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Assessment of the effect of malaria infection on hepatic clearance of dihydroartemisinin using rat liver perfusions and microsomes

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1 The clearance of dihydroartemisinin (DHA) in control and malaria-infected (MI) rats was investigated using the isolated perfused rat liver (IPRL) model and hepatic microsomal studies.

2 In the recirculating IPRL, clearance of DHA was reduced from a mean (s.d.) of 8.2 ± 1.8 ml min^{-1} in controls ($n=8$) to 6.0 ± 1.0 ml min^{-1} in MI ($n=8$; $P<0.01$). Clearance in control livers was similar to the perfusion flow rate, suggesting a high hepatic extraction ratio for DHA.

3 Single-pass IPRL studies in controls ($n=8$) showed that DHA bioavailability at 1.3, 8 and 38 μM was 0.026 ± 0.020, 0.043 ± 0.025 and 0.14 ± 0.06, respectively ($P<0.001$ for 8 μM vs 38 μM). In MI livers ($n=5$), DHA bioavailability at 8 and 38 μM was 0.18 ± 0.07 and 0.40 ± 0.08, respectively ($P=0.002$). Bioavailability was higher in the MI group than in controls ($P=0.01$ at 8 μM and $P<0.001$ at 38 μM). DHA-glucuronide was the sole biliary metabolite.

4 Hepatic microsomal studies of DHA-glucuronide formation showed a significantly lower V_{max}, but no significant change in K_m, in MI compared to control livers ($n=6$). Intrinsic metabolic clearance (V_{max}/K_m) was higher in control than in MI livers (5.2 ± 1.3 and 2.5 ± 1.4 μl min^{-1} mg^{-1}, respectively; $P=0.006$).

5 These studies demonstrate that DHA has a high, concentration-dependent hepatic extraction ratio that is reduced by 20–30% in the *P. berghei* rodent malaria model. The impaired hepatic clearance of DHA in MI is attributable to a reduction in intrinsic metabolic clearance.

Keywords: Malaria; dihydroartemisinin; pharmacokinetics; bioavailability; metabolism; isolated perfused rat liver

Introduction

Artemisinin ('qinghaosu', Figure 1) is the first clinically important antimalarial drug to be isolated from a plant source since powdered Cinchona bark (quinine) was brought to Europe in the Seventeenth century (Klayman, 1985; Hien & White, 1993). Artemisinin and its semi-synthetic derivatives are potent, well-tolerated drugs that have become first-line treatment for falciparum malaria in many tropical countries (World Health Organization, 1995; De Vries & Dien, 1996). Dihydroartemisinin (DHA, Figure 1) is the active metabolite of the artemisinin derivatives, artesunate, artemether and arteether, that are currently in clinical use (De Vries & Dien, 1996; Lee & Hufford, 1990). In the case of artesunate, which is the only water-soluble derivative and thus the only one that can be given by intravenous injection, rapid hydrolysis *in vivo* means that DHA is likely to be of greater clinical importance than the parent drug. Indeed, DHA has been formulated recently as tablets and suppositories which are presently undergoing clinical evaluation at doses of 120 mg initially, followed by 60 mg daily for up to 1 week (Looareesuwan et al., 1996).

DHA has a short elimination half-life (approximately 40 min (Batty *et al*., 1996, 1998a,b; Benakis *et al*., 1997)) but little is known of its metabolism. The supposition that DHA is metabolized to inactive compounds has been supported by a recent study in which pharmacokinetic parameters for DHA were calculated from plasma concentration-time data that were obtained by bioassay (Bethell *et al*., 1997). The pharmacokinetic parameters were consistent with data reported from similar clinical studies using selective high performance liquid chromatography (h.p.l.c.) assays (Batty *et al*., 1996, 1998a,b; Benakis *et al*., 1997).

In vitro studies in rodents suggest that the liver is the primary site of DHA metabolism (Niu *et al*., 1985; Lee & Hufford, 1990) but the complete metabolic pathway is yet to be elucidated. Using rat and hamster liver microsomes Leskovac & Theoharides (1991) found that DHA was metabolized predominantly to four mono-hydroxylated derivatives. By contrast, Maggs *et al*. (1997) reported recently that the principal metabolite of DHA in rat bile was the 12-glucuronide (Figure 1).

In humans, malaria infection (MI) can cause hepatic impairment and influence the disposition of many drugs (Warrell *et al*., 1990). Clearance of quinoline antimalarials such as quinine (White *et al*., 1982) and mefloquine (Boudreau *et al*., 1990; Karbwang & White, 1990) is reduced in patients with MI compared to that in healthy volunteers. In addition, *in vitro* studies using the isolated perfused rat liver (IPRL) model have shown that MI decreases hepatic clearance of quinoline drugs (Mihaly *et al*., 1987; Mansor *et al*., 1990). Furthermore, the IPRL model has been used to demonstrate that MI significantly impairs glucuronide conjugation of several xenobiotics, including paracetamol, salbutamol, harmol and phenol (Murdoch *et al*., 1991, 1992; Glazier *et al*., 1994).

