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In Vitro-In Vivo Correlation and Translation to the Clinical Outcome for CJ-13,610, a Novel Inhibitor of 5-Lipoxygenase

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ABSTRACT:

The metabolism of the 5-lipoxygenase inhibitor, 4-(3-(4-(2-methyl-1*H*imidazol-1-yl)phenylthio)phenyl)-tetrahydro-2*H*-pyran-4-carboxamide (CJ-13,610), was investigated in liver microsomes from human and preclinical species in an effort to compare metabolite profiles and evaluate the in vitro-in vivo correlation for metabolic clearance. Overall, the metabolite profile of CJ-13,610 was comparable across the species tested with multiple oxidative metabolites observed, including sulfoxidation. The sulfoxidation kinetics characterized in rat, dog, and human liver microsomes (HLM) indicated a low apparent Michaelis-Menten constant ($K_{m, app}$) of 4 to 5 μ M. Results from cDNA-expressed cytochrome P450 (P450) studies indicated that the metabolism in HLM was primarily mediated by CYP3A4 and 3A5. A subsequent in vitro study using ketoconazole as an inhibitor of CJ-13,610 sulfoxidation corroborated the CYP3A4/5-mediated pathway (IC₅₀ = 7 nM). Assessment of multiple methods for predicting the

4-(3-(4-(2-Methyl-1H-imidazol-1-yl)phenylthio)phenyl)-tetrahydro-2H-pyran-4-carboxamide (CJ 13,610) (Fig. 1) is a novel reversibleinhibitor of 5-lipoxygenase (5-LOX) (Fischer et al., 2004), a criticalenzyme involved in the initial step of the arachidonic acid cascade thatresults in the ultimate formation of numerous proinflammatory bioactive leukotrienes such as LTA₄, B₄, C₄, D₄, and E₄ (Samuelsson,1983). Leukotrienes have been clearly shown to be potent chemoattractants for neutrophils, eosinophils, and monocytes in response toinflammatory stimuli, and thus it is expected that inhibition of the5-LOX enzyme may lead to successful therapeutic intervention inseveral inflammation-based diseases, including asthma, rheumatoidarthritis, and cardiovascular disease (Werz and Steinhilber, 2006).

Zileuton (Zyflo) is currently the only 5-LOX inhibitor available on the market for the treatment of asthma (Liu et al., 1996; Wenzel and

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human pharmacokinetic profile observed with CJ-13,610 after a 30-mg single oral dose indicated that clearance scaled from human liver microsomes yielded a better prediction when coupled with a Vd_{ss} term that was scaled from dog [area under the concentration-time curve (AUC) and half-life within 1.3-fold of actual] versus a Vd_{ss} term obtained from rat. Single-species allometric scaling of clearance and Vd_{ss} from dog pharmacokinetic studies was equally predictive, whereas scaling from rat resulted in underpredictions of both AUC and maximal concentration (C_{max}). Results from these studies support the strategy of predicting human pharmacokinetics using human liver microsomal intrinsic clearance data. More importantly, results from the present investigation enabled the selection of alternative drug candidates from the chemical series via in vitro screening, while subsequently eliminating costly routine preclinical in vivo studies.

Kamada, 1996). Although efficacious in the treatment of asthma, treatment with zileuton is fraught with issues such as a high daily dose (2400 mg/day) and deleterious effects on the liver, requiring repeated monitoring of liver enzyme levels. In addition, zileuton has been shown to be a mechanism-based inactivator of CYP1A2 (Lu et al., 2003), resulting in the potential for drug-drug interactions with coadministered CYP1A2 substrates such as theophylline (Granneman et al., 1995). An alternative therapeutic strategy includes use of LTB_{4} receptor antagonists, such as montelukast (Singulair), which has been shown to be effective and safe in the treatment of asthma, including the pediatric population (Reiss et al., 1996; Becker, 2000). In lieu of the favorable safety profile of LTB₄ receptor antagonists such as montelukasst, upstream inhibition of the 5-LOX enzyme, theoretically preventing formation of all of the aforementioned leukotrienes, presents a potential opportunity for enhanced clinical efficacy in multiple therapeutic areas. Thus, there remains a desire for a potent 5-LOX inhibitor that can be administered to patients at a lower therapeutic dose while maintaining the desired safety of LTB_4 receptor antagonists.

The use of in vitro biotransformation systems to predict the pharmacokinetic behavior of drug molecules remains an intense area of

ABBREVIATIONS: CJ 13,610, 4-(3-(4-(2-methyl-1*H*-imidazol-1-yl)phenylthio)phenyl)-tetrahydro-2*H*-pyran-4-carboxamide; 5-LOX, 5-lipoxygenase; LT, leukotriene; P450, cytochrome P450; CP-680179, 4-(3-(4-(2-methyl-1*H*-imidazol-1-yl)phenylsulfinyl)phenyl)-tetrahydro-2*H*-pyran-4-carboxamide; LC, liquid chromatography; MS/MS, mass spectrometry; FMO, flavin monooxygenase; HPLC, high-performance liquid chromatography; AUC, area under the concentration-time curve; MRT, mean residence time; hFMO, human flavin monooxygenase; HLM, human liver microsome(s); SSS, single-species allometric scaling; CL_n, hepatic clearance.



