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PHARMACOKINETICS AND DISPOSITION

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Neither dapsone hydroxylation nor cortisol 6β-hydroxylation detects the inhibition of CYP3A4 by HIV-1 protease inhibitors

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Abstract *Objective*: This study examined the use of dapsone *N*-hydroxylation and cortisol 6β-hydroxylation, well accepted in vivo probes of cytochrome P4503A4 (CYP3A4) activity, on defining the effect of three HIV protease inhibitors on CYP3A4 activity.
Methods: Subjects from University Hospital Infectious Disease Clinic about to be started on indinavir, and subjects from two clinical studies, one using ritonavir and the other using amprenavir, were recruited to participate in the study. Subjects received dapsone 100 mg p.o. followed by an 8-h urine collection for dapsone, dapsone *N*-hydroxylamine, cortisol, and 6β-hydroxycortisol concentrations before HIV protease inhibitor administration, and 3–4 weeks into receiving HIV protease inhibitors.
Results: None of the HIV protease inhibitors demonstrated statistically significant alterations in dapsone recovery ratio and 6β-hydroxycortisol/cortisol ratio. In fact, with ritonavir, the dapsone recovery ratio tended to increase rather than decrease, suggesting induction. These negative results were found despite evidence of CYP3A4 inhibition by these three HIV protease inhibitors via published drug-drug interactions with drugs that are substrates for CYP3A4.
Conclusions: These in vivo assays used to probe CYP3A4 activity are suboptimal, most likely because of the presence of extrahepatic sites of metabolism for both dapsone and cortisol, and multiple CYP isozymes involved in dapsone *N*-hydroxylation.

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Introduction

The past several years have seen significant advances in the pharmacologic approach to treatment of HIV infection. The institution of triple drug regimens combining an HIV protease inhibitor with two reverse transcriptase inhibitors has resulted in dramatic reductions in viral load, increases in CD_4 counts, and improved clinical outcome in HIV infected people [1–7]. By cleaving the large polypeptide precursor containing viral capsid proteins and reverse transcriptase, the HIV protease is essential for the formation of mature infectious virions [8, 9]. HIV protease inhibitors are peptidomimetic large lipophilic compounds with numerous aromatic side chains. Because of limited water solubility, these organic bases are extensively bound to α_1 acid glycoproteins. In addition, these drugs have to undergo extensive hepatic oxidation for final elimination through the bile and kidney [10, 11]. These large lipophilic molecules are not only attracted to the cytochrome P450 3A4 for metabolism, but also competitively inhibit the activity of this enzyme. To date, comparative data on the inhibitory effects of various protease inhibitors on cytochrome P450 3A4 (CYP 3A4) metabolism is only defined in vitro [12, 13]. Ritonavir is known to be a potent inhibitor of the CYP 3A4 [14–16]. Although less information is available concerning indinavir and nelfinavir, both appear to have a moderate inhibitory effect on CYP 3A activity [17, 18]. In addition, recently released data on amprenavir also indicates a moderate inhibitory effect on CYP 3A4 as measured by the erythromycin breath test [19].

Drug interactions with HIV protease inhibitors at CYP3A4 are a significant issue in the treatment of HIV infected patients because of the numerous drugs utilized in the treatment and prophylaxis of opportunistic infections (itraconazole, fluconazole, ketoconazole, clarithromycin, rifabutin, rifampin). In addition, other drugs

commonly used in the treatment of concomitant non-infectious diseases in HIV-infected patients (i.e. midazolam, terfenadine, calcium channel antagonists, tamoxifen) utilize CYP3A4 for metabolism.

Several methods have been proposed for measuring CYP 3A4 activity in vivo. Dapsone, a diphenylsulfone, is metabolized by both oxidative and conjugative processes, forming dapsone hydroxylamine (DNH) and *N*-acetylated metabolites [20, 21]. The hydroxylamine is the best characterized metabolite of dapsone, and greater than 90% of dapsone hydroxylation in human liver microsomes is reported to be mediated by CYP 3A4 [22]. In healthy volunteers, dapsone *N*-hydroxylation exhibits a unimodal distribution [23]. The overall efficiency of this metabolic pathway can be measured in vivo by the fractional metabolic clearance to DNH [24], and the dapsone and DNH recovery ratio provides a phenotypic measure of this fractional clearance [25].