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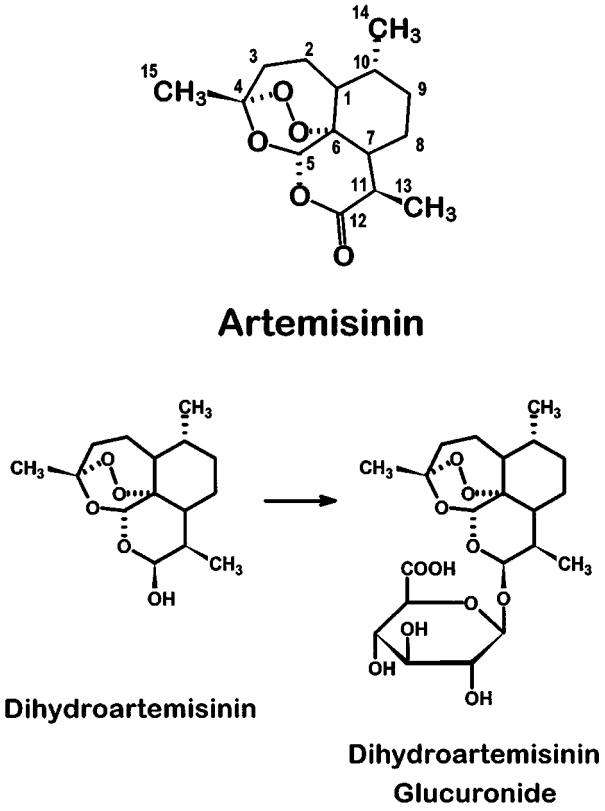

Figure 1 Chemical structure of artemisinin, dihydroartemisinin and the principal metabolite, dihydroartemisinin glucuronide.

We have investigated the hypothesis that MI decreases hepatic clearance of DHA, using both the IPRL model and hepatic microsomal studies.

Methods

Materials

DHA was a gift from the Mediplantex Company, Hanoi, Vietnam. Purity was verified by h.p.l.c. analysis (Batty *et al*., 1996). [12−^{3}H]-DHA (1.4 Ci mmol^{-1}) was purchased from Moravek Biochemicals Inc, California, U.S.A. Chemical and radiochemical purity (99.8%) were confirmed by h.p.l.c.

Human O+ blood (35–50 days post-collection) was obtained from the WA Blood Transfusion Service of the Australian Red Cross. Bovine serum albumin (Fraction V), sodium taurocholate, uridine 5′-diphosphoglucuronic acid (UDPGA), β-glucuronidase (Type H1 from *Helix pomatia*; 400,000 units g^{-1}), polyoxyethylene 20 cetyl ether (Brij 58®) and D-saccharic acid-1,4-lactone (saccharolactone) were purchased from the Sigma Chemical Company, Missouri, U.S.A. Other chemicals included: sodium pentobarbitone injection (Nembutal®, 60 mg ml^{-1}; Boehringer Ingelheim Pty Ltd, NSW, Australia); [^{3}H]-H_2O (100 mCi ml^{-1}) and ^{14}C-sucrose (200 μCi ml^{-1}; Amersham Life Sciences, Buckinghamshire, U.K.); Starscint® liquid scintillation fluid (Packard Instrument B.V. - Chemical Operations, Groningen, Netherlands); compound sodium lactate (Hartman's) infusion (Baxter Healthcare, NSW, Australia); sterile heparinized saline injection, 50 units in 5 ml (Delta West, Western Australia). All other laboratory chemicals and solvents were of analytical grade.

Plasmodium berghei *malaria model*

This study was approved by the University of Western Australia Animal Experimentation and Ethics Committee. *Plasmodium berghei* infection was established in 8–10 week old Balb/c mice (Animal Resources Centre, Western Australia) using cryopreserved *P. berghei* parasites (ANKA strain; Australian Army Malaria Institute, QLD, Australia). A thin blood smear was prepared on alternate days and the peripheral parasitaemia, expressed as a percentage of total erythrocytes, was determined by microscopic examination of 10 high power fields (Seiverd, 1983). Parasites were passaged to healthy mice within 12 days of inoculation or when the parasitaemia exceeded 40% (Mihaly *et al*., 1987).

Four-week old male Wistar rats (Animal Resources Centre, Western Australia) were housed in chaff-lined cages (2 per cage) and pairs of rats were allocated randomly to control or MI groups. Animals in the MI group were administered an i.p. injection of 10^7 parasitized red blood cells from Balb/c mice. Control rats received an i.p. injection of 0.9% sodium chloride solution. The rats were monitored regularly, and the livers removed for perfusion or microsomal enzyme experiments 12–14 days post-inoculation.

Isolated perfused rat liver (IPRL) preparation

Rats were anaesthetised with sodium pentobarbitone (60–120 mg kg^{-1}) and IPRL preparations were established according to standard techniques (Miller, 1973; Jones *et al*., 1984; Mihaly *et al*., 1987; Mansor *et al*., 1990). The perfusate comprised 10% human O+ red blood cells and 1% bovine serum albumin in modified Krebs-Henseleit buffer (composition: Na^+ 141 mM, K^+ 5.4 mM, Mg^{2+} 1.2 mM, Ca^{2+} 2.5 mM, Cl^- 125 mM, $H_2PO_4^{2-}$ 1.2 mM, HCO_3^- 25 mM, SO_4^{2-} 1.2 mM, dextrose 10 mM). Red blood cells in the human donor blood were washed (×3) with an equal volume of 0.9% sodium chloride solution, followed by centrifugation at 1200 *g* for 10 min (4°C) and aspiration of the supernatant and buffy coat.

