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Differential protein binding of indinavir and saquinavir in matched maternal and umbilical cord plasma

Sreeja Sudhakaran,[1] Craig R. Rayner,[1] Jian Li,[1] David C. M. Kong,[1] Neil M. Gude[2] & Roger L. Nation[1]

[1]*Facility for Anti-Infective Drug Development & Innovation, Monash University and* [2]*Department of Perinatal Medicine, Royal Women's Hospital, Melbourne, Australia*

Correspondence
Professor Roger L. Nation, Facility for Anti-Infective Drug Development and Innovation, Monash University, 381 Royal Parade, Parkville, 3052, Melbourne, Australia.
Tel: + 61 3 9903 9061
Fax: + 61 3 9903 9629
E-mail: roger.nation@vcp.monash.edu.au

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Aims
To determine whether lower umbilical cord than maternal binding of indinavir and saquinavir contributed to the low cord : maternal (C : M) total concentration ratios reported previously.

Methods
Indinavir and saquinavir unbound fraction (*f*u) was determined using equilibrium dialysis. Buffer solutions of human serum albumin (HSA) (20.0, 30.0, 40.0 g l^{-1}) and α_1-acid glycoprotein (AAG) (0.20, 0.60, 2.00 g l^{-1}) were spiked with indinavir (1.00 and 8.00 mg l^{-1}) or saquinavir (0.15 and 1.50 mg l^{-1}). Matched maternal and umbilical cord plasma was spiked with 1.00 mg l^{-1} indinavir (n = 12) or 0.15 mg l^{-1} saquinavir (n = 20). Spiked protein/plasma solutions were dialyzed against isotonic phosphate buffer, at 37 °C. At equilibrium, indinavir and saquinavir concentrations were quantified, and the f_u determined.

Results
Indinavir and saquinavir demonstrated protein concentration-dependent binding in buffer solutions of HSA and AAG. Indinavir f_u was significantly higher in umbilical cord (0.53 ± 0.12) compared with maternal (0.36 ± 0.11) plasma (95% CI of the difference –0.26, –0.097). Similarly, saquinavir *f*u was different between umbilical cord (0.0090 ± 0.0046) and maternal plasma (0.0066 ± 0.0039) (95% CI of the difference –0.0032, –0.0016). The transplacental AAG concentration gradient contributed significantly to the binding differential of both drugs.

Conclusions
The differential plasma binding of both drugs, which was largely the result of the transplacental AAG concentration gradient, would contribute to the low C : M total plasma concentration ratios observed previously. Unbound concentrations of indinavir and saquinavir are likely to be substantially lower in umbilical cord than maternal plasma.

Introduction

The relative extent of binding of a drug in maternal and fetal plasma is an important determinant of transplacental pharmacokinetics. Only the unbound drug is available to traverse the placenta and exert pharmacological effects [1]. Nevertheless, total drug concentrations are usually measured in plasma from mother and fetus to indicate the extent of placental transfer [2]. The composition of plasma proteins, endogenous ligands and hormones varies substantially between maternal and fetal plasma [3], resulting in differential protein binding of several drugs [4]. Therefore, total drug concentrations may not accurately reflect the unbound concentrations on either side of the placenta [5].

HIV protease inhibitors (PIs) are potent antiretrovirals, demonstrating prolonged suppression of HIV replication to below detectable levels [6]. In healthy volunteers, indinavir is 60% protein bound, whilst saquinavir and other PIs are >98% protein bound [7]. Although the PIs are routinely administered in pregnancy [8], the protein binding of PIs in pregnant women is not known. It is recognized that in addition to treating the maternal HIV infection, antiretroviral prophylaxis in the fetus is important in decreasing mother to child transmission (MTCT) of HIV [9]. Therefore, preloading the fetus with PIs via placental transfer, may further protect against MTCT of HIV [10, 11].

The placental transfer of PIs has been studied using matched maternal and umbilical cord plasma collected at delivery [12–14]. Total PI concentrations in umbilical cord relative to maternal plasma (C : M ratios) were below unity, and this has been attributed to low placental transfer [12–14]. However, the influence of differential protein binding on these ratios has not been considered. Measurement of the unbound fraction of PIs in maternal and umbilical cord plasma will establish whether lower umbilical cord binding contributes to the low C : M total concentration ratios. The unbound fraction will also be useful when interpreting total concentrations in maternal and umbilical cord plasma, and predicting whether the unbound concentrations will be effective in preventing MTCT of HIV.