FIG. 1. Structure of CJ-13,610.

research within the drug discovery continuum, with much of the seminal work initiated by Houston (1994) and Iwatsubo et al. (1997). In particular, the use of hepatic microsomes as an in vitro tool for quantitatively predicting metabolic clearance, as well as the contribution of specific drug-metabolizing enzymes, is routinely applied in drug discovery to diagnose liabilities associated with drug disposition before nomination of drug candidates for development. To this end, the primary objectives of the present investigations were to 1) profile the in vitro metabolic pathways of CJ-13,610 in liver microsomes from multiple species, 2) compare the kinetics of liver microsomal metabolism in vitro and the in vivo distribution of CJ-13,610 across species in an effort to assess in vitro-in vivo correlation, and 3) estimate the contribution of the specific human enzymes involved in the metabolism of CJ-13,610. This work was a retrospective analysis to investigate potential methodologies for predicting the disposition properties of CJ-13,610 as it relates to the potential for pharmacokinetic variability and drug-drug interaction risk of this potential therapeutic agent in humans. Results from these analyses will prove useful for any drug discovery program evaluating new chemical entities within this chemical class of compounds.

Materials and Methods

Chemicals. CJ-13,610 and the sulfoxide metabolite standard (CP-680179) were obtained from the Pfizer Global Research and Development chemical bank. Potassium phosphate buffer, NADPH, magnesium chloride, tolbutamide, bupropion, coumarin, dextromethorphan, diclofenac, midazolam, omeprazole, paclitaxel, astemizole, tacrine, ketoconazole, and benzydamine were purchased from Sigma-Aldrich (St. Louis, MO). Pooled human liver microsomes (50 donors), dog liver microsomes, and recombinant human CYP2B6, 2C8, 2J2, and 3A5 Supersomes were purchased from BD Biosciences (San Jose, CA). Rat liver microsomes were purchased from XenoTech, LLC (Kansas City, MO), and recombinant human CYP1A2, 2D6, 2C9, 2C19, and 3A4 Baculosomes were purchased from Invitrogen (Carlsbad, CA). All other chemicals were obtained from commercial sources and were of the highest purity available.

In Vitro Incubations. The intrinsic clearance of CJ-13,610 (1 μ M) was compared in human (0.8 mg/ml), rat (0.5 mg/ml), and dog (0.5 mg/ml) liver microsomes using substrate depletion methodologies. Incubations were performed in 100 mM potassium phosphate, pH 7.4, buffer (1 mM MgCl₂) at 37°C and were initiated after a 5-min preincubation period by addition of NADPH (1 mM). At selected time intervals (0, 2, 5, 10, 20, and 30 min), 50-µl aliquots were taken and subsequently placed into a 96-well plate containing 150 µl of cold acetonitrile with internal standard (0.2 µM tolbutamide). Plates were then centrifuged at 3000 rpm (4°C) for 10 min, and the supernatant was transferred to a separate 96-well plate for LC-MS/MS analysis. The in vitro half-life $(t_{1/2})$ was calculated by linear regression of the natural log percentage remaining of substrate as a function of incubation time using GraphPad Prism (GraphPad Software Inc., San Diego, CA) software according to the equation $t_{1/2} = \ln 2$ /slope. In an effort to identify the enzymes involved in the metabolism of CJ-13,610 (1 µM), incubations with recombinant heterologously expressed P450 enzymes (150 pmol/ml and 15 pmol/ml for 3A4), and recombinant human flavin monooxygenase 3 (FMO3) (50 µg/ml) were conducted in the presence of 100 mM potassium phosphate, pH 7.4, buffer (1 mM MgCl₂) and NADPH (1 mM) in a total volume of 1 ml (0.2 ml for FMO3 incubation). Reactions were initiated by addition of NADPH (1 mM) and incubated on an Eppendorf Thermomixer at 37°C for 30 min. Incubations with FMO3 were subjected to a 5-min preincubation period in the presence of NADPH, and the reactions were initiated by addition of CJ-13,610 or benzydamine as a positive control. Incubations were terminated by addition of ice-cold acetonitrile containing 0.2 µM tolbutamide and centrifuged at 3000 rpm for 10 min. Samples were subsequently analyzed by LC-MS/MS for determination of substrate depletion after transfer of supernatant to a separate 96-well plate. Positive control probe substrates for each individual P450 were included to confirm enzyme activity. Inhibition of CJ-13,610 sulfoxidation by the CYP3A inhibitor ketoconazole was tested with incubation conditions identical to those of enzyme kinetic studies described above in human liver microsomes, except that ketoconazole (0–5 μ M) was also included in the incubation, with each concentration in triplicate. CJ-13,610 was incubated at a single concentration equal to the estimated $K_{\rm m}$ (5 μ M), and the total organic in each incubation was 1% (v/v), and the sulfoxide metabolite (CP-680179) was measured by LC-MS/MS.

