PHARMACOKINETICS, PHARMACODYNAMICS AND DRUG METABOLISM

N-(3,4-Dimethoxyphenethyl)-4-(6,7-Dimethoxy-3,4-Dihydroisoquinolin-2[1H]-yl)-6,7-Dimethoxyquinazolin-2-Amine (CP-100,356) as a “Chemical Knock-out Equivalent” to Assess the Impact of Efflux Transporters on Oral Drug Absorption in the Rat

AMIT S. KALGUTKAR,1 KOSEA S. FREDERICK,1 JONATHAN CHUPKA,1 BO FENG,1 SARAH KEMPSHALL,2 ROCHELLE J. MIRELES,1 KATHERINE S. FENNER,2 MATTHEW D. TROUTMAN1

1Pharmacokinetics, Dynamics and Metabolism Department, Pfizer Global Research and Development, Eastern Point Road, Groton, Connecticut 06340
2Pharmacokinetics, Dynamics and Metabolism Department, Pfizer Global Research and Development, Ramsgate Road, Sandwich, Kent CT139NJ, UK

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ABSTRACT: The utility of the diaminoquinazoline derivative CP-100,356 as an in vivo probe to selectively assess MDR1/BCRP-mediated drug efflux was examined in the rat. CP-100,356 was devoid of inhibition (IC50 > 50 μM) against major human P450 enzymes including P4503A4. In human MDR1-transfected MDCKII cells, CP-100,356 inhibited acetoxymethyl calcein (calcein-AM) uptake (IC50 = 0.5 ± 0.07 μM) and digoxin transport (IC50 = 1.2 ± 0.1 μM). Inhibition of prazosin transport (IC50 = 1.5 ± 0.3 μM) in human BCRP-transfected MDCKII cells by CP-100,356 confirmed the dual MDR1/BCRP inhibitory properties. CP-100,356 was a weak inhibitor of OATP1B1 (IC50 = 66 ± 1.1 μM) and was devoid of MRP2 inhibition (IC50 > 15 μM). In vivo inhibitory effects of CP-100,356 in rats were examined after coadministration with MDR1 substrate fexofenadine and dual MDR1/BCRP substrate prazosin. Coadministration with increasing doses of CP-100,356 resulted in dramatic increases in systemic exposure of fexofenadine (36- and 80-fold increase in Cmax and AUC at a CP-100,356 dose of 24 mg/kg). Significant differences in prazosin pharmacokinetics were also discernible in CP-100,356-pretreated rats as reflected from a 2.6-fold increase in AUC. Coadministration of CP-100,356 and P4503A substrate midazolam did not result in elevations in systemic exposure of midazolam in the rat. The in vivo methodology should have utility in drug discovery in selective and facile assessment of the role of MDR1 and BCRP efflux transporters in oral absorption of new drug candidates. © 2009 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 98:4914–4927, 2009

Keywords: MDR1; BCRP; CP-100,356; drug absorption; bioavailability; P450; efflux; drug–drug interaction
INTRODUCTION

During initial stages of hit-to-lead phase in drug discovery, most new chemical entities (NCEs) suffer from poor systemic exposure after oral (p.o.) dosing in animals, necessitating a need for identifying issues limiting oral absorption. Besides first pass metabolism, other factors such as aqueous solubility, membrane permeability, and active drug efflux can attenuate oral absorption. With regards to active drug efflux, members of the ATP-binding cassette (ABC) superfamily of efflux transporters can significantly modulate the systemic exposure of NCEs.1–5 These primary active efflux transporters are expressed at the major barriers within the body (e.g., small intestine, blood–brain barrier, placenta, kidney, and liver), where they reduce the uptake or enhance the systemic clearance of drugs. Multidrug resistance protein (human MDR1/rodent Mdr1, P-glycoprotein, ABCB1) is the prototypical ABC transporter first identified through studies on multidrug resistance. Besides its role in multidrug resistance, MDR1 has been shown to reduce the intestinal absorption of drugs like digoxin, tamoxifen, paclitaxel, fexofenadine, imatinib, abacavir, and vinblastine amongst others by transporting these drugs back to the apical surface of the intestinal enterocyte.2–4 Breast cancer resistance protein (human BCRP/rodent Bcrp, ABCG1), which confers drug resistance to a variety of chemotherapeutic agents,5–7 has also been shown to influence the intestinal absorption of the anti-inflammatory agent sulfasalazine and anticancer drugs including topotecan, imatinib, and lapatinib.8–11 The putative role of these efflux transporters in drug disposition has also led to some concern regarding the potential for drug–drug interactions (DDIs), in particular for drugs with a narrow therapeutic index.12–15