Indices of liver viability

Livers were perfused for an equilibration period of 15–20 min and 100% O_2 was fed into the oxygenator throughout the perfusion. Liver viability was assessed by visual appearance, portal vein pressure, bile flow rate, and oxygen consumption (Gores *et al*., 1986). Bile flow rate was determined by collecting bile into pre-weighed microcentrifuge tubes and weighing at the end of each experiment (assuming specific gravity = 1 *g* ml^{-1}). Oxygen consumption was determined from blood gas determinations of inflow and outflow perfusate (Equations 1–3; Appendix) that was drawn into blood gas syringes (Bard-Parker 1 ml arterial blood gas sampling kits; Becton-Dickinson, New Jersey, U.S.A.) during the experiments. Blood gas analyses were performed using a NOVA StatProfile® Plus 5 blood gas analyzer (NOVA Biomedical, Waltham, MA, U.S.A.).

Recirculating IPRL system

Perfusate (total reservoir volume 150 ml) was recirculated through the liver at 8 ml min^{-1}. Sodium taurocholate solution (30 μmol ml^{-1}) was infused into the perfusate reservoir at 1 ml h^{-1}. Samples (500 μl) for blood gas determination were drawn at 0, 30 and 60 min after the equilibration period. DHA (3 mg ml^{-1} in ethanol, 100 μl) was added to the perfusate to give a total concentration of 7 μM, which is equivalent to the

peak plasma concentration found in humans after conventional therapeutic doses (120 mg) of artesunate (Batty *et al*., 1998a).

Samples (1.5 ml) were drawn from the perfusate reservoir at 5, 10, 15, 20, 25, 30, 40, 50 and 60 min after addition of DHA, stored on ice until the end of the experiment, centrifuged (10,000 *g* for 1 min) and the supernatant stored frozen at −25°C. Bile was collected for 60 min after addition of DHA to the perfusate and also stored at −25°C. At the completion of each perfusion, the livers were flushed with chilled 0.9% sodium chloride solution, dried between absorbent towels and weighed. DHA in the perfusate samples was analysed by h.p.l.c. The between-day coefficient of variation ($n=15$) for this assay was 14.2% at 2240 nM.

Single-pass IPRL system

Livers were perfused at 10 ml min^{-1}. For control livers, DHA (0.4 or 4 mg ml^{-1} in ethanol) and [^{3}H]-DHA (41 μg ml^{-1} (200 μCi ml^{-1}) in ethanol; 200 μl) were added to each perfusate reservoir (400 ml) to give total concentrations of approximately 1, 8 or 38 μM, respectively. For MI livers, the perfusion concentrations of DHA were 8 and 38 μM. The order of perfusion was randomized according to a pre-determined schedule, with drug-free perfusate passed through the liver for 15 min between experiments. Samples (500 μl) for blood gas determination were drawn at 0, 45, 90 and 135 min after the equilibration period. Samples for DHA analysis (2 ml) were drawn from the perfusate reservoir and collected from the hepatic outflow cannula at 0, 15, 17.5 and 20 min. The perfusate was centrifuged and the supernatant stored at −25°C until analysed. Bile was collected for 45 min periods, comprising the 30 min perfusion and subsequent 15 min washout.

DHA concentration in inflow and outflow samples was determined by h.p.l.c. using the method of Batty *et al*. (1996), with minor modifications. Briefly, the sample was filtered (Centrisart C4 10,000 MW microcentrifuge filters; Sartorius AG, Göttingen, Germany) and the supernatant (100 μl) injected directly onto the column. Eluent fractions were collected every 15 s using an LKB 2212 HeliRac Fraction Collector (LKB-Produkter AB, Bromma, Sweden). DHA was quantified by liquid scintillation counting of both α- and β-^{3}H-DHA peaks (retention times 6 and 7.25 min, respectively) using a Tri-Carb 1500 Liquid Scintillation Analyzer (Packard Instrument Co., CT, U.S.A.).