This study was undertaken to examine the role of differential protein binding in the transplacental disposition of two PIs, indinavir and saquinavir. The first objective was to measure the extent of binding to isolated fractions of human serum albumin (HSA) and α_1-acid glycoprotein (AAG), secondly, to measure the unbound fraction in matched maternal and umbilical cord plasma and finally, to assess the influence of the HSA and AAG concentration differential [3] on the extent of binding in matched samples.

Methods

Materials

Indinavir sulphate and saquinavir mesylate were donated by Merck Research Laboratories (New Jersey, USA) and Roche (Basel, Switzerland), respectively. [^{3}H]-Saquinavir (specific activity 1.90 Ci mmol^{-1}, radiochemical purity 98.2%) was purchased from Moravek Biochemicals Inc. (California, USA). HSA (essentially globulin free) and human AAG (orosomucoid) were from Sigma-Aldrich (Castle Hill, Australia). All other chemicals were analytical grade.

Patients and clinical study protocol

Thirty-two non-HIV infected pregnant women were enrolled into the study. The inclusion criteria were women who had a normal gestation and delivered by elective Caesarean section. Women were excluded if they had previous medical conditions or were treated with chronic medications. The study protocol was approved by the Ethics Committees of the Royal Women's Hospital and Monash University, and written informed consent was obtained from all participants prior to enrolment.

Blood samples (10 ml) were collected by venepuncture from a maternal peripheral vein prior to elective Caesarean section, and mixed arterial-venous blood (4–10 ml) from the clamped umbilical cord after delivery of the placenta. The interval between collection of the two samples was less than 25 min. Blood was collected into plastic tubes containing EDTA, and centrifuged. The resulting plasma was stored at −20 °C until required for plasma protein binding studies.

Determination of protein binding

The extent of binding of indinavir and saquinavir was determined by equilibrium dialysis. Perspex dialysis cells were separated by a semipermeable membrane (molecular weight cut-off 12,000–14 000; Spectrum Medical Industries, California, USA). Concentrations of HSA (20.0, 30.0, 40.0 g l^{-1}) and AAG (0.20, 0.60 g l^{-1}) expected in maternal and fetal plasma [3], and an AAG concentration (2.00 g l^{-1}) representative of HIV/AIDS patients [15] were prepared in isotonic phosphate buffer (0.067 M; pH 7.4). Mixed protein solutions (HSA 30.0 g l^{-1} and AAG 0.20–2.00 g l^{-1}) were also prepared. Protein solutions were spiked to obtain therapeutic concentrations of indinavir (1.00 and 8.00 mg l^{-1}) [16] or saquinavir (0.15 and 1.50 mg l^{-1}) [17, 18]. Matched maternal and umbilical cord plasma samples were spiked with 1.00 mg l^{-1} indinavir (n = 12) or 0.15 mg l^{-1} saquinavir (n = 20).

Aliquots (2 ml) of spiked protein/plasma solutions were dialyzed against an equal volume of isotonic phos-

phate buffer. Dialysis cells were gently rotated in a 37 °C thermostatically controlled water bath until equilibrium was attained (6 and 8 h for indinavir and saquinavir, respectively). At equilibrium, aliquots of buffer and protein/plasma solutions were stored at −20 °C until the time of analysis. The unbound fraction (f_u) was calculated at distribution equilibrium by the ratio of drug in the buffer to the protein/plasma compartment. Non-specific adsorption onto the dialysis units was minimal for both indinavir (10.1%) and saquinavir (12.5%). The reproducibility of determination of the f_u was measured using plasma from healthy volunteers (Red Cross Blood Bank, Melbourne, Australia); the coefficient of variation was less than 15% for indinavir ($n = 6$) and saquinavir ($n = 6$).