Both MDR1 and BCRP exhibit broad substrate specificity making it difficult to predict a priori as to which NCE will be a substrate for these transporters.16,17 In drug discovery, identifying the potential for drug efflux usually relies on the measure of asymmetric permeability in flux ratio from basolateral-to-apical (BA) side/apical-to-basolateral (AB) side in the Caco-2 assay and/or in Madin–Darby canine kidney (MDCK) cell lines transfected with individual efflux transporters. Because in vitro MDR1-MDCK or Caco-2 assays can reveal efflux under prototypic experimental conditions involving drug concentrations in the low μM range, and since, concentrations in vivo can easily reach mM levels resulting in saturation of active efflux transporters in the gut lumen,18 the true impact of active efflux on absorption and elimination remains unanswered until the compound of interest is tested for its pharmacokinetic properties (e.g., oral bioavailability assessments) in rodents. In cases where low oral absorption is discerned, follow-on in vivo mechanistic studies in transport-deficient mice (e.g., Mdr1 and Bcrp knock-out mice) are often used as surrogate markers to assess the impact of efflux on substrate pharmacokinetics and pharmacology in humans.

Since the rat is commonly used for oral pharmacokinetic assessments in drug discovery, we were interested in developing a “chemical knock-out equivalent” rat model for rapidly and selectively assessing drug efflux mediated by MDR1 and/or BCRP as a cause for poor oral absorption, particularly in scenarios where issues dealing with aqueous solubility, intrinsic permeability, and first pass metabolism are not in question. As such, this idea represents an extension of previous work by Strelevitz et al.,18 wherein, the general P450 suicide inactivator 1-aminobenzotriazole was used as a selective in vivo tool in rats to elucidate the role of intestinal and hepatic CYP3A metabolism as a cause of poor oral bioavailability. Commercially available MDR1 inhibitors (e.g., ketoconazole, cyclosporin A, ritonavir, verapamil, quinidine, and mibebradil) are plentiful, however, most of these compounds also display “off-target” activity such as cytochrome P4503A4 inhibition.18–25 Because P4503A is expressed in the small intestine and liver and can limit oral absorption of drugs via first pass metabolism in rodents and humans,24,25 the utility of commercial MDR1 inhibitors as selective in vivo probes of active efflux comes into question. Furthermore, some MDR1 inhibitors (e.g., cyclosporin A) can inhibit influx and/or efflux transporters such as organic anion transporting polypeptide (OATP) and multidrug resistance-associated protein2 (MRP2), an ABC-transporter of the MDR1-related protein subfamily.18,26

Against this backdrop, N-(3,4-Dimethoxyphenethyl)-4-((6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)-6,7-dimethoxyquinazolin-2-amine (CPI-100,356) (Fig. 1), an initial lead from the Pfizer MDR inhibitor program to overcome multidrug resistance,27,28 seemed attractive as a “chemical knock-out equivalent” because of a literature report commenting on its potential selectivity for MDR1 inhibition relative to P4503A4 inhibi-
In order to assess the viability of CP-100,356 as a selective in vivo probe of MDR1/BCRP-mediated drug efflux, our initial objectives were to compare the in vitro MDR1/BCRP inhibitory specificity of CP-100,356 relative to major P450 isoforms, MRP2 and OATP1B1 transporters. MDR1/BCRP inhibitory selectivity versus MRP2 and OATP1B1 was added as a criterion since these proteins modulate intestinal efflux and hepatobiliary uptake/efflux of certain anionic drugs including the MDR1 substrate fexofenadine, which was used as a model “victim” drug in our studies. The next objective was to assess MDR1/BCRP inhibition in rats via DDI studies with prototypic substrates fexofenadine and prazosin (see Fig. 1). Pharmacokinetic interaction(s) of CP-100,356 and midazolam (Fig. 1), a P4503A4 but not MDR1/BCRP substrate, was also examined in rats to further confirm the in vitro results on the lack of P4503A inhibition by CP-100,356. The collective findings are summarized, herein.