At the completion of each perfusion, bolus doses of [^{3}H]-H_2O and ^{14}C-sucrose were used to determine the cellular ('functional') volume of the liver (St-Pierre *et al*., 1989). Whilst ^3H_2O distributes throughout the total water space of the liver, ^{14}C-sucrose does not cross membranes and, in the case of the liver, the mean transit time of this tracer is used to quantify the sinusoidal volume and the Space of Disse (St-Pierre *et al*., 1989). The difference between mean transit times of ^3H_2O and ^{14}C-sucrose was used to determine the cellular volume of the liver. [^{3}H]-H_2O (5.3 μCi μl^{-1}; 2 μl) and ^{14}C-sucrose (0.2 μCi μl^{-1}; 2 μl) were added to separate 200 μl aliquots of perfusate and equilibrated for at least 1 h before injecting the tracers (100 μl) into the portal vein cannula of the IPRL circuit. Perfusate outflow fractions were collected into microcentrifuge tubes (1.2 s intervals for 96 s for ^{14}C-sucrose; 3 s intervals for 240 s for [^{3}H]-H_2O) using a Gilson FC 203B Fraction Collector (Gilson Medical Electronics, WI, U.S.A.). The fractions were centrifuged (10,000 *g* for 15 s) and the activity in aliquots of supernatant (100 μl for ^{14}C-sucrose and 200 μl for [^{3}H]-H_2O) was quantified by liquid scintillation counting. The mean transit times of the tracers through the liver was calculated (Equations 4–8; Appendix) and corrected for the mean transit time through the system without a liver (14.6 ± 1.6 s; $n=8$). Cellular water space (functional volume) was determined from the difference between total water space (obtained from [^{3}H]-H_2O mean transit time) and the sinusoidal plus Disse volume (obtained from ^{14}C-sucrose mean transit time) (St-Pierre *et al*., 1989). At the completion of each perfusion, the livers were flushed with chilled 0.9% sodium chloride solution, dried between absorbent towels and weighed.

Analysis of biliary metabolites

Aliquots of bile (20 μl from the recirculating perfusions; 2 μl from the single-pass perfusions, 8 μM and 38 μM concentrations only) were injected onto an Ultracarb C_8 h.p.l.c. column (5 μm, 250 × 4.6 mm, Phenomenex, Cheshire, U.K.) and eluted with a gradient of acetonitrile (20–35% over 15 min, 35–70% over 10 min) in 0.1 M ammonium acetate (pH 6.9) at a flow rate of 0.9 ml min^{-1}. Radiochromatograms of the biliary metabolites excreted during the single-pass perfusions were obtained with an A-200 Flow-One/β radioactivity detector (Canberra-Packard, Berkshire, U.K.) located in parallel to the mass spectrometer (95 : 5 split flow). Flo-Scint A liquid scintillation fluid (Canberra-Packard) was mixed with the eluate at 1 ml min^{-1}. Positive-ion electrospray mass spectra were acquired with a Quattro II tandem quadrupole instrument (Micromass Ltd., Manchester, U.K.). Low concentrations of metabolite were detected by selected ion monitoring as described previously (Madden *et al*., 1996).

In addition, DHA-glucuronide in the bile was quantified by liquid scintillation counting of aliquots of bile and perfusate from the single-pass perfusions (8 μM and 38 μM concentrations only). The proportion of the DHA dose recovered in bile was estimated by relating the specific activity of the bile to the amount of DHA that was passed through the liver during the perfusion, assuming that all of the ^{3}H in the bile was DHA-glucuronide.

Microsomal glucuronidation of DHA

Rats were anaesthetized with sodium pentobarbitone (60–120 mg kg^{-1}). The portal vein was cannulated and the liver flushed with 5–8 ml of heparinized saline (4°C), followed immediately by 10 ml of 1.15% KCl in 0.01 M sodium phosphate buffer (pH 7.4, 4°C). The liver was removed and homogenized in approximately 3 volumes of 1.15% KCl in 0.01 M sodium phosphate buffer (Ultra-Turrax homogenizer; John Morris Scientific, NSW, Australia) and the homogenate centrifuged at 9,000 *g* for 20 min (Avanti J25I centrifuge; Beckman Instruments Inc., CA, U.S.A.). The supernatant was centrifuged at 240,000 *g* for 26 min ((McManus & Ilett, 1976); Beckman TL-100 ultracentrifuge) and the microsomal pellet re-suspended in 20% glycerol in 0.1 M sodium phosphate buffer. Protein concentration (Bradford, 1976) and cytochrome P450 content (Omura & Sato, 1964; Mazel, 1971) of the microsomal suspensions were determined prior to storage at −80°C.

Incubation mixtures (200 μl; in duplicate) contained microsomes (0.7 mg protein), 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 5 mM saccharolactone, 0.1 mg Brij 58® and 3 mM UDPGA. Solutions of DHA and [^{3}H]-DHA in ethanol (50 μM–40 mM, 4 nCi μl^{-1}) were used to give final concentrations of DHA in the incubation mixture of 5–4000 μM. Incubations were carried out under linear conditions (7 min at

37°C) and reactions were stopped by adding 200 μl acetonitrile (−20°C), vortexing and centrifuging at 10,000 *g* for 1 min. Supernatant (80 μl) was assayed by h.p.l.c. (Batty *et al*., 1996) using a mobile phase of 45% acetonitrile in 0.1 M acetate buffer (pH 4.8) at 0.7 ml min^{-1}. Column eluate was collected in 1 min fractions and [^{3}H]-DHA-glucuronide was quantified by liquid scintillation counting. Retention times for DHA-glucuronide, α-DHA and β-DHA were 2.5, 8.5 and 12 min, respectively. Formation of DHA-glucuronide was confirmed by quantitative conversion to DHA with β-glucuronidase (approximately 1100 units, 1 h incubation) and by the essential requirement of UDPGA for production of DHA-glucuronide in the reaction mixture.