6β-Hydroxycortisol (6β-OHF) is a polar metabolite of cortisol formed in the endoplasmic reticulum of the hepatocyte during phase I biotransformation reactions. It is the major unconjugated urinary product of cortisol, and constitutes approximately 1% of the daily cortisol secretion [26]. Urinary 6β-hydroxycortisol excretion has been proposed to be a specific marker of CYP3A4 induction [27]. There appears to be a significant positive correlation between 6β-hydroxycortisol/free cortisol urinary ratio (6β-OHF/free cortisol), and both liver microsomal cortisol 6β-hydroxylase activity and CYP3A liver content [27].

The purpose of our present study was to evaluate and compare the effect of three HIV protease inhibitors with known inhibitory activity on CYP 3A4, in clinical use at our institution, on CYP 3A4 activity. To accomplish this goal, we utilized dapsone *N*-hydroxylation and cortisol 6β-hydroxylation as in vivo probes for CYP 3A activity. Both of these probes have been used previously to evaluate CYP 3A4 activity in vivo. Our data are descriptive in nature, therefore data on all the study subjects are presented.

Materials and methods

Subjects

HIV infected patients attending the University Hospital Infectious Disease Clinic who were starting therapy with an HIV protease inhibitor, as well as participants of two ongoing clinical protocols involving protease inhibitors (ACTG 315 utilizing ritonavir and a phase I trial evaluating the safety, pharmacokinetics and antiviral activity of amprenavir after multiple dosing in patients with HIV infection), were recruited for study participation. Patients were excluded if there was a history of intolerance to dapsone; if they were currently on dapsone for any reason; if they had undergone any change in drug therapy related to HIV or opportunistic infections within 2 weeks prior to the initiation of the study or if they were unable to give informed consent. The protocol was approved by the Colorado Multiple Institution Review Board and all patients gave informed consent prior to entry into the study. All experiments were conducted in accordance with the ethical guidelines specified by the Colorado Multiple Institutional Review Board.

Protocol

The study was conducted in two parts, prior to and 3–4 weeks after the initiation of protease inhibitor therapy. The night before the study, subjects were asked to fast overnight except for water, but the subjects were instructed to take their usual doses of antiretroviral medications on the day of the study. On arrival at the clinic, patients were asked to empty their bladder and dapsone 100 mg was given orally with 12 oz Coca Cola. The subjects were asked to fast for an additional 3 h and to collect urine for 8 h after dapsone administration for the measurement of dapsone, DNH, 6β-hydroxycortisol and cortisol. To prevent the degradation of the DNH, the urine was collected in containers with 5 g ascorbic acid and stored at 4 °C until aliquotted. After measurement of total volume, a 100 ml aliquot was stored at −20 °C until analysis. Thorough drug histories were obtained at both time points to ensure that the HIV protease inhibitor was the only drug added during the time period of the study.

Assays

The concentration of dapsone and its *N*-hydroxylamine metabolite in the urine were determined by reversed-phase HPLC analysis according to a published procedure [24]. Dapsone *N*-hydroxylamine standard was generously provided to us by Dr. J. Uetrecht, University of Toronto, Toronto, Canada. 3-Aminophenylsulfone was used as internal standard for both dapsone and DNH assays. In samples from patients taking trimethoprim-sulfamethoxazole, an interfering peak with retention time similar to that of the internal standard in the DNH assay was found. For these samples, the HPLC was repeated using a standard curve formulated from urine containing known amounts of DNH.

The 0 to 8-h dapsone recovery ratio (RR_{DDS}) was calculated from the following equation:

$$\frac{HDA_{\mu}}{HDA_{\mu} + DDS_{\mu}}$$

where HDA_{μ} is the urinary recovery of the DNH, and DDS_{μ} is the urinary dapsone recovery in 8 h.

Urinary free cortisol levels were measured in the UCHSC Endocrinology laboratory using solid phase radioimmunoassay methodology after an extraction with methylene chloride [28, 29]. Urinary 6β-hydroxycortisol was measured by a commercially available ELISA assay (Stabiligen, Nancy, France) based on the method of Zhiri et al. [30]. The ratio 6β-hydroxycortisol to free cortisol (6β-OHF/free cortisol) in the urine was then calculated for each sample.