**EXPERIMENTAL**

**Materials**

CP-100,356 (chemical and isomeric purity >99%) was synthesized at Pfizer Global Research and Development (Groton, CT). Detailed synthetic procedure for CP-100,356 and related analogs has been published. Solvents used for analysis were of analytical or HPLC grade (Fisher Scientific, Pittsburgh, PA). NADPH, fexofenadine, digoxin, and prazosin were purchased from Sigma–Aldrich Research (St. Louis, MO). Pooled human liver microsomes, probe P450 substrates (phenacetin, diclofenac, S-mephenytoin, bufuralol, testosterone, and midazolam) and inhibitors (ketoconazole, quinidine, sulfaphenazole, and furafylline) were purchased from BD Gentest (Woburn, MA). The selective and reversible CYP2C19 inhibitor (+)-N-3-Benzyl nirvanol was synthesized at Pfizer. Cell culture reagents, transport buffer used for transwell assays (Hanks’ balanced salt solution (HBSS) with 10 mM HEPES and 25 mM D-glucose, 1.25 mM CaCl₂, and 0.5 mM MgCl₂) were purchased from Invitrogen (Carlsbad, CA).

**P450 Inhibition Assays**

The ability of CP-100,356 to function as a competitive inhibitor of the five major CYP enzymes namely P4501A2, 2C9, 2C19, 2D6, and 3A4 was examined in triplicate in human liver microsomes as follows: Phenacetin (50 μM) (a probe substrate for P4501A2), or diclofenac (10 μM) (a probe substrate for P4502C9), or S-mephenytoin (50 μM) (a probe substrate for CYP2C19), or bufuralol (10 μM) (a probe substrate for P4502D6), or testosterone (50 μM) or midazolam (5 μM) (probe substrates for P4503A4) was incubated with human liver microsomes (protein concentration = 0.03 mg/mL), 3.3 mM MgCl₂, and 1.3 mM NADPH in a total volume of 0.6 mL of 100 mM phosphate buffer pH 7.4, in the presence and absence of CP-100,356 at a concentration range of 0–50 μM. Low protein concentration was employed in order to keep nonspecific microsomal binding of CP-100,356 to a minimum. The reactions were initiated by the addition of NADPH, and incubations were conducted in a
shaking water bath at 37 °C for 10 min in the case of diclofenac, bufuralol, testosterone, and midazolam or 30 min in the case of phenacetin and S-mephentoin. All reactions were terminated by the addition of acetonitrile (200 μL) and the samples were centrifuged at 2500g for 5 min prior to analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Analytical procedures for the quantitation of metabolites of probe P450 substrates utilized in the present study have been described in detail elsewhere.\textsuperscript{34} Specific CYP enzyme inhibitors, ketoconazole (P4503A4), quinidine (P4502D6), sulfaphenazole (P4502C9), (+)-N-3-Benzyl nirvanol (P4502C19), and furafylline (P4501A2) were used as positive controls in the competitive inhibition studies.

MDR1, MRP2, and BCRP Inhibition Assays

Wild-type Madin–Darby canine kidney (MDCK) II and MDR1- or MRP2-transfected MDCK cells were grown in minimum essential medium α with supplements at 37 °C, 5% CO₂, and 95% humidity. The cells were harvested using trypsin and plated at a density of $2 \times 10^6$ cells/cm² in Falcon/BD 96-well insert plates (Bedford, MA) with a 1 μm pore polyethylene terephthalate filter. Seeded inserts were then placed into prefilled Falcon/BD feeder trays containing 37 mL of complete growth medium. The plates were incubated at 37 °C with 95% humidity and 5% CO₂ for 4 days and subsequently used for transwell assays. For the inhibition studies with CP-100,356, each cell line (50,000 cells/well) was plated into Costar 3904 black 96-well plates (Perkin-Elmer, Waltham, MA) with 100 μL of medium supplemented with 1% fetal bovine serum and allowed to become confluent overnight. CP-100,356 (0–15 μM) was added to monolayers in 10 μL of HBSS containing 1% DMSO as solvent. Plates were incubated at 37 °C for 30 min. Calcein-AM (Invitrogen) was added in 10 μL of HBSS to yield a final concentration of 0.1 μM. Incubations were conducted in duplicate. For assessment of MDR1-mediated digoxin transport, calcein-AM was replaced with digoxin (5 μM). Plates were incubated for another 60 min. Cells were then washed three times with ice-cold PBS. PBS was added to the cells, and the cells were read with a Victor fluorometer (Perkin-Elmer) at excitation and emission wavelengths of 485 and 535 nm, respectively. MDR-1 and MRP2 inhibition was calculated using the following equation:

$$\text{% Inhibition} = \frac{(\text{the amount of inhibition})_{\text{treated}}}{(\text{the amount of efflux})_{\text{untreated}}} \times 100$$

where “The amount of efflux” was defined as the fluorescence from MDCK cells subtracted by that from the MDR1, MRP2-MDCK cells, and “The amount of inhibition” was (The amount of efflux)\text{untreated}–(The amount of efflux)\text{treated}. The IC\text{50} values were determined by fitting the percentage of inhibition–concentration data into the Hill equation using the ordinary least square method of the LabStats Excel Add-in (Pfizer, Inc., Groton, CT). The Hill slope, the minimum and the maximum % inhibition were not fixed, and the % inhibition data were not weighted. The IC\text{50} was based on the concentration at which the apparent 50% inhibition was achieved (i.e., apparent IC\text{50}). For inhibition of digoxin transport, experiments were conducted in triplicate. Cyclosporin A, a known inhibitor of MDR1 and MRP2, was used as a positive control in this evaluation.