Pharmacokinetic and statistical analysis

Pharmacokinetic parameters ($AUC_{0-\infty}$, k, t, and CL) from the recirculating experiments were determined from the perfusate concentration-time data using non-compartmental analysis (Heinzel *et al*., 1993; Rowland & Tozer, 1995). For single-pass experiments, equations 9–12 (Appendix) were used. Intrinsic clearance (CL_{int}; Equation 13; Appendix) was calculated from the equation for the venous equilibrium, or well-stirred, model of hepatic elimination (Rowland & Tozer, 1995) which has been shown to be the most appropriate for a drug with a high hepatic extraction ratio (Smallwood *et al*., 1988). For the microsomal studies, V_{max} and K_m were determined by fitting the Michaelis-Menten equation to the reaction velocity-concentration data (Houston, 1994; Quinton *et al*., 1995). Intrinsic metabolic clearance was calculated as $CL_{int} = V_{max} \div K_m$ (Houston, 1994).

Differences between means were analysed by Student's *t*-test, unless otherwise indicated. Data are expressed as mean ± s.d. or median and interquartile range, as appropriate.

Results

Recirculating IPRL studies

The age-matched control rats weighed significantly more than the MI group, required a larger dose of pentobarbitone and had smaller livers (Table 1). The mean parasitaemia in the MI rats was 40 ± 16% (range 14–63%). Whilst the absolute flow rates did not vary between the groups, perfusate flow in controls (0.8 ± 0.1 ml min^{-1} g^{-1}) was significantly higher than MI livers (0.6 ± 0.1 ml min^{-1} g^{-1}; $P = 0.009$), when normalized for liver weight. Portal vein pressure was similar in the MI and control groups (Table 1). Bile flow rates and oxygen consumption were stable throughout the experiments and significantly lower in the MI livers than the controls (Table 1).

The concentration-time profiles of DHA in perfusate from the two groups are shown in Figure 2 and pharmacokinetic parameters derived from these data are given in Table 1. Clearance of DHA was the same as the perfusate flow rate in controls. By contrast, clearance of DHA was significantly lower than perfusate flow rate in the MI group.

Single-pass IPRL studies

Physical data for the rats and the indices of liver viability for the single-pass IPRL experiments are given in Table 2. The mean parasitaemia in the MI rats was 21 ± 25% (range 1–55%). Although the control rats weighed significantly more than those in the MI group, mean liver weights and cellular volume of the livers were comparable. The cellular volumes of the livers were 49 ± 8% (controls) and 53 ± 8% (MI) of the wet weight of the livers and were used as the denominator in all subsequent pharmacokinetic calculations.

Table 1 Details of rats, indices of viability and pharmacokinetic parameters for dihydroartemisinin from recirculating IPRL experiments ($n = 8$ in each group). Data are mean ± s.d.

Parameter	*Control*	*Malaria-infected*	P
Rat weight (g)	214 ± 38	151 ± 21	0.001
Pentobarbitone dose (mg kg^{-1})	94 ± 19	60 ± 7	0.001
Liver weight (g)	11.2 ± 1.9	13.5 ± 1.9	0.03
Perfusate flow rate[a] (ml min^{-1})	8.2 ± 0.5	7.6 ± 0.9	n.s.
Portal vein pressure (cm)	4.2 ± 1.0	7.3 ± 5.1	n.s.
Bile flow (μl min^{-1} g^{-1} liver)	0.80 ± 0.17	0.48 ± 0.25	0.02
Oxygen consumption[b] (μmol O_2 min^{-1} g^{-1} liver)	1.7 ± 0.2	1.3 ± 0.2	<0.001
DHA concentration at baseline; Co (nM)	6040 ± 1630	6260 ± 930	n.s.
DHA clearance[a] (ml min^{-1})	8.2 ± 1.8	6.0 ± 1.0	0.009
DHA $t_{1/2}$ (min)	9.1 ± 0.8	12.1 ± 1.9	0.001

[a]Perfusate flow rate and DHA clearance were significantly different in the malaria-infected group ($P = 0.01$). [b]Oxygen consumption data from 0, 30 and 60 min were not significantly different within each group and were pooled for analysis.

Table 2 Details of rats and indices of variability from single-pass IPRL experiments. Data are mean ± s.d.

Parameter	*Control* ($n = 8$)	*Malaria-infected* ($n = 5$)	P
Rat weight (g)	267 ± 57	161 ± 23	0.001
Pentobarbiton dose (mg kg^{-1})	131 ± 35	71 ± 11	0.004
Liver weight (g)	10.6 ± 2.4	10.2 ± 1.1	n.s.
Cellular volume of liver; V_L[a] (ml)	5.1 ± 1.1	5.4 ± 0.6	n.s.
Perfusate flow rate (ml min^{-1} ml^{-1} V_L[a])	1.8 ± 0.4	1.7 ± 0.2	n.s.
Portal vein pressure (cm)	10.2 ± 1.0	11.8 ± 1.0	0.02
Bile flow[b] (μl min^{-1} g^{-1} liver)	1.03 ± 0.41	0.82 ± 0.26	n.s.
Oxygen consumption[b] (μmol O_2 min^{-1} g^{-1} liver)	1.8 ± 0.3	1.4 ± 0.2	<0.001

[a]V_L is cellular volume of the liver. [b]Bile flow and oxygen consumption were not significantly different across the collection periods within each group. Therefore, bile flow was taken as the mean of three collection periods for control livers and two for MI livers, and oxygen consumption as the mean of four sampling times for control livers and three for MI livers.

