Hepatic Clearance Prediction of Nine Human Immunodeficiency Virus Protease Inhibitors in Rat

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ABSTRACT: This study aimed to determine the rate-limiting step in the overall hepatic clearance of the marketed human immunodeficiency virus (HIV) protease inhibitors (PI) in rats by predicting the experimentally determined hepatic *in vivo* clearance of these drugs based on *in vitro* clearance values for uptake and/or metabolism. *In vitro* uptake and metabolic clearance values were determined in suspended rat hepatocytes and rat liver microsomes, respectively. *In vivo* hepatic clearance was determined after intravenous bolus administration in rats. Excellent *in vitro–in vivo* correlation (IVIVC; $R^2 = 0.80$) was observed when metabolic intrinsic Cl values were used, which were determined *in vitro* at a single concentration corresponding to the blood concentration observed in rats *in vivo* at the mean residence time. On the contrary, poor IVIVC was observed when *in vitro* metabolic Cl values based on full Michaelis–Menten profiles were used. In addition, the use of uptake Cl values or a combination of both uptake and metabolic clearance data led to poor predictions of *in vivo* clearance. Although our findings indicate a key role for metabolism in the hepatic clearance of several HIV PI in rats, subsequent simulations revealed that inhibition of hepatic uptake can lead to altered hepatic clearance for several of these drugs. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: *in vitro*/*in vivo* correlations (IVIVC); hepatic clearance; hepatic metabolism; hepatic transport; transporters; drug transport; drug metabolizing enzymes; HIV/AIDS

INTRODUCTION

Prediction of *in vivo* clearance values of drug candidates based on *in vitro* data [or *in vitro–in vivo* extrapolation (IVIVE) for drug clearance] has become an essential activity during the preclinical phases of contemporary drug development programs. As reported in the companion paper by De Bruyn et al., clearance predictions with *in vitro* models derived from human liver tissue allow estimating a safe first-in-human dose to initiate clinical trials.[1] *In vivo* drug clearance prediction in rats on the contrary, as applied in the present paper, is useful from different perspectives. First of all, accurate knowledge of the pharmacokinetic behavior of drug candidates in preclinical species (as compared with man) supports optimal design of toxicity studies with sensible use of animals. Second, as *in vivo* pharmacokinetic profiles can also be obtained in animals via various routes of administration (including the intravenous route) and under standardized conditions for a series of compounds (the present study), *in vitro–in vivo* comparisons become highly relevant and may provide opportunities to fine-tune IVIVE algorithms for drug clearance.

For compounds undergoing primarily hepatic elimination, *in vivo* clearance predictions are based on the extrapolation of *in vitro* data covering all hepatic drug elimination processes. These processes may include hepatic sinusoidal uptake, metabolism, and/or biliary excretion, resulting in an entangled interplay that complicates the extrapolation to the *in vivo* situation.[2,3] Additionally, drug binding and sequestration equilibria will affect the overall impact of these processes.

Both microsomes and suspended hepatocytes have successfully been used for predicting the metabolic clearance of many compounds.[4–8] However, these model systems fail to quantitatively predict the *in vivo* clearance of drugs for which active uptake transport forms the rate-limiting step in the overall hepatic clearance.[9] For these compounds, better *in vitro–in vivo* correlations are observed when *in vitro* uptake clearance values are measured and extrapolated to *in vivo* parameters.[10] Thus, with the recognition that transport proteins can significantly influence hepatic clearance (even for compounds that largely depend on metabolic enzymes for their clearance), typical pharmacokinetic models used for IVIVE of hepatic clearance had to be modified.[11] This has led to the development of IVIVE models implementing multiple hepatic disposition processes measured with various *in vitro* methods.[12,13] Consequently, such models can be used to gain information about the interplay between various hepatic elimination processes and the rate-limiting step in the overall hepatic clearance of drugs.

For the purpose of the present study on drug clearance prediction in rat, all marketed human immunodeficiency virus (HIV) protease inhibitors (PIs) were included as model compounds. As the predominant role of drug metabolizing enzymes in hepatobiliary disposition of HIV PIs is generally considered as obvious, the potential role of transporters in disposition of these antiretroviral drugs has remained somewhat controversial. Nevertheless, interactions of HIV PIs with transporters have been described. HIV PIs are potent inhibitors of the sodium taurocholate-cotransporting polypeptide[14] and several members of the organic anion transporting polypeptide family[15] in rat hepatocytes. In addition, HIV PIs are

Abbreviations used: FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; HIV, human immunodeficiency virus; IVIVE, *in vitro–in vivo* extrapolation; PI, protease inhibitor

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substrates of several efflux transporters.[16,17] Extensive metabolism by cytochrome P450 enzymes has been explored in rat liver microsomes.[18,19] Thus, a complex interplay between multiple processes defines the hepatic disposition and clearance of HIV PIs in rats. By directly comparing the rate of uptake into rat hepatocytes and the rate of metabolism in rat hepatocytes and rat liver microsomes, Parker and Houston[20] indicated that at low-substrate concentrations, hepatic uptake is the rate-limiting step in the overall hepatic clearance of nelfinavir, saquinavir, and ritonavir in rats. In addition, they showed that this uptake process is extremely rapid and therefore suggested that the hepatic clearance of these three HIV PIs is still blood-flow limited in the *in vivo* situation.