Methodology for evaluating human BCRP-mediated prazosin transport in BCRP-MDCKII cells have been previously published.\textsuperscript{30} Inhibition of prazosin transport (2 μM) by CP-100,356 was tested in triplicate at a minimum of eight concentrations spanning 0–15 μM. Bioanalytical methodology for prazosin quantitation by LC-MS/MS has been described previously.\textsuperscript{30} The IC\text{50} values were determined by fitting the percentage of inhibition–concentration data into the Hill equation using the ordinary least square method of the LabStats Excel Add-in (Pfizer, Inc.) as described above.

OATP1B1 Inhibition Assay

OATP1B1-HEK, human OATP1B1 transporter stably transfected into human embryonic kidney (HEK293) cells, were obtained from Prof. Dietrich Keppler (DKFZ-Heidelberg, Germany). OATP1B1-HEK cells were grown in DMEM, 10% heat inactivated FBS, 1% Gentamicin, and 400 μg/mL Genetin. Wild-type HEK293 cells were grown in DMEM, 10% heat inactivated FBS, 1% Penicillin/Streptomycin, and 100 μg/mL Zeocin. On day one of the study, 3.0 × 10^3 OATP1B1-HEK and HEK wild-type cells were seeded on a 24-well poly-d-lysine coated plate (Biocoat, BD) and incubated (37 °C, 95% humidity, 5% CO₂) for...
after dosing. Blood samples from the various were collected at 0.15, 0.5, 1, 2, 4, 6, 8, and 24 h prior to p.o. administration then serial samples or vehicle. Blood samples (250 μL) were coadministered with CP-100,356. For the DDI studies, Fexofenadine or prazosin or midazolam (10 mg/kg) were all administered orally (po) as suspensions in 0.5% methylcellulose. Fexofenadine was administered in 50% saline/50% PEG400 whereas prazosin and midazolam were administered in 95% glycerol formal containing 5% DMSO. CP-100,356 (6.0, 12, and 24 mg/kg), fexofenadine (10 mg/kg), prazosin (2.5 mg/kg), and midazolam (10 mg/kg) were all administered intravenously (i.v.) via the jugular vein of rats over 30 s at 1.0, 0.5, 1.0 mg/kg, respectively, and serial blood samples were collected before dosing and 0.083, 0.15, 0.5, 1, 2, 4, 6, 8, and 24 h after dosing. Fexofenadine was administered in 50% saline/50% PEG400 whereas prazosin and midazolam were administered in 95% glycerol formal containing 5% DMSO. CP-100,356 (6.0, 12, and 24 mg/kg), fexofenadine (10 mg/kg), prazosin (2.5 mg/kg), and midazolam (10 mg/kg) were all administered orally (po) as suspensions in 0.5% methylcellulose. For the DDI studies, Fexofenadine or prazosin or midazolam were coadministered with CP-100,356 or vehicle. Blood samples (250 μL) were taken prior to p.o. administration then serial samples were collected at 0.15, 0.5, 1, 2, 4, 6, 8, and 24 h after dosing. Blood samples from the various pharmacokinetic studies were centrifuged at 3000 rpm for 10 min at 4°C to generate plasma. All plasma samples were kept frozen until analysis. Aliquots of plasma (100 μL) were transferred to 96-well blocks and acetonitrile (200 μL) containing trazodone as internal standard (50 ng/mL) was added to each well. Samples were prepared for analysis by solid phase extraction using a Waters Oasis® MAX (10 mg) extraction plate (Waters Corp, MA), following the manufacturer's directions. Following extraction, the samples were then analyzed by LC-MS/MS and concentrations of fexofenadine, prazosin, and midazolam in plasma were determined by interpolation from a standard curve.