Drug and protein analysis

Indinavir concentrations in buffer and protein/plasma solutions were determined using a validated HPLC assay [19]. Protein precipitation, rather than solid-phase extraction was utilized. The measured concentration of the quality control (QC) samples in buffer (0.25, 1.0, 5.0 mg l^{-1}) and plasma (0.25, 1.0, 10.0 mg l^{-1}) were within 10% of the nominal concentrations, and the coefficient of variation was less than 10% for all QCs. [^{3}H]-Saquinavir concentrations in buffer and protein/plasma solutions were determined using liquid scintillation counting. HSA and AAG concentrations in maternal and umbilical cord plasma were measured using radial immunodiffusion (Nor Partigen®, Marburg, Germany).

Statistical analysis

Group data are expressed as mean ± SD. Differences in the f_u and plasma protein concentrations between maternal and umbilical cord plasma were assessed using the two-sided Student's *t*-tests for paired data, accepting $P < 0.05$ as statistically significant. Relationships between the indinavir and saquinavir f_u and plasma protein concentrations were investigated using linear, partial and multiple regression analysis (SPSS for Windows, version 11.5, Chicago, IL, USA).

Results

The indinavir and saquinavir f_u in buffer solutions of HSA and/or AAG are presented in Table 1. Upon increasing HSA concentrations from 20 to 40 g l^{-1}, saquinavir f_u decreased; there was little or no difference in indinavir f_u across this range of HSA concentrations. At any given drug concentration, when increasing AAG concentrations, the f_u of both PIs decreased. A similar trend was observed when increasing only the AAG concentrations in the mixed protein solutions. Furthermore, the indinavir and saquinavir f_u was usually less in the mixed protein solutions, compared with the solution containing an equivalent concentration of AAG alone.

The f_u of indinavir and saquinavir in matched maternal and umbilical cord plasma are summarized in Table 2. Mean indinavir fu was significantly different in umbilical cord compared with maternal plasma (95% CI of the difference −0.26, −0.097; $P = 0.001$); the C : M ratio of f_u ($f_{uC:M}$) was 1.58 ± 0.42. In all matched pairs, except pair 4, indinavir f_u was higher in umbilical cord than maternal plasma. For saquinavir, the f_u in umbilical cord and maternal plasma was different (95% CI of the difference −0.0032, −0.0016; $P < 0.001$); the $f_{uC:M}$ was 1.50 ± 0.40. In every matched pair, the saquinavir f_u was higher in umbilical cord than maternal plasma.

Table 1
Mean f_u of indinavir ($n = 2$) and saquinavir ($n = 2$) in buffer solutions of HSA and/or AAG

	f_u			
Protein concentration (g l^{-1}) in isotonic phosphate buffer	Indinavir 1.00 mg l^{-1}	Indinavir 8.00 mg l^{-1}	Saquinavir 0.15 mg l^{-1}	Saquinavir 1.50 mg l^{-1}
HSA 20.0	0.73	0.62	0.035	0.020
HSA 30.0	0.64	0.60	0.026	0.019
HSA 40.0	0.66	0.59	0.011	0.014
AAG 0.20	0.81	0.82	0.070	0.14
AAG 0.60	0.74	0.68	0.016	0.017
AAG 2.00	0.34	0.26	0.0052	0.0073
HSA 30.0/AAG 0.20	0.68	0.58	0.0060	0.021
HSA 30.0/AAG 0.60	0.51	0.56	0.0065	0.015
HSA 30.0/AAG 2.00	0.29	0.31	0.0029	0.0038

Table 2
Mean ± SD for the f_u, and concentrations of HSA and AAG in matched maternal (M) and umbilical cord (C) plasma for indinavir and saquinavir data sets

PI	f_u			HSA (g l^{-1})			AAG (g l^{-1})		
	M	C	C : M	M	C	C : M	M	C	C : M
Indinavir	0.36 ± 0.11	0.53* ± 0.12	1.58 ± 0.42	33.1 ± 2.76	37.1* ± 4.67	1.13 ± 0.17	0.77 ± 0.24	0.26* ± 0.11	0.35 ± 0.11
Saquinavir	0.0066 ± 0.0039	0.0090* ± 0.0046	1.50 ± 0.40	33.1 ± 5.04	40.1* ± 2.88	1.25 ± 0.33	0.76 ± 0.24	0.27* ± 0.08	0.40 ± 0.18