Plasma and Microsomal Protein Binding. Plasma protein binding and nonspecific microsomal binding in human, rat, and dog were performed in triplicate using a 96-well equilibrium dialysis apparatus according to published methods (Banker et al., 2003). To prepare the dialysis block, dialysis membranes were soaked in phosphate-buffered saline, pH 7.4, for 60 min. After hydration, membranes were rinsed with 100 mM phosphate, pH 7.4, buffer (1 mM MgCl₂), separated, and allowed to soak for a minimum of 1 h. Human, rat, and dog liver microsomes were diluted to a final concentration of 0.8, 0.5, and 0.5 mg/ml in 100 mM phosphate buffer, pH 7.4, respectively. Heparinized human, rat, and dog plasma (Bioreclamation, Hicksville, NY) was collected from at least three male and female fasted donors and frozen at -70°C until use. The pH of the plasma was determined and adjusted to pH 7.4 by drop-wise addition of dilute phosphoric acid. The final concentration of CJ-13,610 was 1 µM in both microsomal binding (ketoconazole and midazolam as controls) and plasma protein binding (S-warfarin as control) studies. The conditioned membrane strips were then placed into the 96-well dialysis apparatus. The dialysate side of the 96-well equilibrium dialysis block was loaded with 150 µl of phosphate buffer (100 mM), pH 7.4, to prevent dehydration of the membrane. The same volume of plasma or diluted microsomes spiked with compound was placed into the sample side. Once the dialysis block was loaded with plasma or microsomal samples and buffer, an adhesive sealing film was used to cover the block to prevent evaporation. The equilibrium block was incubated at 37°C for 4 h in a 5% CO₂ incubator to ensure that equilibrium conditions were achieved, as verified in preliminary experiments. After the incubation, a $50-\mu l$ aliquot of the postdialysis plasma (or microsomes) and buffer samples were placed in a 96-well microtiter plate and then precipitated with 3 volumes of an acetonitrile-containing internal standard. The extraction plate was centrifuged for 5 min at 3000 rpm, and 90 µl was transferred to a fresh analytical plate for LC-MS/MS analysis.

In Vitro Metabolite Profiling of CJ-13,610. The in vitro metabolite profile of CJ-13,610 was investigated in rat, dog, and human liver microsomes. A potassium phosphate-buffered 1-ml reaction (100 mM, pH 7.4) with CJ-13,610 (20 μ M), liver microsomes (1 mg/ml), and MgCl₂ (1 mM) was initiated by addition of NADPH (2 mM) and incubated at 37°C for 60 min in 13 × 100-mm borosilicate glass test tubes. Protein was precipitated by addition of 2 volumes of cold acetonitrile, and the resulting mixture underwent centrifugation at 3000 rpm (4°C) for 15 min. The supernatants were drawn off, placed into clean glass test tubes, and subsequently dried under a gentle stream of nitrogen (N₂) gas. Dried samples were then reconstituted in 200 μ l of mobile phase [85:15 (v/v) ammonium formate (10 mM, pH 4.1)-acetonitrile] for LC-MS/MS analysis.

Enzyme Kinetics. The kinetics of CJ-13,610 sulfoxidation was investigated in rat, dog, and human liver microsomes. Preliminary experiments established the linear conditions for CJ-13,610 sulfoxidation with respect to incubation time (0–20 min) and protein concentration (0–0.5 mg/ml microsomal protein). In brief, multiple concentrations of CJ-13,610 (0–100 μ M) in triplicate were incubated in 100 mM potassium phosphate, pH 7.4, buffer (1 mM MgCl₂) with rat liver microsomes (0.05 mg/ml), human liver microsomes (0.05 mg/ml), and dog liver microsomes (0.1 mg/ml) for 15 min at 37°C in a shaking water bath (total volume 200 μ l) after addition of 2 mM NADPH. Incubation reactions were subsequently quenched by addition of 200 μ l of ice-cold acetonitrile containing tolbutamide (0.2 μ M) as internal standard. Incubation plates were then centrifuged at 3000 rpm (4°C), and supernatant was transferred to an analytical 96-well plate for LC-MS/MS analysis.

Pharmacokinetic Studies. Male Sprague-Dawley rats were purchased from Charles River Laboratories, Inc. (Wilmington, MA) and acclimated to their surroundings for approximately 1 week with food and water provided ad libitum. On the day before the study, animals were anesthetized with isoflurane (to effect) and then implanted with BASi vascular catheters in the carotid artery and jugular vein. Animals were acclimated in Culex cages overnight before dosing. Patency of the carotid artery catheter was maintained using the "tend" function of a Culex ABS. Three rats were dosed intravenously with a 10% ethanol-56% PEG-400-34% phosphate-buffered saline (pH 7.4) at 0.5 mg/kg via the jugular vein catheter at a dose volume of 0.5 ml/kg. Blood collections were performed by the Culex ABS predose and at 2, 5, 15, and 30 min and 1, 2, 4, 6, 8, 12, 18, and 24 h postdose; samples were collected into chilled heparinized tubes and centrifuged for 10 min at 3000 rpm, and the resulting plasma was aliquoted to 96-well plates for LC-MS/MS analysis. Male beagle dogs (approximately 1-3 years of age and weighing between 9 and 12 kg at study initiation) were obtained from Marshall Farms (North Rose, NY). Dogs (n = 3) were dosed intravenously using a butterfly tubing set and syringe into the cephalic vein. The intravenous dose was administered at 0.1 mg/kg (1 mg/ml) in 10% ethanol-50% PEG-400-40% phosphate-buffered saline, pH 7.4. The dogs were placed in metabolism cages after dosing and for 24 h postdose. All dogs were fasted overnight, but water was allowed ad libitum. Food was returned 4 h postdose. All dogs were weighed before dosing. A catheter was then inserted into the cephalic vein in one leg of each dog for blood collection. Blood was collected predose and at 5, 15, and 30 min and 1, 2, 4, 6, 8, 10, and 24 h postdose. Blood was obtained from the catheter for collection times up to 2 h. After 2 h, the catheters were removed, and blood was collected from the jugular vein for the remaining time points using a needle and syringe. Approximately 0.6 ml of blood was collected at each time point into lithium heparin blood tubes, mixed thoroughly, and chilled. Plasma was obtained by centrifugation at 5200 rpm for 15 min at 4°C. In addition, urine samples were collected at 0 to 10 and 10 to 24 h postdose. Plasma and urine samples were stored at -10°C until analysis by LC-MS/MS. All animal studies were approved by the St. Louis Pfizer Institutional Animal Care and Use Committee. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