**Analytical Procedures**

Analyte and internal standard concentrations in plasma were monitored on a Sciex API model 4000 Qtrap triple quadrupole mass spectrometer (PE Sciex, Ontario, ON, Canada). Analytes were chromatographically separated using a Shimadzu LC20AD Prominance (Shimadzu, Inc, Torrance, CA) HPLC system. An autosampler was programmed to inject 10 μL on a Phenomenex Luna 5 μ, C18(2) 50 x 2.0 mm. The aqueous mobile phase was 95% 10 mM ammonium acetate containing 1% (v/v) isopropyl alcohol and 0.1% (v/v) formic acid/5% acetonitrile. The organic mobile phase was 5% 10 mM ammonium acetate containing 1% (v/v) isopropyl alcohol and 0.1% (v/v) formic acid/90% acetonitrile containing 0.1% (v/v) formic acid. Samples were eluted using a constant flow rate of 0.3 mL/min. The LC gradient started at 5% B for 0.5 min, ramped linearly to 95% B over 2.5 min then returned to the initial condition over 1 min. Ionization was conducted in the positive ion mode at the ionspray interface temperature of 500°C, using nitrogen for nebulizing and heating gas. Fexofenadine, prazosin, midazolam, and trazodone were analyzed in the MRM mode using the transitions m/z 502 → 466, 385 → 95, 326 → 291, and 372 → 148, respectively. Calibration curves were prepared by plotting the appropriate peak area ratios against the concentrations of analyte in plasma using 1/C weighting of analyte/internal standard peak height ratios. The concentration of the analytes in the plasma samples was determined by interpolation from the standard curve. The dynamic range of the assay was 5–2500 ng/mL.
Pharmaceutic Data Analysis

Plasma concentration–time profiles were analyzed using the well-established non-compartmental method in WinNonLin v2.1 (Pharsight, Mountain View, CA). Plasma Clearance (CLp) was calculated as the i.v. dose divided by the area under the plasma concentration–time curve from zero to infinity (AUC0–∞). AUC0–∞ was calculated by the linear trapezoid rule. The terminal slope of the ln (concentration) versus time plot was calculated by linear least-squares regression and the half-life was calculated as 0.693 divided by the absolute value of the slope. The steady state volume of distribution (Vdss) was determined using non-compartmental analysis as follows:

\[ V_{dss} = \frac{\text{i.v. dose} \times AUMC}{(AUC)^2} \]

where AUMC is the total area under the first moment of the drug concentration–time curve from time zero to infinity.

The relative bioavailability (F) of the p.o. doses was calculated by using the following equation:

\[ F = \frac{AUC_{0–\infty}^{\text{i.v.}}}{AUC_{0–\infty}^{\text{p.o.}}} \times \frac{\text{Dose}_{\text{i.v.}}}{\text{Dose}_{\text{p.o.}}} \]

Statistical Analysis

An unpaired two-tailed Student’s t-test was used to assess significance of differences in various pharmacokinetic parameters in the DDI studies across groups. In instances where parameters possessed unequal variances, statistical analysis was performed with Welch’s correction. One-way ANOVA with a Benferroni post-test comparison was used to assess difference in pharmacokinetic parameters for fexofenadine and prazosin across groups. In all cases, p < 0.05 was predetermined as the criterion for significance. All Statistical analysis was performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Cytochrome P450 Inhibition by CP-100,356

The ability of CP-100,356 to function as a competitive inhibitor of the five major human P450 enzymes namely P4501A2, 2C9, 2C19, 2D6, and 3A4 was examined in human liver microsomes. Considering that P4503A4 inhibition is dependent on substrate used in the microsomal assay, two structurally distinct probe substrates namely testosterone and midazolam were chosen for analysis of P4503A4 inhibition by CP-100,356. Virtually no inhibition (IC50 > 50 μM) of the catalytic activity of the individual P450 enzymes was observed with CP-100,356 at the concentration range employed (0–50 μM) in the competitive inhibition study. Under the present experimental conditions, the enzyme-specific inhibitors furafylline, sulfaphenazole, (+)-N-3-Benzylirvanol, quinidine, and ketoconazole demonstrated potent competitive inhibition of P4501A2, 2C9, 2C19, 2D6, and 3A4 activity, respectively, in human liver microsomes. The corresponding IC50 values of 0.016 μM for midazolam and 0.018 μM for testosterone (ketoconazole), 0.068 μM (quinidine), 0.414 μM ( (+)-N-3-Benzylirvanol), 0.169 μM (sulfaphenazole), and 2.1 μM (furafylline) are consistent with those previously published by Walsky and Obach.34