Perfusate flow rates were not significantly different between the two groups, whether normalized according to liver weight (controls = 1.0 ± 0.2 ml min^{-1} g^{-1}; MI = 1.0 ± 0.1 ml min^{-1} g^{-1}) or cellular volume (Table 2). Within both groups, portal vein pressure, bile flow and oxygen consumption were stable during the perfusions. However, as with the recirculating perfusions, mean oxygen consumption in the MI group was significantly lower than controls (Table 2).

The bioavailability of DHA was concentration-dependent and significantly lower in controls than in MI livers (Table 3). Clearance and intrinsic clearance were found to be concentration-dependent, with significant differences between controls and MI livers, and within the control group at 8 and 38 μM (Table 3). The extraction ratios in control livers were 0.86 ± 0.06, 0.96 ± 0.03 and 0.97 ± 0.02 at 38, 8 and 1.3 μM, respectively. In MI livers the extraction ratios were 0.60 ± 0.26 and 0.76 ± 0.31 at 38 and 8 μM, respectively.

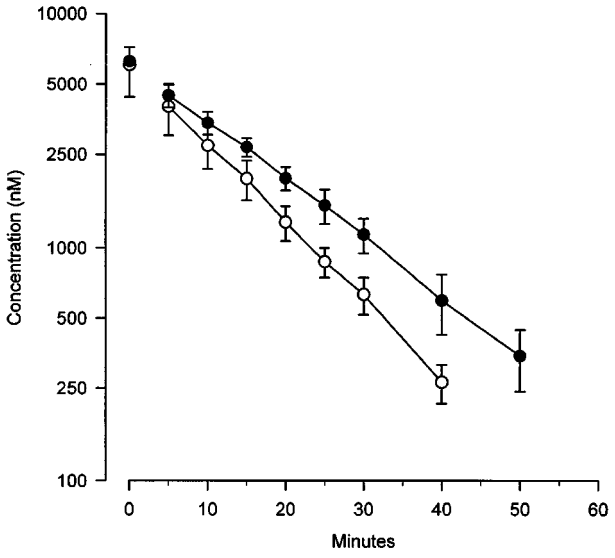

Figure 2 Perfusate concentration-time profile for DHA in control (○; $n = 8$) and malaria-infected (●; $n = 8$) isolated perfused rat livers (recirculating design). DHA concentration at baseline (Co) was determined by extrapolation. Data are mean ± s.d.

Biliary metabolites

Liquid chromatography-mass spectrometry analysis of bile from the recirculating IPRL experiments revealed only the presence of DHA-glucuronide. In addition, only one radiolabelled metabolite of [^{3}H]-DHA was identified in the bile from the single-pass perfusions of both control and MI livers. No [^{3}H]-DHA was detected. The metabolite corresponded to a peak in the mass chromatogram representing the ammonium adduct of glucuronidated DHA ($[M + NH_4]^+$, m/z 478) and yielded DHA (identified by chromatographic and mass spectral comparisons (Maggs *et al*., 1997)) when the bile was pre-incubated with β-glucuronidase. The two isomers of DHA-glucuronide found as minor metabolites in the bile of rats given DHA intravenously (Maggs *et al*., 1997), namely the glucuronide of 3-hydroxydesoxydihydroartemisinin and that of a tetrahydrofuran acetate isomer, were absent from bile in all except one of the control liver perfusions. From that perfusion, the tetrahydrofuran acetate glucuronide isomer represented 5.5 and 2% of the biliary radioactivity from the 8 and 38 μM perfusions, respectively.

Recovery of DHA-glucuronide in the bile (single-pass perfusions; Table 3), combined with bioavailability data (4 and 14% in controls, 18 and 40% in MI, from 8 μM and 38 μM perfusions, respectively; Table 3) indicates that approximately 70% of the DHA dose could be accounted for in these studies. Biliary clearance of DHA, as DHA-glucuronide, was significantly impaired in MI livers during the 38 μM perfusions (Table 3).

Microsomal glucuronidation of DHA

Data for the rat microsomal enzyme studies are given in Table 4. The mean parasitaemia in the MI rats was 48 ± 7% (range 39 – 57%). The cytochrome P450 content and the V_{max} for DHA-glucuronide formation were lower in MI than in control livers while K_m values were not significantly different (Table 4). Consistent with data from the IPRL studies, CL_{int} was significantly lower in the MI livers.

Discussion

Due to the rapid development of multi-drug resistant falciparum malaria in some tropical countries, the artemisinin

Table 3 Pharmacokinetic data for dihydroartemisinin from single-pass IPRL experiments. Data are mean ± s.d.