Dosing of CJ-13,610 in Humans. The phase I first-in-human study was a placebo-controlled, randomized parallel group study of increasing doses of CJ-13,610 administered sequentially, at least 1 week apart. The study protocol and informed consent documents were approved by the institutional review board. All subjects signed an approved written informed consent form before any study-related activities for this clinical study. A higher dose was given only if the preceding dose did not result in any safety concerns. There were six subjects in each dose group, with four randomized to active study drug and two to a matching placebo. CJ-13,610 was administered as a solution to fasted male subjects at doses of 1, 3, 10, 30, 100, and 300 mg. A light meal was provided 4 h after dosing. Blood samples were collected at intervals for up to 96 h postdose for the assay of plasma CJ-13,610 and plasma LTB₄. Urine was collected from 0 to 24 and 25 to 96 h, the volume was measured, and aliquots were sent to Pfizer Laboratories for bioanalysis of CJ-13,610. Blood pressure, pulse rate, respiratory rate, and oral temperature were measured at intervals for 24 h and subsequently every 24 h for the duration of the study.

Liquid Chromatography-Mass Spectrometry Analysis. CJ-13,610 and sulfoxide (CP-780179) were both analyzed on a PE Sciex API-3000 triple quadrupole instrument. The mass spectrometer was equipped with an electrospray ionization interface connected in line with a Shimadzu LC20AD pump and a CTC PAL (Leap Technologies, Carrboro, NC) autosampler. Analytes were separated using a Zorbax 3.5- μ m Eclipse Plus C₁₈ 2.1 × 50-mm column with a gradient elution profile. The mobile phase was flowing at 0.45 ml/min, and the gradient was initiated and held for the first 0.8 min at 95% A-5% B (A, 0.1% formic acid in H₂O; B, 0.1% formic acid in acetonitrile) and was then ramped linearly to 5% A-95% B over the next 2.3 min and held for 1.2 min. The profile was then immediately returned to initial conditions and allowed to reequilibrate for 2 min. The source temperature was set to 400°C, and mass spectral analyses were performed using multiple reaction monitoring, with transitions for CJ-13,610 (m/z 393.9/319.3) or sulfoxide metabolite (m/z 410.1/

158.3) using a TurboIonSpray source in positive ionization mode (3.5 kV spray voltage). The limit of quantitation (signal/noise ratio, 3:1) for CJ-13,610 was 0.0015 to 0.0024 μ M for in vivo studies (rat, dog, and human) and 0.0049 μ M for in vitro studies. The limit of quantitation of CP-680179 for enzyme kinetic studies was 0.0024 μ M. Low, medium, and high quality control samples were included in all bioanalyses, and each quality control sample was calculated to be within 15% of nominal concentrations (data not shown). All data were analyzed using PE Sciex Analyst 1.4.2 software. For in vitro profiling of CJ-13,610 metabolites, an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) was coupled to a Discovery C_{18} column (5- μ m, 3.8 \times 150-mm; Supelco, Bellefonte, PA). Solvent A was 10 mM ammonium formate (pH 4.1) and solvent B was acetonitrile. The initial mobile phase was 85:15 A-B (v/v) and by linear gradient transitioned to 20:80 A-B over 20 min. The flow rate was 0.40 ml/min. The HPLC eluent was introduced via electrospray ionization directly into a Finnigan LCQ Deca XPPLUS ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) operated in the positive ion mode. Ionization was assisted with sheath and auxiliary gas (nitrogen) set at 70 and 15 psi, respectively. The electrospray voltage was set at 5 kV with the heated ion transfer capillary set at 350°C and 15 V. Relative collision energies of 25 to 40% were used when MS/MS operations were performed with the ion trap.

Enzyme Kinetic and Pharmacokinetic Analysis. In vitro intrinsic clearance (CL_{int}) was calculated from rat, dog, and human liver microsomes using substrate depletion data and the standard values of 45 mg of microsomal protein/g of liver for all species, and 40, 32, and 20 g of liver/kg b.wt. for rat, dog, and human, respectively. In turn, hepatic clearance (CL_h) was predicted using the well stirred model, factoring in plasma protein binding (f_u), nonspecific microsomal binding [$f_{u(microsomes)}$], and liver blood flow (Q) (70, 40, and 20.7 ml/min/kg for rat, dog, and human, respectively), according to eq. 1:

$$CL_{h} = \frac{Q \cdot f_{u} \cdot \frac{CL_{int}}{f_{u(microsomes)}}}{Q + f_{u} \cdot \frac{CL_{int}}{f_{u(microsomes)}}}$$
(1)