MDR1, BCRP, and MRP2 Inhibition by CP-100,356

The inhibitory effects of CP-100,356 (concentration range 0.1–15 μM) against human MDR1 and human MRP2 were assessed in MDR1-and MRP2-MDCKII monolayers by determining the calcein-AM uptake.36 Cyclosporin A was used a control in the calcein-AM inhibition study for MDR1 and MRP2 activity, respectively.37–38 Cyclosporin A yielded IC50 values of 2.7 ± 0.1 and 13 ± 3.0 μM for inhibition of calcein-AM uptake mediated by MDR1 and MRP2, respectively. Under these experimental conditions, CP-100,356 inhibited the MDR1-mediated transport of Calcein-AM, yielding an IC50 value of 0.50 ± 0.07 μM (Fig. 2, panel A). Virtually, no inhibition was discernible in calcein-AM uptake in MRP2-MDCK cells at the highest CP-100,356 concentration tested of 15 μM (concentrations greater than 15 μM proved to be cytotoxic to cells). MDR1 inhibition by CP-100,356 was also assessed in MDR1-MDCKII monolayers by determining effects on the B → A transport of prototypic MDR1 substrate digoxin. CP-100,356 inhibited digoxin B → A transport in the across MDR1-MDCKII monolayers with an IC50 of 1.2 ± 0.1 μM (Fig. 2, panel B). The inhibitory effects of CP-100,356 (concentration range 0.1–15 μM) against human BCRP were assessed in BCRP-MDCKII monolayers by determining the B → A transport of prototypic BCRP substrate and
anti-hypertensive agent prazosin.\textsuperscript{30,40} CP-100,356 decreased the BCRP-mediated transport of prazosin, yielding an IC\textsubscript{50} value of 1.5 ± 0.3 μM (Fig. 3).

**Inhibition of OATP1B1-Mediated Uptake of Estradiol 17β-D-Glucuronide by CP-100,356**

The plot of percentage inhibition of OATP1B1-mediated transport of estradiol 17β-D-glucuronide by CP-100,356 is shown in Figure 4. The results indicate weak inhibition of OATP1B1 by CP-100,356 (IC\textsubscript{50} = 66 ± 1.1 μM). Rifamycin, a positive control, inhibited OATP1B1-mediated transport of estradiol 17β-D-glucuronide with an IC\textsubscript{50} of 0.23 ± 0.07 μM.

**In Vivo Pharmacokinetic Interactions Between CP-100,356 and Fexofenadine in Sprague–Dawley Rats**

The pharmacokinetic parameters describing the disposition of fexofenadine in Sprague–Dawley rats after intravenous administration and after oral administration in the absence or presence of CP-100,356 are shown in Tables 1 and 2, respectively. The mean concentration versus time profiles after i.v. and p.o. dosing is shown in Figure 5. Following a single i.v. dose of 1 mg/kg, fexofenadine exhibited a high CL\textsubscript{p} (93 ± 8.8 mL/min/kg) which exceeded the hepatic blood flow in this species (70 mL/min/kg) and a moderate V\textsubscript{dss} (1.8 ± 0.3 L/kg) resulting in a mean elimination half-life of 0.48 ± 0.012 h. The corresponding AUC\textsubscript{0–∞} was 180 ± 19 ng h/mL. Following a...
single p.o. dose of 10 mg/kg, systemic exposure of fexofenadine as ascertained from maximal plasma concentration ($C_{\text{max}}$) and AUC$_{0-\infty}$ was $10 \pm 0.7$ ng/mL and $14 \pm 6.8$ ng h/mL, respectively. The corresponding oral bioavailability of fexofenadine at the 10 mg/kg dose was less than 1%. As depicted in Table 2 and Figure 5, concomitant p.o. administration of fexofenadine at 10 mg/kg and CP-100,356 at doses of 6, 12, and 24 mg/kg to rats resulted in a dramatic increase in systemic exposure of fexofenadine as ascertained from $C_{\text{max}}$ to AUC$_{0-\infty}$. At a CP-100,356 dose of 24 mg/kg, fexofenadine $C_{\text{max}}$ and AUC increased by $\sim$36- and $\sim$78-fold, respectively, when compared with the values in the absence of CP-100,356. Coadministration of fexofenadine with CP-100,356 doses >24 mg/kg did not result in further significant increase in fexofenadine systemic exposure.