**P < 0.05 for difference between the maternal and the respective umbilical cord value.*

HSA and AAG concentrations were significantly different between matched samples. HSA concentrations were higher in umbilical cord than maternal plasma for indinavir (95% CI of the difference −7.38, −0.54; $P = 0.027$) and saquinavir (95% CI of the difference −10.0, −3.83; $P < 0.001$) data sets; the C : M ratio of HSA concentrations ($HSA_{C:M}$) was 1.13 ± 0.17 and 1.25 ± 0.33 in the indinavir and saquinavir study, respectively. In contrast, AAG concentrations were lower in umbilical cord compared with maternal plasma for indinavir (95% CI of the difference 0.39, 0.63; $P < 0.001$) and saquinavir (95% CI of the difference 0.37, 0.61; $P < 0.001$) data sets; the C : M ratio of AAG concentrations ($AAG_{C:M}$) was 0.35 ± 0.11 and 0.40 ± 0.18 in the indinavir and saquinavir study, respectively.

The influence of HSA and AAG in explaining the variability in the f_u of the two PIs was examined. There was a significant relationship between the indinavir f_u and AAG concentrations across the pooled umbilical cord and maternal samples ($r^2 = 0.38$, $P = 0.001$, $n = 24$), but not between the indinavir f_u and HSA concentrations ($P = 0.28$). The combined influence of HSA and AAG on the indinavir f_u marginally improved the relationship ($r^2 = 0.39$, $P = 0.005$, $n = 24$). The significant influence of AAG concentrations on the indinavir f_u ($P = 0.001$) was confirmed using partial regression analysis, when HSA concentrations were held constant. The relationship between indinavir $f_{uC:M}$ *vs.* $HSA_{C:M}$ and $AAG_{C:M}$ was not significant ($P = 0.46$).

The saquinavir f_u showed considerable interindividual variability in umbilical cord (0.0090 ± 0.0046) and maternal (0.0066 ± 0.0039) plasma. There was less variability in the $f_{uC:M}$ of saquinavir (1.50 ± 0.40), and therefore the regression analysis was performed using $f_{uC:M}$ *vs.* $HSA_{C:M}$ and $AAG_{C:M}$. There was a significant relationship between the $f_{uC:M}$ and $AAG_{C:M}$ ($r^2 = 0.26$, $P = 0.021$, $n = 10$), but not between the $f_{uC:M}$ and $HSA_{C:M}$ ($P = 0.46$). The simultaneous influence of $HSA_{C:M}$ and $AAG_{C:M}$ on the $f_{uC:M}$ produced a multiple r^2 of 0.34 ($P = 0.029$, $n = 10$). Partial regression analysis verified the significant influence of $AAG_{C:M}$ on the saquinavir $f_{uC:M}$ ($P = 0.004$), when $HSA_{C:M}$ was held constant.

Discussion

The protein binding characteristics of two PIs were examined in maternal and umbilical cord plasma. Indinavir and saquinavir were chosen as model PIs based on their varying extents of binding to plasma proteins [7]. Both PIs were significantly less bound in umbilical cord than maternal plasma, indicating that the low C : M ratios of total drug [12–14] were influenced by differ-

ential protein binding. Furthermore, the AAG concentration differential across the placenta, contributed to the binding differential of indinavir and saquinavir in matched samples.

The extent of binding of indinavir and saquinavir to isolated fractions of HSA and AAG was measured. Upon increasing the concentration of AAG in buffer, the f_u of each PI decreased, consistent with protein concentration-dependent binding; with increasing HSA concentration, the f_u of saquinavir decreased but that of indinavir was not greatly affected. When increasing only the AAG concentration in the mixed protein solutions, the f_u of both PIs decreased. These results demonstrated that at physiologically relevant protein concentrations, indinavir and saquinavir were bound to both HSA and AAG, although AAG appeared to be the major binding protein. This is consistent with binding data for other PIs including, VX-478 (amprenavir) [20], KNI-272 [21] and lopinavir [22].