Enzyme kinetic parameters for CJ-13,610 sulfoxidation were estimated using nonlinear regression within GraphPad Prism software, and the standard Michaelis-Menten velocity equation (eq. 2):

(

$$v = \frac{V_{\max} \cdot [S]}{K_{\mathrm{m}} + [S]} \tag{2}$$

Intrinsic clearance from enzyme kinetic studies (CL $'_{int}$) was defined according to eq. 3:

$$CL'_{int} = \frac{V_{max}}{K_m}$$
(3)

Inhibition of CJ-13,610 sulfoxidation in human liver microsomes was expressed as IC_{50} and estimated according to eq. 4, where Top is the maximum percent control activity and Bottom is the minimum percent control activity:

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(X - LogIC_{50})}}$$
(4)

Pharmacokinetic parameters after intravenous and oral dosing to rats and dogs and oral dosing to humans were estimated using noncompartmental analysis methods (WinNonlin version 5.2; Pharsight, Mountain View, CA). Total body clearance was calculated using the standard equation (eq. 5), where AUC is area under the plasma concentration-time curve, calculated using the linear trapezoidal rule:

$$Clearance = \frac{Dose}{AUC_{(0-\infty)}}$$
(5)

Volume of distribution at steady-state (Vd_{ss}) was calculated using the following equation (eq. 6), where MRT is mean residence time and clearance is total body clearance:

$$Vdss = MRT \cdot Clearance$$
(6)

Human pharmacokinetic simulations were conducted using WinNonlin and a one-compartment first-order absorption and elimination (no lag) pharmacokinetic model. Human clearance was predicted using CL_h estimated either from in vitro human liver microsomal experiments as described above or from single-species scaling from rat and dog pharmacokinetic studies (Hosea et al., 2009) using eq. 7:

$$CLi.v._{predicted} = \left(\frac{BW_{human}}{BW_{animal}}\right)^{0.75} \cdot \left(CLi.v._{animal} \cdot \frac{BW_{animal}}{Fu_{animal}}\right) \cdot \left(\frac{Fu_{human}}{BW_{human}}\right) \quad (7)$$

where CLi.v. is clearance after intravenous administration, BW is body weight, and Fu is fraction unbound in plasma. Vd_{ss} was scaled and predicted using pharmacokinetic data generated from intravenous studies in rat and dog using eq. 8:

$$Vd_{predicted} = Vd_{animal} \cdot \frac{Fu_{human}}{Fu_{animal}}$$
(8)

Absorption rate $(k_a, hour^{-1})$ for human pharmacokinetic simulations was estimated using modeled rat oral pharmacokinetic data, consistent with reported data suggesting use of rats for predicting human absorption of orally administered drugs (Chiou and Barve, 1998).

Results

In Vitro Metabolism of CJ-13,610. The rates of in vitro liver microsomal metabolism of CJ-13,610 in rat, dog, and human liver microsomes, as well as plasma and microsomal binding are presented in Table 1. CL_{int} determined from substrate depletion methodologies was highest for rat and lowest for dog (rat > human > dog), whereas plasma protein binding appeared to be highest in rat ($f_u = 0.07$). The predicted CL_h using the well stirred model overestimated plasma clearance when neither plasma nor nonspecific microsomal protein binding was considered. Nonspecific binding to liver microsomes in vitro appears to be low [$f_{u(microsomes)} \sim 0.8$] and thus, did not have a major impact on the calculated hepatic clearance values (Table 1). However, factoring in both plasma and microsomal binding resulted in CL_h estimates that more closely resembled total plasma clearance observed after intravenous dosing to rats and dogs (Table 1).

Characterization of CJ-13,610 and Metabolites In Vitro in Liver Microsomes. The LC-mass spectrometry-UV data demonstrat-

TABLE 1

Summary of substrate depletion kinetics of CJ-13,610 and enzyme kinetic parameters for the sulfoxidation metabolic pathway in rat, dog, and human liver microsomes

Numbers in parentheses indicate S.E. of nonlinear regression analysis. Reaction velocities were estimated as described under *Materials and Methods*.

Rat	Dog	Human
26	71	28
0.07	0.12	0.13
0.81	0.79	0.83
54	19	31
40.6	16.5	11.7
6.2	3.1	3.1
7.4	3.8	3.6
11.5	4.4	
4.1 (0.55)	5.4 (0.32)	4.8 (0.59)
247 (8.1)	77.9 (1.1)	184 (5.4)
60	14	38
	Rat 26 0.07 0.81 54 40.6 6.2 7.4 11.5 4.1 (0.55) 247 (8.1) 60	Rat Dog 26 71 0.07 0.12 0.81 0.79 54 19 40.6 16.5 6.2 3.1 7.4 3.8 11.5 4.4 4.1 (0.55) 5.4 (0.32) 247 (8.1) 77.9 (1.1) 60 14

^a No plasma or microsomal binding correction (eq. 1).

^b Corrected for plasma protein binding only (eq. 1).

^c Corrected for both plasma and nonspecific microsomal binding (eq. 1).