Statistical analysis

Comparisons of the dapsone recovery ratios and 6β-hydroxycortisol to free cortisol ratios were calculated for each patient before and 3–4 weeks after the initiation of HIV protease inhibitor therapy. The change in the ratios for each subject was analysed as a group and for each of the HIV protease inhibitors. A paired Student's *t*-test was used to examine the difference in the ratios before and during HIV protease inhibitor therapy. The small sample size made statistical significance difficult to establish with the individual protease inhibitors.

Results

Patient characteristics

Twenty-two subjects enrolled in the study but four subjects did not return their urine collection during the

study. Characteristics of the 18 patients who completed both parts of the study were as follows. Sixteen (89%) were male; two (11%) were female. The mean age was 40.6 ± 9.4 years. The mean CD_4 count at study entry was 315/mm^3 ± 206 with a mean viral load at study entry of 4.21 ± 1.10 $\log_{10}$ RNA copies/ml. Review of medication history over the course of the study revealed that in 13 patients the protease inhibitor was the only medication added over the course of the study. In five patients ZDV, 3TC or D4T was added or substituted along with the HIV protease inhibitor. (None of these drugs is a known substrate or inhibitor of CYP 3A4.) Review of viral load data at the time of second urine collection showed a 1.3 $\log_{10}$ decline in viral load compared to levels at the start of the study ($P = 0.000012$; $n = 17$). Sixteen of the 18 patients had at least 0.5 log reduction in plasma viral load. With the patients on indinavir, six out of nine had plasma viral load <400 copies/ml. In addition, data on serum bilirubin concentration before and after initiation of indinavir was available for six of nine patients. This demonstrated that serum bilirubin concentrations increased significantly after the initiation of the drug ($P = 0.006$; $n = 6$). Both the viral load data and the bilirubin increase were strong, albeit indirect, evidence of adherence with HIV protease inhibitor therapy.

Table 1 shows the results of the dapsone recovery ratio for all patients pre- and post-initiation of protease inhibitor. In five patients, who were also receiving trimethoprim-sulfamethoxazole for *Pneumocystis carinii* pneumonia prophylaxis, there was an interfering peak in the urine that overlapped with that of the internal standard. This peak was subsequently identified as due to N_4-acetyl sulfamethoxazole, based on HPLC retention time. These samples were repeated using external standards as described in materials and methods. No significant decrease in the dapsone recovery ratio was seen with any of the HIV protease inhibitors. For all drugs but indinavir, the ratio increased, although the results did not reach statistical significance; however, the sample size was very small. The data with the amprenavir administration is difficult to interpret because patient 17 did not generate any measurable DNH in the urine prior to HIV protease inhibitor administration. This patient was not on any medications at the time to explain this observation. In addition, the only medication added during the second phase of the study was amprenavir. Nonetheless, when data for all drugs were analyzed together, there was no difference between dapsone recovery ratios before and after initiation of protease inhibitors ($P = 0.1164$).

Similar results were seen for the urinary 6β-OHF/free cortisol ratio as shown in Table 2. Although there was variability in the results, none of the HIV protease inhibitors showed a consistent change in the urinary ratio when analyzed for individual HIV protease inhibitors or when the data were combined for all subjects ($P = 0.2326$).