The aim of this study was to accurately delineate the respective roles of enzymes and transporters in the overall hepatic elimination of all commercially available HIV PIs in rats. Therefore, *in vitro* uptake data in suspended rat hepatocytes and metabolic data in rat liver microsomes were extrapolated to the hepatic *in vivo* clearance for all HIV PIs and compared with experimentally determined *in vivo* blood clearance in rats. The same approach was applied for this series of compounds in man (see companion paper De Bruyn et al.)[1], providing unique data sets for detailed cross-species comparison of pharmacokinetic behavior of these compounds. At the same time, possible species-specific aspects of clearance prediction algorithms (IVIVE) may be identified.

MATERIALS AND METHODS

Chemicals

Ritonavir, indinavir sulfate, saquinavir mesylate, and nelfinavir mesylate were obtained from Hetero Drugs Limited (Hyderabad, India). Atazanavir was provided by Bristol-Myers Squibb (New Brunswick, New Jersey) and amprenavir, darunavir, lopinavir, and tipranavir were obtained through the NIH AIDS Reagent Program. William's E medium, Hanks' balanced salt solution, L-glutamine, fetal bovine serum (FBS), penicillin–streptomycin mixture (contains 10,000 IU potassium penicillin and 10,000 μg streptomycin sulfate per milliliter in 0.85% saline) and trypsin EDTA were purchased from Lonza SPRL (Verviers, Belgium). HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] was purchased from MP Biochemical (Illkirch, France). Mineral oil was purchased from Acros Organics (Thermo Scientific, Geel, Belgium). Collagenase (type IV), cyclosporin A, NADPH, glucose-6-phosphate, $MgCl_2$, and silicon oil were obtained from Sigma–Aldrich (Schnelldorf, Germany).

Animals

All rats were housed according to the Belgian and European laws, guidelines, and policies for animal experiments, housing, and care in the central animal facilities of the university. The Institutional Ethical Committee for Animal Experimentation granted approval for this project.

Isolation of Rat Hepatocytes

Hepatocytes were isolated from male Wistar (170–200 g) rats using a two-step collagenase perfusion, as described previously.[21] After isolation, cells were centrifuged (50*g*) for 3 min at 4°C and the pellet was resuspended in William's E medium supplemented with 5% FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Hepatocytes were counted using a hemocytometer and cell viability (>90%) was determined using Trypan blue exclusion. Freshly-isolated rat hepatocytes were resuspended in Krebs–Henseleit buffer (NaCl 130 mM, KCl 5.17 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 1.2 mM, HEPES 12.5 mM, glucose 11.1 mM, Na-pyruvate 5 mM; pH 7.4) and kept on ice until the start of the experiment.

Uptake Experiments with Suspended Hepatocytes

All experiments with suspended hepatocytes were conducted as described previously.[15] Briefly, 500 μL of a double-concentrated cell suspension (2 million cells/mL) was preincubated for 10 min at 37°C. Subsequently, 500 μL of a double-concentrated substrate solution (2 μM) were added to initiate the incubation at 37°C (or 4°C to determine the nonsaturable uptake component). After an incubation period of 30 s, triplicate 200 μL aliquots of the suspension were immediately transferred to 1.5 mL ice-cold microcentrifuge tubes, containing 700 μL of an oil layer (silicone/mineral oil mixture; density, 1.015) above 300 μL of 8% NaCl solution. Subsequently, the tubes were centrifuged for 2 min at 16,000 *g* using a tabletop centrifuge (Eppendorf 5415 C, Hamburg Germany). After freezing microcentrifuge tubes in dry ice, the conical tube ends were cut and the contents diluted in 300 μL of a 70%/30% methanol–water mixture. Samples were stored at −20°C until analysis.

Preparation of Rat Liver Microsomes

Briefly, three male Wistar rats (197–200 g) were fasted for 24 h prior to the preparation of rat liver microsomes. After rats were anesthetized with an intraperitoneal injection of 120 mg/kg of ketamine and 24 mg/kg xylazine, the livers were perfused with ice-cold oxygenated homogenization buffer [5 mM 3-(N-morpholino)-propanesulfonic acid MOPS, 1 mM EDTA, 250 mM sucrose, pH 7.4] to remove all blood. Subsequently, livers were excised, minced with surgical scissors, and added to ice-cold homogenization buffer (3 mL/g tissue). The tissue mixture was further homogenized in a glass/Teflon© potter at 4°C. The pooled tissue solution was sampled for CYP content determination and centrifuged (4,000 *g*) for 10 min at 4°C. After suspending the resulting pellet with homogenization buffer and repeating the centrifugation step, the supernatants were combined and transferred to polycarbonate ultracentrifuge tubes (Fisher Scientific, Landsmeer, The Netherlands). This solution was subjected to another centrifugation step (15,000*g*) for 20 min at 4°C, after which the pellet was discarded and the supernatant was ultracentrifuged (150,000*g*) for 70 min at 4°C. The resulting microsomal pellet was washed with homogenization buffer and ultracentrifugation was repeated (150,000*g*) for 35 min at 4°C. The microsomal pellet was homogenized in microsomal storage buffer (5 mM MOPS, 1 mM EDTA, 20%, w/v, glycerol) with a glass/Teflon© potter homogenizer (800 rpm). A sample of the microsomes was taken for protein content determination and aliquots (500 μL) were stored at −80°C.

Determination of Metabolic Clearance Using Rat Liver Microsomes

Incubations were performed in 48-well plates in a Thermomixer (Eppendorf AG, Hamburg, Germany) set at 37°C and 350 rpm in a volume of 400 μL. Pooled rat liver microsomes were diluted in phosphate buffer (100 mM, pH 7.4) containing glucose-6-phosphate (3 mM) and $MgCl_2$ (3 mM). Parent depletion by rat