**In Vivo Pharmacokinetic Interactions Between CP-100,356 and Prazosin in Sprague–Dawley Rats**

The pharmacokinetic parameters describing the disposition of prazosin in Sprague–Dawley rats after intravenous administration and after oral administration in the absence or presence of CP-100,356 are shown in Tables 1 and 3, respectively. The mean concentration versus time profiles after i.v. and p.o. dosing is shown in Figure 6. Following a single i.v. dose of 0.5 mg/kg, prazosin exhibited a moderate $C_{\text{lp}}$ (36 ± 6.8 mL/min/kg) and a moderate $V_{\text{dss}}$ (2.2 ± 0.2 L/kg) resulting in a mean elimination half-life of 1.3 ± 0.2 h. The corresponding AUC$_{0-\infty}$ was 240 ± 39 ng h/mL. Following a single p.o. dose of 2.5 mg/kg, systemic exposure of prazosin as ascertained from maximal plasma concentration ($C_{\text{max}}$) and AUC$_{0-\infty}$ was 61 ± 10 ng/mL and 240 ± 51 ng h/mL, respectively. The corresponding oral bioavailability of prazosin was $\sim$20 ± 4.3%. As shown in Table 3 and Figure 6, oral coadministration of prazosin (2.5 mg/kg) and CP-100,356 (24 mg/kg) resulted in a statistically significant increase in the $C_{\text{max}}$ and AUC$_{0-\infty}$ of prazosin.

**DISCUSSION**

An initial literature survey for selecting MDR1 inhibitors in this work was not encouraging...
considering that many inhibitors possessed additional “off-target” activity including inhibition of P4503A4 and/or other transport proteins like MRP2 and OATP1B1 which are also known to be involved in active efflux or influx. Therefore, we focused our attention on the diaminoquinazoline derivative CP-100,356, an optimized lead compound developed in-house as a MDR1 inhibitor. Of particular interest was the finding on a greater than 100-fold selectivity of CP-100,356 as an MDR1 inhibitor over P4503A4 inhibition. In that same study, prototypic MDR1 inhibitors such as quinidine, verapamil, and cyclosporin A exhibited MDR1 selectivity ratios < 20. Our present results confirmed the lack of P4503A4 inhibition by CP-100,356 even when microsomal incubations were conducted in the presence of structurally diverse P4503A4 probe substrates midazolam and testosterone. In addition, CP-100,356 was devoid of inhibitory attributes against other major human P450 enzymes including P4501A2, 2C9, 2C19, and 2D6. Lack of P4503A4 inhibition by CP-100,356 was consistent with lack of DDIs between CP-100,356 and midazolam in rats. This was anticipated to some extent since midazolam is not a MDR1/BCRP substrate in rodents and humans; the principal clearance mechanism of midazolam in rats and humans involves intestinal and hepatic metabolism mediated by P4503A isozymes (P4503A1/2 and P4503A4 in rat and human, respectively). Consistent with these observations, is the finding that coadministration with selective P4503A inhibitor ketoconazole results in significant increases in midazolam oral bioavailability in rats and human.

Table 3. Pharmacokinetic Parameters of Prazosin and Midazolam After Single Oral Doses of 2.5 and 10 mg/kg, Respectively, to Male Sprague–Dawley Rats in the Presence of Vehicle or CP-100,356 at an Oral Dose of 24 mg/kg

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</th>
<th>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (ng h/mL)</th>
<th>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>F (%)</th>
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<tbody>
<tr>
<td>Prazosin</td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>61 ± 10</td>
<td>2.7 ± 1.2</td>
<td>240 ± 51</td>
<td>ND</td>
<td>20 ± 4.3</td>
</tr>
<tr>
<td>CP-100,356 (24 mg/kg)</td>
<td>85 ± 12</td>
<td>2.8 ± 2.0</td>
<td>620 ± 120**</td>
<td>ND</td>
<td>52 ± 10</td>
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<tr>
<td>Midazolam</td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>400 ± 64.0</td>
<td>0.33 ± 0.14</td>
<td>412 ± 31.0</td>
<td>ND</td>
<td>10 ± 0.7</td>
</tr>
<tr>
<td>CP-100,356 (24 mg/kg)</td>
<td>510 ± 96.0</td>
<td>0.33 ± 0.14</td>
<td>570 ± 130</td>
<td>ND</td>
<td>14 ± 2.5</td>
</tr>
</tbody>
</table>

CP-100,356 was coadministered with prazosin or midazolam. Data are presented as mean ± SD from three male rats and were derived from non-compartmental analysis. ND, not determined.