Parameter	*Concentration* (μM)	*Conrol* ($n = 8$)	*Malaria-infected* ($n = 5$)	P
Bioavailability	38	0.14 ± 0.06[a]	0.40 ± 0.08[a]	< 0.001
	8	0.04 ± 0.02[a]	0.18 ± 0.07[a]	0.01
	1.3	0.03 ± 0.02	–	–
Clearance	38	1.5 ± 0.3[a]	1.0 ± 0.2[a]	0.008
(ml min^{-1} ml^{-1} V_L[c])	8	1.7 ± 0.3[a]	1.4 ± 0.2[a]	n.s.
	1.3	1.8 ± 0.4	–	–
Intrinsic clearance[d]	38	27 ± 14[b]	6 ± 2	0.003
(ml min^{-1} ml^{-1} V_L[c])	8	127 ± 91[b]	21 ± 14	0.01
	1.3	268 ± 258	–	–
DHA-glucuronide recovery in bile	38	55 ± 14	28 ± 15	0.02
(% of total dose)	8	69 ± 14	41 ± 16	0.02
Biliary clearance of DHA[e]	38	253 ± 66	115 ± 44	0.006
(ml h^{-1})	8	318 ± 62	214 ± 102	n.s.

[a]Significant differences within groups ($P < 0.005$; One way repeated measures ANOVA). [b]Significant differences within groups ($P < 0.05$; One way repeated measures ANOVA). [c]V_L is cellular volume of the liver. [d]Determined from equation 13 (Appendix). [e]Determined from equation 12 (Appendix). – Not determined.

Table 4 Details of rats, cytochrome P450 content, Michaelis-Menten parameters and intrinsic metabolic clearance data from microsomal enzyme studies.

Parameter	*Control* ($n=6$)	*Malaria-infected* ($n=6$)	P
Rat weight (g)	224±19	156±15	<0.001
Liver weight (g)	10.2±1.5	10.7±1.5	n.s.
Cytochrome P450 content (nmol mg^{-1} protein)	0.86±0.11	0.20±0.02	<0.001
V_{max} (nmol min^{-1} mg^{-1} protein)	3.4±0.7	2.1±0.6	0.005
K_m (μM)	608 (599–759)[a]	965 (895–1144)[a]	n.s.
Intrinsic metabolic clearance[b] (μl min^{-1} mg^{-1} protein)	5.2±1.3	2.5±1.4	0.006

[a]Data are mean±s.d. or median (interquartile range) as appropriate. [b]Determined from $CL_{int} = V_{max}/K_m$ (Houston, 1994).

derivatives have become an important alternative to quinoline drugs for the management of severe and complicated human malaria. However, empirical use of artemisinin, artesunate and artemether in a variety of clinical settings has preceded reliable reports of pharmacokinetic and metabolic data for these drugs (De Vries & Dien, 1996; Hien & White, 1993). We have evaluated the clearance of DHA, a potent metabolite of the semi-synthetic artemisinin derivatives, and a compound that is now manufactured and formulated for oral and rectal administration in the treatment of falciparum malaria.

The IPRL model is an established technique for investigation of drug clearance by the intact liver, but the validity of data is dependent on organ viability during perfusions (Miller, 1973; Gores *et al*., 1986; Cheung *et al*., 1996; Meijer *et al*., 1981). In the present study, portal vein pressure (normally 5–12 cm at perfusate flow rates of 5–15 ml min^{-1}) and bile flow rates (at least 0.3–0.5 μl min^{-1} g^{-1} liver weight) for control livers were consistent with those in previous reports (Miller, 1973; Gores *et al*., 1986; Cheung *et al*., 1996). Oxygen consumption in control IPRLs was similar throughout the experiments, albeit slightly lower than in some previous studies (normally at least 1.9 μmol O_2 min^{-1} g^{-1} liver (Gores *et al*., 1986; Cheung *et al*., 1996)). As in previous reports (Mansor *et al*., 1990; Murdoch *et al*., 1991, 1992), indices of liver viability were affected by MI, with bile flow and oxygen consumption significantly lower in MI compared to control livers (Tables 1 and 2). Overall, these data confirm that the experimental conditions for our IPRL studies were acceptable.

Due to the reported changes to hepatic architecture in MI (Francis & Warrell, 1993; Garnham, 1966), we used the multiple indicator technique (St-Pierre *et al*., 1989) to determine cellular volume of the livers in the single-pass perfusions. We found that the cellular volume of the liver comprised 49% (controls) and 53% (MI) of liver wet weight. Thus, if there were changes in hepatic architecture due to sinusoidal congestion by Kupffer cells and parasitized erythrocytes, our data suggest that significant collateral circulation did not develop in the MI livers. Changes in intrinsic metabolic clearance might therefore be the principal cause of reduced hepatic clearance of DHA, an hypothesis that was subsequently tested with microsomal enzyme studies.

In vitro studies in rodents have suggested that both oxidation and glucuronidation are involved in DHA clearance. Leskovac & Theoharides (1991) used rat and hamster liver microsomes with an NADPH-generating system and found four mono-hydroxylated derivatives of DHA, one mono-hydroxylated derivative of deoxy-DHA and two unidentified compounds. By contrast, Maggs *et al*. (1997) found that DHA-glucuronide was the principal metabolite recovered from the bile of rats given ^{14}C-DHA by intravenous injection. No hydroxylated metabolites were identified.