^d Calculated using eq. 3.

ing the metabolic profile of CJ-13,610 in human liver microsomes is provided in Fig. 2A. The protonated $[M + H]^+$ molecular ion for CJ-13,610 was observed at m/z 394. Fragmentation of CJ-13,610 produced a key fragment ion at m/z 349 that corresponded to the loss of the amide moiety (Fig. 2B). A subsequent ion produced from m/z349 was observed at m/z 320 and corresponded to the further fragmentation of the tetrahydropyran moiety. The fragment ion at m/z 189 corresponded to the cleavage of the arylsulfide and proved useful in the identification of metabolites of CJ-13,610 (e.g., M5). Overall, the metabolite profile was consistent across the species tested (rat, dog, and human; data not shown). The proposed metabolic pathways of CJ-13,610 in human is depicted in Fig. 3.

Metabolite M1. Oxidation of the imidazole moiety resulted in the proposed acetamidine metabolite, M1 ($[M + H]^+$ at m/z 370), based in part on the major fragmentation ion at m/z 353 that corresponded to a loss of 17 Da (NH₃). The loss of ammonia was not observed in the parent CJ-13,610 mass spectrum; furthermore, the presence of an ion at m/z 325 (-45 Da) indicated that the amide moiety was intact.

Metabolite M2. The oxidized metabolite producing a $[M + H]^+$ at m/z 410 was proposed as the sulfoxide, M2. A loss of both 18 Da $(-H_2O)$ and 17 Da (-OH) is consistent with sulfoxidation. The presence of an ion at m/z 365 indicated that the amide moiety was intact. The fragmentation pattern of M2 was consistent with the synthetically prepared sulfoxide standard (CP-680179).

Metabolite M3. The metabolite producing an $[M + H]^+$ at m/z 410 was proposed to bear a hydroxylation at the tetrahydropyran moiety (M3). The major dehydrated ion at m/z 392 (50% relative abundance) was indicative of an aliphatic hydroxylation. The presence of an ion at m/z 365 indicated that the amide moiety was intact.

Metabolite M4. The metabolite bearing an addition of 32 Da over that of parent CJ-13,610 produced an $[M + H]^+$ at m/z 426. The presence of fragment ions at 408 and 407 Da was indicative of a loss of H₂O and hydroxyl (–OH) and similar to that observed with M2. The ion at m/z 220 represented an oxidation of the fragment ion of CJ-13,610 at m/z 189. Together these data indicate M4 to be a sulfone metabolite (a secondary metabolite of M2).

Metabolite M5. The metabolite bearing an addition of 34 Da over that of parent CJ-13,610 produced an $[M + H]^+$ at m/z 428. Proposed as the dihydrodiolimidazole metabolite, M5, the collision-induced fragment at m/z 370 (-58 Da) indicated a biotransformation event at the 4,5-position of the imidazole moiety, incidentally producing a protonated fragment ion isobaric to that of M1. A secondary fragment of the ion at m/z 370 was observed at m/z 353 and corresponded to the loss of ammonia (-17 Da) from the resulting acetamidine moiety.

Metabolite M6. The dihydropyran metabolite M6 produced an $[M + H]^+$ at m/z 392. The collision-induced fragmentation of this metabolite produced an ion at m/z 364, indicating a facile loss of ethylene (-28 Da) from the dihydropyran moiety. The loss of formamide at m/z 348 and the cleavage of the arylsulfide linkage indicated that the remainder of the compound had been unaltered.

Metabolite M7. The metabolite producing an $[M + H]^+$ at m/z 408 bore biotransformation events of the arylsulfide (sulfoxidation) and the tetrahydropyran moieties. Consistent with that of M2, a major fragment ion observed was at m/z 391 and corresponded to the loss of 17 Da (–OH), a pattern consistent with sulfoxidation. The overall mass of the remainder scaffold being 2 Da less than that of M2 and parent CJ-13,610 implicated the corresponding oxidation of the pyran moiety to the dihydropyran oxidation state.

CJ-13,610 Sulfoxidation Kinetics. The sulfoxide metabolite of CJ-13,610 (CP-680179) was available as a synthetic standard, which afforded the ability to compare the enzyme kinetics for this predominant metabolic pathway across species. The enzyme kinetics of



FIG. 2. A, representative LC-mass spectrometry (I) and HPLC/UV (II) profiles of CJ-13,610 metabolites obtained from human liver microsomes. B, mass spectrum of CJ-13,610 obtained by LC-MS/ MS. Conditions are as described under *Materials and Methods*.



HN



FIG. 3. Proposed metabolic scheme of CJ-13,610 in human liver microsomes.

NH₂

NH₂

NH,

8 М7

4

。 М2

oo

Μ4



FIG. 4. Substrate saturation plots of CJ-13,610 sulfoxidation in rat (RLM, \blacksquare), dog (DLM, \blacktriangle), and human (HLM, \bullet) liver microsomes. Incubation conditions consistent with Michaelis-Menten steady-state enzyme kinetics are described under *Materials and Methods*.

CJ-13,610 sulfoxidation in human liver microsomes was described by the classic Michaelis-Menten equation (Fig. 4), with the apparent kinetic constants shown in Table 1 for rat, dog, and human. The apparent Michaelis-Menten constant ($K_{m, app}$) was similar across species (4.1–5.4 μ M). However, maximal rates (V_{max}) varied by greater than 3-fold, with the highest V_{max} observed in rat liver microsomes. CL'_{int} was determined by dividing V_{max} by $K_{m, app}$ (eq. 3), and the rank order was rat > human > dog (Table 1). CL'_{int} estimates from sulfoxidation kinetics closely resembled CL_{int} values derived from substrate depletion studies (Table 1).