Discussion

The initial purpose of this study was to identify an in vivo probe that would provide a means of evaluating and comparing the activity of CYP 3A4 during treatment with HIV protease inhibitors. An accurate, minimally invasive in vivo probe for CYP 3A4 would be important to establish, so that the extent of drug-drug interactions could be prospectively identified and toxicity therefore averted. This study failed to show any consistent effect of each of three protease inhibitors (ritonavir, indinavir, amprenavir) on CYP 3A4 activity as determined by two established in vivo probes, dapsone *N*-hydroxylation and cortisol β-hydroxylation. These results are unexpected, especially for ritonavir, since that drug appears to be one of the most potent inhibitors of CYP 3A4, as demonstrated by the high level of inhibition of rifabutin and desacetyl rifabutin metabolism [31] as well as complete inhibition of clarithromycin 14-hydroxylation [32]. In addition, ritonavir is also a very potent inhibitor of saquinavir (a well established substrate of CYP 3A4) metabolism, increasing saquinavir's AUC 20–50 fold [16, 33]. In liver microsomes, ritonavir's inhibition of CYP 3A4 occurs at a very low K_i [34]. Indinavir has also been demonstrated to be an inhibitor of CYP 3A4 in both liver microsomes as well as in in vivo drug interaction studies with substrates of CYP 3A4 [17, 34–36]. A recently published abstract indicates that amprenavir also has a significant inhibitory effect on CYP 3A4 as evaluated by the erythromycin breath test [19]. Significant decreases in both dapsone *N*-hydroxylation (as measured by the dapsone recovery ratio) and cortisol β-hydroxylation (as measured by the 6β-hydroxycortisol to free cortisol ratio) were expected at least in those patients taking ritonavir if these probes have specificity towards CYP 3A4. Smaller changes in the ratios were predicted with indinavir and amprenavir. Regardless of whether all patients were analyzed together or grouped according to the HIV protease inhibitor, no significant differences in the ratios were observed upon initiating treatment with the HIV protease inhibitors. Since the sample size was small, the data from all subjects are presented in the tables. As can be clearly seen, not one subject on ritonavir had a decrease in the dapsone recovery ratio. If anything, ritonavir demonstrated stimulatory activity on dapsone hydroxylamine formation. In the study, each subject served as his own control, reducing intersubject variability, and the only drugs added between the two periods that could affect CYP 3A4 activity were the HIV protease inhibitors. Thus, given the in vivo and in vitro data on ritonavir's inhibitory potential on the CYP 3A4 isozyme, the specificity of the CYP 3A4 probes used in this study must be addressed. Although we did not have a direct measure of adherence with HIV protease inhibitors like plasma concentration of the protease inhibitors at the time of urine collection, the plasma viral load data for the three HIV protease inhibitors and

Table 1 Urinary analysis of dapsone recovery ratio

Protease inhibitor	Patient no.	Dapsone recovery ratio		
		Pre-PI		Post-PI
Indinavir	3	0.612		0.725
	4	0.618		0.544
	6	0.705		0.700
	7	0.799[a]		0.891[a]
	9	0.675[a]		0.930[a]
	10	0.814		0.863
	11	0.583[a]		0.608[a]
	12	0.681[a]		0.360[a]
	16	0.533		0.115
Mean RR-DDS ± SD		0.667 ± 0.091		0.6373 ± 0.267
P value			$P = 0.3464$	
Ritonavir	5	0.787[a]		0.810[a]
	8	0.526		0.792
	13	0.545		0.846
	14	0.716		0.781
	18	0.887		0.879
Mean RR-DDS ± SD		0.6922 ± 0.156		0.8216 ± 0.040
P value			$P = 0.0571$	
Amprenavir	1	0.785		0.640
	2	0.780		0.956
	15	0.724		0.860
	17	0.000		0.979
Mean RR-DDS ± SD		0.5723 ± 0.383		0.8588 ± 0.155
P value			$P = 0.1605$	
All drugs				0.7377 ± 0.222
Mean RR-DDS ± SD		0.6528 ± 0.194		
P-value			$P = 0.1164$	

[a] Chromatogram for hydroxylamine contained interfering peaks. Assay repeated with external standard as described in text

Table 2 Urinary analysis of 6β-OHF/free cortisol

Protease inhibitor	Patient no.	6β-OHF/free cortisol (ng/ml)		
		Pre-PI		Post-PI
Indinavir	3	3.285		2.137
	4	2.177		2.225
	6	3.985		2.805
	7	2.429		1.502
	9	2.358		3.077
	10	3.397		2.949
	11	15.939		5.395
	12	2.539		2.643
	16	5.803		6.902
Mean 6β-OHF/free cortisol ± SD		4.72 ± 4.36		3.29 ± 1.73
P value			$P = 0.1298$	
Ritonavir	5	7.134		1.865
	8	3.674		1.580
	13	1.237		2.130
	14	3.646		4.389
	18	3.808		2.828
Mean 6β-OHF/free cortisol ± SD		3.94 ± 2.10		2.56 ± 1.12
P value			$P = 0.1406$	
Amprenavir	1	1.970		1.875
	2	3.891		4.292
	15	3.518		7.605
	17	4.690		9.540
Mean 6β-OHF/free cortisol ± SD		3.52 ± 1.14		5.83 ± 3.41
P value			$P = 0.0820$	
All drugs				
Mean 6β-OHF/free cortisol ± SD		4.23 ± 3.24		3.65 ± 2.30
P-value			$P = 0.2326$	

serum bilirubin concentration effect for indinavir suggest good adherence. Several studies have failed to show correlation between the proposed 'gold standard' probe of in vivo CYP 3A activity, the erythromycin breath test (ERMBT) and either RR_{DDS} or 6β-OHF/free cortisol ratio [37–39], thus questioning the use of these probes to evaluate drug metabolism via CYP3A4.