We appear to be the first to investigate the relative extent of binding of indinavir and saquinavir in matched maternal and umbilical cord plasma. A key finding was that the mean f_u of both PIs was significantly higher in umbilical cord than maternal plasma. In addition, HSA concentrations were higher, while AAG concentrations were substantially lower, in umbilical cord compared with maternal plasma. Similar transplacental concentration gradients for HSA [23–25] and AAG [26–28] have been shown to contribute to the differential protein binding of drugs in the maternal-fetal unit.

There was no apparent relationship between the indinavir f_u and HSA concentrations in the matched maternal and fetal plasma samples. There was, however, a significant relationship between the indinavir f_u and AAG concentrations; this improved marginally when HSA concentrations were also considered. The influence of the transplacental gradients of HSA and AAG on the saquinavir f_u was investigated. The significant relationship between the $f_{uC:M}$ and $AAG_{C:M}$ improved slightly when the combined effects of $HSA_{C:M}$ and $AAG_{C:M}$ were examined. Thus, HSA was less important than AAG for indinavir and saquinavir binding in maternal and umbilical cord plasma, which is consistent with the binding to isolated protein fractions in this study and previous findings [29, 30].

The present results indicate the AAG concentration differential contributes significantly to the differential binding of indinavir and saquinavir in matched samples. This is in agreement with other basic drugs including pethidine [26], lignocaine [27] and disopyramide [28], which were less bound in umbilical cord than maternal plasma. In the current study, the combined effects of AAG and HSA did not fully explain the variability in the f_u of the two PIs. There is a transplacental lipoprotein concentration gradient [26], and amprenavir is marginally bound to these proteins [20], although, the extent of indinavir and saquinavir binding to lipoproteins has not been determined. Endogenous ligands (e.g. free fatty acids) may influence drug binding to HSA through competitive or allosteric interactions [4]. However, the results from the present and previous studies [29, 30] indicate HSA accounts for a small proportion of the total plasma binding of PIs.

The placental transfer of PIs has been studied previously using matched maternal and umbilical cord plasma collected at delivery [12–14]. The C : M total concentration ratios for the PIs were below unity (<0.30), and this has been interpreted to be the result of low placental transfer [12–14]. The present study supports the hypothesis that the low ratios for indinavir and saquinavir are partly due to lower umbilical cord than maternal plasma binding. Factors other than differential binding may also contribute to the low C : M total concentration ratios for the PIs. In the case of indinavir, assuming a C : M total concentration ratio of 0.30 [12–14], and using the mean f_u determined in the present study, the C : M unbound concentration ratio is 0.44. This two- to three-fold difference in the calculated unbound concentrations in matched samples is consistent with data from the isolated perfused human placenta, where the maternal to fetal transfer clearance of indinavir was three-fold lower than the fetal to maternal transfer clearance [19]. Thus, differential transfer clearance also contributes to the low C : M total concentration ratio for indinavir. The PIs are substrates for the efflux transporter, P-glycoprotein (P-gp) [31, 32], and studies reveal P-gp restricts maternal to fetal transfer of xenobiotics [11, 33]. Therefore, P-gp mediated efflux is likely to also contribute to the low C : M total concentration ratios observed for the PIs [12–14].

Administration of PIs during pregnancy has significantly decreased MTCT of HIV [8]. Anti-retroviral prophylaxis in the fetus is recognized to protect further against potential HIV infection [9], and therefore effective fetal PI concentrations are desired. As discussed above, following administration of PI to the mother the unbound concentration in fetal plasma would be expected to be approximately half that in maternal plasma. It may be important to recognize this difference in unbound concentrations between the fetus and the mother in order to achieve fetal PI concentrations greater than the IC_{50} for HIV [34].

In conclusion, the f_u of indinavir and saquinavir was significantly higher in umbilical cord than matched

maternal plasma, indicating that differential binding was a determinant of the low C : M total concentration ratios reported previously [12–14]. Furthermore, the transplacental AAG concentration gradient was shown to contribute to the binding differential of both PIs. Knowledge of the f_u of PIs in umbilical cord and maternal plasma will be useful when evaluating total concentrations, and predicting whether the respective unbound concentrations will be effective against HIV infection.

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