Cytochrome P450 Identification. Data illustrating substrate depletion (percent remaining) of CJ-13,610 after incubation for 30 min with a battery of recombinant human P450s (1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2J2, 3A4, and 3A5), recombinant human FMO3, and human liver microsomes are shown in Fig. 5A. It is apparent that

CYP3A4 and 3A5 play a predominant role in the metabolism of CJ-13,610, whereas CYP2C8 and 2D6 demonstrated minimal activity toward CJ-13,610 (84 ± 0.07 and 85 ± 0.21 remaining, respectively). Negligible activity was observed for the remaining P450 enzymes tested. Considering that the sulfoxidation of CJ-13,610 was observed as a predominant pathway of metabolism, the role of human flavin monooxygenase (hFMO3) was investigated. We were surprised to find that hFMO3 did not show activity toward CJ-13,610, whereas the positive control substrate, benzydamine, was rapidly metabolized (data not shown). Last, ketoconazole, an established potent inhibitor of CYP3A in vitro, was coincubated with CJ-13,610 in human liver microsomes (Fig. 5B) and was shown to potently inhibit the formation of the sulfoxide metabolite from CJ-13,610 (IC₅₀ = 7 nM).

Preclinical and Clinical Pharmacokinetics of CJ-13,610. The pharmacokinetics of CJ-13,610 in rats and dogs were modeled using a noncompartmental analysis approach (Table 2). Pharmacokinetics in the rat were described by total body clearance of 11.5 ml/min/kg, Vd_{ss} of 3.6 l/kg, and MRT of 5.2 h. Pharmacokinetics in the dog were described by total body clearance of 4.4 ml/min/kg, Vd_{ss} of 2.9 l/kg, and MRT of 11 h. Less than 1% of parent CJ-13,610 was recovered in urine after intravenous dosing in both rats and dogs, which indicated negligible urinary clearance in the disposition of CJ-13,610. Pharmacokinetic parameters after single oral doses of CJ-13,610 in humans (n = 4) are shown in Table 3. Exposure as estimated by AUC and the maximal clearance after an oral dose (C_{max}) was linear and proportional up to a dose of 300 mg (Fig. 6), and the overall mean plasma elimination half-life ($t_{1/2}$) was comparable at all dose levels (Table 3).

Pharmacokinetic Simulations. In an effort to model the human pharmacokinetic profile of CJ-13,610, multiple methods were applied, ranging from in vitro scaling to single-species allometric scaling from rat and dog. The primary objective of this investigation was to



FIG. 5. In vitro characterization of the enzymes involved in the metabolism of CJ-13,610. A, activity across a panel of recombinantly expressed P450 enzymes, recombinant human FMO3, and HLM. B, inhibition of CJ-13,610 sulfoxidation in human liver microsomes by the CYP3A inhibitor ketoconazole (IC₅₀ = 7 nM).

Summary of estimated pharmacokinetic parameters after intravenous administration of CJ-13,610 to rats and dogs (n = 3) using noncompartmental pharmacokinetic analysis

Pharmacokinetic Parameter	Rat	Dog	
Dose (mg/kg)	0.5	0.1	
AUC $(\mu M \cdot h)$	1.83 (0.11)	0.94 (0.04)	
Clearance (ml/min/kg)	11.5 (0.69)	4.4 (0.17)	
Vd _{ss} (l/kg)	3.6 (0.17)	2.9 (0.29)	
MRT (h)	5.2 (0.4)	11 (1.5)	

demonstrate that scaling in vitro intrinsic clearance using human liver microsomes would accurately estimate clearance in human subjects. In support of this approach, an in vitro-in vivo correlation using liver microsomal intrinsic clearance was assessed in rat and dog. As demonstrated in Table 1, predicted CL_h in rat and dog very closely resembled total plasma clearance observed in pharmacokinetic studies (within 2-fold). When clearance was scaled from either human liver microsomes (Vd_{ss} scaled from dog) or single-species allometric scaling from dog (CL = 3.2 ml/min/kg), the pharmacokinetic profile in humans was closely predicted, with the AUC after a 30-mg single oral dose predicted within 10%, roughly comparable to the observed S.D. in exposure observed among four subjects (Table 4; Fig. 7). Likewise, the predicted C_{max} and half-life were within 1 to 1.5 S.D. of observed pharmacokinetic parameters (Table 4). When Vd_{ss} scaled from rat pharmacokinetics studies (6.7 l/kg) was combined with clearance scaled from human liver microsomes, the AUC was closely predicted, but the C_{max} was underpredicted and the half-life was overpredicted. Single-species allometric scaling of clearance and Vd_{ss} from rat pharmacokinetic studies was less accurate, with the predicted AUC accounting for roughly 60% of that observed in human subjects and the predicted C_{max} being ~33% of the observed value (Table 4; Fig. 7).

Discussion

CJ-13,610 is a novel reversible inhibitor of 5-LOX, a critical enzyme in the arachidonic acid cascade that catalyzes the initial step in the ultimate formation of numerous proinflammatory leukotrienes. Preclinical pharmacology data suggest that chemical modulation of the 5-LOX pathway by CJ-13,610 has potential therapeutic benefits in disease areas such as asthma, liver fibrosis, and pain (Werz and Steinhilber, 2006; Horrillo et al., 2007; Zweifel et al., 2008; Cortes-Burgos et al., 2009). In the investigations described herein, in vitro studies were conducted with CJ-13,610 to delineate the in vitro hepatic metabolism of this compound in an effort to evaluate the in vitro-in vivo correlation for metabolic clearance and enable the prediction of the pharmacokinetic profile of CJ-13,610 observed in humans after single-dose administration.