Though in vitro assays may show evidence of a specific enzymatic pathway of drug metabolism in liver microsomes, this does not exclude other pathways of metabolism [40]. CYP 3A enzymes are found extrahepatically especially in the small bowel [41–43]; thus, an orally administered probe may undergo significant metabolism in the bowel. If inducers or inhibitors of hepatic CYP 3A4 do not have comparable effects on intestinal enzyme activity, and an oral probe measures the sum of intestinal and hepatic enzyme activity, while ERMBT only measures hepatic CYP 3A4 activity, then oral and intravenous probes may not always correlate when evaluating CYP 3A4 activity.

Additionally, there may be alternate pathways of metabolism in vivo that are not apparent with in vitro studies. Recent studies of dapsone metabolism indicate that multiple CYPs play a significant role in *N*-hydroxylation. In one study, no correlation was seen between dapsone *N*-hydroxylation and CYP 3A levels in a panel of six human livers [44]. Their data suggested that multiple CYPs including CYP 2C9 and CYP 3A, contribute to dapsone hydroxylation but still do not account for all metabolism, implicating involvement of other enzymes. Inhibition of dapsone *N*-hydroxylation by high concentrations of INH suggests that CYP 2E1 may play a role. Mitra et al. showed that CYP 3A4 and CYP 2E1 accounted largely for dapsone *N*-hydroxylation [45]. CYP 3A4 was a high capacity low affinity isozyme (K_m = 100 μM), while the CYP 2E1 had low capacity and high affinity for dapsone *N*-hydroxylation with a K_m of 5 μM. There was also evidence that CYP 2C isoforms contribute to dapsone metabolism at low substrate concentrations. In the original studies implicating CYP 3A4 as the predominant route of dapsone *N*-hydroxylation, dapsone concentrations of 100 μmol l were used in vitro [22]. However, peak steady state plasma concentrations of dapsone at the usual dose are in the range of 8–13 μmol l [46]; thus at dapsone concentrations achieved in vivo, the enzyme accounting for the major part of dapsone *N*-hydroxylation may well be CYP 2E1, with a lesser contribution by CYP 2C, and CYP 3A4 not at all. Our data showing some stimulatory effect of ritonavir on the dapsone hydroxylamine formation are also consistent with the concept that CYP isozymes other than 3A4 are involved in dapsone hydroxylamine formation, since ritonavir does stimulate a host of microsomal enzymes including CYP2E1, while inhibiting CYP 3A4 [47–49].

Similarly, it is unclear if CYP 3A4 is the sole metabolic pathway for the 6β-hydroxylation of cortisol in vivo. Although rifampin increases the urinary ratio of hydroxycortisol to free cortisol, the increase does not correlate with that seen in the ERMBT in patients receiving both tests [27, 50]. Troleandomycin, a specific CYP 3A4 inhibitor, had no consistent effects on the ratio of 6β-OHF/cortisol in a small number of patients [50], and studies in an anhepatic patient undergoing liver transplantation found that the ratio fell by only 50%, suggesting an extrahepatic site of cortisol hydroxylation [50].

In conclusion, our study failed to show significant inhibition of dapsone *N*-hydroxylation and cortisol β-hydroxylation by three HIV protease inhibitors as measured by the urinary dapsone recovery ratio or 6β-hydroxycortisol to free cortisol ratio. Since the evidence suggests that the three protease inhibitors used in this study are inhibitors of CYP 3A4 based on both in vitro work, and in vivo drug interaction studies, neither dapsone hydroxylamine formation, nor cortisol hydroxylation can be used as in vivo probes to evaluate CYP 3A4 activity. Since CYP 3A4 is central to the metabolism of many drugs in clinical use today, further studies are needed to develop a non-invasive probe that is both specific and sensitive to CYP 3A4 activity to allow prediction of drug toxicity and critical interactions with HIV protease inhibitors. A recent study examining the effect of delavirdine on CYP 3A4 activity in vivo utilizing ERMBT as a probe, did demonstrate a significant inhibition consistent with previous drug interaction studies with delavirdine [51]. However, as previously described, intravenously administered ^{14}C-erythromycin for the ERMBT has shortcomings in assessing the contribution of small intestinal CYP 3A4 activity in drug metabolism.

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