*p < 0.05.
**p < 0.001.
Although CP-100,356 was devoid of P450 inhibitory properties, the compound emerged as a potent human MDR1 inhibitor with low μM IC50 value in the MDR1-MDCKII cell line under two different scenarios which included determination of calcine-AM uptake and digoxin transport. In addition, CP-100,356 also displayed inhibition of human BCRP-mediated prazosin transport in BCRP-MDCKII monolayers with IC50 values comparable to those obtained for inhibition of MDR1-mediated digoxin transport. Given that most MDR1 and BCRP inhibitors share common physiochemical parameters such as lipophilicity, aromaticity, and presence of positive charge at physiological pH, the dual inhibitory properties of CP-100,356 against MDR1 and BCRP were anticipated. However, the finding that MDR1-related ABC transporter MRP2 was not inhibited by CP-100,356 (virtually no inhibition up to 15 μM) was somewhat of a surprise given previous reports on a significant overlapping inhibitory specificity between MRP2 inhibitors and MDR1/BCRP inhibitors. Lack of inhibition of MRP2 by CP-100,356 was important in our case because of recent studies which indicate that MRP2 expression while highest in the liver is not exclusive to this organ. In fact, intestinal enterocytes has also been shown to express this efflux transporter. Furthermore, given that MRP2 plays a role in the biliary efflux of intrinsically anionic drugs including fexofenadine, observed DDIs between CP-100,356 and fexofenadine in the rat would be hard to interpret particularly with respect to the biochemical mechanism(s) affecting absorption.

Overall, based on these in vitro results, we felt that CP-100,356 was a reasonably selective inhibitor of MDR1/BCRP and as such could be used as a “chemical knock-out” equivalent in the rat. To test in vivo MDR1/BCRP inhibition by CP-100,356, we chose the histamine H1-receptor antagonist fexofenadine as the “victim” drug. Hepatic metabolism is of minimal importance in the elimination of fexofenadine in rodents (mice and rats) and human; the drug is subject to active efflux and its major elimination mechanism involves biliary excretion in the unchanged form, mediated principally by MDR1 and MRP2 to some extent. The lack of observed DDIs between the P450 inactivator 1-aminobenzotriazole and fexofenadine in the rat confirms the non-P450 clearance pathway. In addition, fexofenadine also functions as a substrate for hepatic uptake by human and rat OATPs/
theoretical oral bioavailability of ~49% (based on a hepatic extraction ratio of 51%), which is obtained by dividing prazosin CLp by the rat hepatic blood flow of 70 mL/min/kg) could be anticipated for prazosin. However, in subsequent p.o. studies, an oral bioavailability of ~20% was estimated. In the presence of CP-100,356, a statistically significant increase in AUC was observed which afforded an oral bioavailability estimate closer to the maximum possible theoretical value of ~50%. Whether the increase in prazosin systemic exposure was mediated via selective inhibition of MDR1 or BCRP (or a combination of the two processes) remains unclear at the present time. Additional DDI studies with more selective BCRP inhibitors such as the fumitremorgin C analog Ko143 could potentially shed light on the issue. ^61,64

A potential weakness in our studies is that assessment of CP-100,356 inhibitory activity for MDR1, BCRP, MRP2, and OATP1B1 were all conducted with human isoforms whereas DDI studies were conducted in the rat. We acknowledge that there may be unique cases where NCEs will exhibit species differences in substrate properties for influx/exflux transporters, however, we feel that the methodology will be effective enough for the vast majority of the compounds. This is due to several reasons such as the high sequence homology of the efflux transporters between rodents and humans (~80–90% sequence identity of rat and mice Mdr1/Bcrp with human MDR1/BCRP transporters), ^62,63 our internal work on >3300 compounds indicating a high correlation (R² = 0.92) when tested for substrate properties against human MDR1 and mouse Mdr1, respectively, ^64 and our in-house side-by-side comparison of the human versus mouse BCRP substrate properties of ~20 marketed human BCRP substrates, which reveal minimal species differences in substrate specificity (Feng, B. Pfizer internal data). Nevertheless, studies are ongoing in our laboratory to characterize CP-100,356 inhibition of rodent Mdr1, Bcrp, Mrp2, and Oatp transporters. In conclusion, we provide data to show that CP-100,356 is an efficient chemical knock-out probe to investigate active drug efflux by MDR1 and BCRP. The methodology has potential applications in early drug discovery to rapidly assess in vivo impact of MDR1/BCRP efflux on oral absorption of NCEs and bypass time- and cost-prohibitive studies in MDR1 and/or BCRP knockout mice. We envision a scenario wherein CP-100,356 would be coadministered with new compounds which are of pharmacological interest but carry a liability by virtue of active efflux as measured in in vitro high-throughput Caco-2 and/or MDR1-MDCKII assays. The rat would be an obvious choice for this exercise since the species is routinely used to “screen” oral absorption potential of new compounds.

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