In the recirculating perfusions of control livers, we found that DHA clearance was equivalent to perfusate flow rate, suggesting that the drug has a high hepatic extraction ratio. Single-pass IPRL studies in control livers confirmed the high hepatic extraction ratio for DHA and also demonstrated that the clearance was concentration-dependent. Moreover, we were able to confirm that DHA-glucuronide was the principal metabolite of DHA (Maggs *et al*., 1997) and the only metabolite eliminated in the bile, accounting for 55 and 69% of the dose from the 38 μM and 8 μM perfusions, respectively. Combined with the DHA bioavailability data, we were able to account for approximately 70% of the DHA dose.

Our IPRL studies also provide novel data on the effect of MI on DHA clearance, demonstrating a 20–35% reduction in total clearance. Since MI and control livers had comparable cellular volumes, it was likely that metabolic rather than structural changes to the liver were responsible for impaired clearance of DHA. MI is known to decrease the cytochrome P450 content in rat liver microsomes (McCarthy *et al*., 1970) and has been shown in the IPRL model to reduce the clearance of several drugs, including the quinoline antimalarials and xenobiotics that are metabolised by glucuronidation (Mihaly *et al*., 1987; Mansor *et al*., 1990; Murdoch *et al*., 1991, 1992; Glazier *et al*., 1994). Biliary clearance of DHA-glucuronide, the principal biliary metabolite of DHA (Maggs *et al*., 1997), also was impaired in MI (Table 3).

There are three possible explanations for reduced biliary clearance of DHA-glucuronide in MI: slower bile flow rate, impaired hepatobiliary transport of the metabolite into the bile canaliculi, and reduced biotransformation of DHA to DHA-glucuronide (Klaassen & Watkins, 1984). Firstly, although slower bile flow from MI livers has been documented previously (Murdoch *et al*., 1992) and in the present study, and may have been a contributing factor, this cannot account completely for the reduced biliary clearance of DHA-glucuronide. Secondly, hepatobiliary transport of glucuronides with a molecular weight above 300–400 ($MW_{DHA\text{-}glucuronide}$ = 460) is an active, carrier-mediated process (Klaassen & Watkins, 1984) which may be impaired in MI due to organelle disruption and inhibition of oxidative phosphorylation reducing the availability of ATP (Riley & Deegan, 1960). However, we found that less than 5% of the total dose was present in the outflow perfusate samples as DHA-glucuronide in both control and MI livers (data not shown), suggesting that hepatobiliary transport was not substantially affected by MI. Finally, reduced metabolism of DHA to DHA-glucuronide is the most plausible explanation for the lower biliary clearance of DHA-glucuronide, because bioavailability of DHA was greater and a lower proportion of the total dose was recovered as DHA-glucuronide in the bile of MI livers compared to controls. Therefore, impaired intrinsic metabolic clearance was

again the most likely reason for reduced total clearance of DHA in MI.

The hepatic microsomal studies of DHA glucuronidation confirmed the lower V_{max} and CL_{int}, with no significant change in K_m, in MI livers. Mechanisms of impaired glucuronidation that have been proposed include reduced concentrations of UDPGA, inhibition of uridine diphosphoglucuronyltransferase (UGT) activity, structural changes to the smooth endoplasmic reticulum and increased levels of β-glucuronidase (Ismail *et al*., 1994). Whilst impaired UDPGA synthesis secondary to depletion of hepatic glycogen stores has been suggested as a possible cause of impaired glucuronidation in MI (Murdoch *et al*., 1992), a causal relationship has not been established conclusively (Mulder, 1992). The principal alternative mechanism for impaired glucuronidation in MI is reduced UGT activity. UGTs are membrane-bound enzymes located in the endoplasmic reticulum and, according to the conformation hypothesis for regulation of UGT, require re-arrangement of their three-dimensional structure for activation (Mulder, 1992; Burchell & Coughtrie, 1989; Zakim & Dannenberg, 1992). Hence, structural changes to cellular organelles that occur in MI (Rosen *et al*., 1967) might decrease UGT activity and reduce glucuronidation (Glazier *et al*., 1994; Ismail *et al*., 1994). Speculation on the cause of impaired glucuronidation in our experiments is beyond the scope of this report. Nevertheless, as constant amounts of microsomal protein, UDPGA and other co-factors were used in our microsomal studies, we conclude that UGT activity is compromised in MI.

Our IPRL studies demonstrate that DHA has a high, concentration-dependent hepatic extraction ratio that was reduced by 20–30% in the *P. berghei* rodent malaria model. Elimination of DHA-glucuronide, the principal biliary metabolite, was reduced by 40–50% in MI. Based on microsomal enzyme studies, the impaired hepatic clearance of DHA in MI can be attributed to a reduction in intrinsic metabolic clearance. Despite the magnitude of these changes, the clinical consequences of impaired glucuronidation of DHA in MI in humans are difficult to predict. Glucuronidation is a high-capacity conjugation reaction (Mulder, 1992), but few drugs are metabolized exclusively by this pathway (Miners & MacKenzie, 1991) and, to the best of our knowledge, none of these has been the subject of detailed evaluation in human MI. Recent studies from our laboratories have shown that DHA-glucuronide also is the principal metabolite of DHA in human liver microsomes (unpublished data, Ilett *et al*.). However, since the UGT isoform(s) responsible for DHA metabolism have not been identified, the potential for drug interactions is unpredictable. Our *in vitro* data highlight the need for studies of DHA disposition in patients with malaria.

Declaration

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