because artemisinin jejunal permeability is high and the absorption of artemisinin is not affected by efflux by P-glycoprotein. It is not likely that the decreased bioavailability of artemisinin after multiple oral administration is due to induction of a P-glycoprotein-mediated transport, induction of gut wall metabolism, or a change in any general jejunal transport mechanism of artemisinin. A more likely explanation is autoinduction of hepatocellular activity.

References