Methodologies for scaling of in vitro metabolism data to predict the

TABLE 3

Summary of pharmacokinetics of CJ-13,610 in healthy fasted male subjects after single oral doses administered as solution

	-			
Dose	$AUC_{0-\infty}^{a}$	$C_{\max}{}^a$	$T_{\rm max}$	t _{1/2}
	$ng \cdot h/ml$	ng/ml		h
1 mg	N.C.	2.1 (0.6)	6.8 (3.0)	N.C.
3 mg	249 (73)	11 (3)	5.0 (3.4)	14.8 (2.0)
10 mg	783 (279)	34 (10)	4.5 (1.0)	16.2 (2.8)
30 mg	1970 (220)	144 (34)	1.8 (1.5)	13.4 (2.1)
100 mg	6240 (2520)	684 (263)	1.5 (0.6)	13.1 (2.9)
300 mg	19,700 (4000)	2110 (1000)	3.5 (0.6)	14.6 (0.9)

N.C., not calculated because of insufficient data.

^{*a*} Mean (S.D.) are geometric for AUC and C_{max}



FIG. 6. Linearity of pharmacokinetic parameters AUC and C_{max} observed after single-dose oral administration of CJ-13,610 to human subjects (n = 4).

in vivo behavior of drugs have been proposed for many years (Houston, 1994; Iwatsubo et al., 1997; Obach, 2001), and there are numerous examples of successful predictions of the in vivo pharmacokinetic properties of drugs using in vitro data (Gilissen et al., 2000; Obach, 2000; Kuperman et al., 2001; Obach et al., 2007). When this type of in vitro-in vivo relationship is investigated with liver microsomes, there are some requisite assumptions: 1) clearance must be primarily mediated by phase I metabolism in the liver (e.g., P450 or FMO) and 2) clearance must not be susceptible to other non-P450-mediated metabolism or excretion. Indeed, results of metabolism studies of CJ-13,610 in humans demonstrated that this compound possesses the aforementioned properties. [Results from rat studies indicated a negligible role for hepatobiliary transport in the disposition of CJ-13,610 (data not shown).] In particular, our data indicated that the in vitro biotransformation of CJ-13,610 results primarily in metabolites arising from S-oxidation (M2, Fig. 2a) and imidazole oxidation (M1, Fig. 2a), whereas oxidation of the tetrahydropyran moiety was also observed (M3) as a principal biotransformation pathway in CJ-13,610 metabolism (Fig. 3). More importantly, metabolic profiling studies in rat and dog microsomes also indicate that the aforementioned biotransformation pathways significantly contribute to the hepatic metabolism of CJ-13,610 (data not shown).

Intrinsic clearance data generated using the traditional substrate depletion approach and the well stirred model (eq. 1) demonstrated that predicted CL_h, when corrected for both plasma protein and nonspecific microsomal binding, was comparable to total plasma clearance (within 1.6-fold) observed for both rat and dog (Table 1). Microsomal binding has been reported to potentially affect estimates of hepatic clearance when microsomal fractions are used, especially for basic lipophilic amines (Obach, 1997, 1999). Although binding to plasma proteins was moderately high across the species tested ($f_u = 0.07-0.13$), nonspecific binding to microsomes was low [$f_{u(microsomes)} \sim 0.8$] and did not have a dramatic impact on the calculated hepatic clearance (still within 2-fold of

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TABLE 4

Comparison of simulated human pharmacokinetic parameters using various scaling methodologies for clearance and Vd_{ss} and pharmacokinetics of CJ-13,610 after a 30-mg single oral dose to healthy male subjects

All pharmacokinetic simulations were conducted using WinNonlin, and an absorption rate constant (k_a) of 0.83 h⁻¹ (estimated by modeling rat oral pharmacokinetic data) was used. High oral bioavailability was also assumed. n = 4.

Scaling Methodology	Clearance	Vd _{ss} ^a	AUC _{0-∞}	C_{\max}	t _{1/2}
	ml/min/kg	l/kg	$ng \cdot h/ml$	ng/ml	h
Human liver microsomes (Vd_{ss} scaled from rat) Human liver microsomes (Vd_{ss} scaled from dog) Scaled from rat pharmacokinetics Scaled from dog pharmacokinetics Single oral dose (30 mg)	3.6^b 3.6^b 5.3^c 3.2^c	6.7 3.2 6.7 3.2	1802 1802 1222 2014 1970 (220)	51 97 48 98 144 (34)	21.7 10.4 14.7 11.6 13.4 (2.1)

^a Predicted Vd_{ss} in human from either rat or dog using eq. 8.

^b Predicted clearance in human using eq. 1.

^c Predicted clearance in human using eq. 7.

total plasma clearance). Nonetheless, in correcting for both plasma and nonspecific microsomal binding, estimates of hepatic clearance were more representative of total in vivo clearance (Table 1).

CL'_{int} for the sulfoxidation of CJ-13,610 calculated by eq. 3 after enzyme kinetic analysis closely resembled total intrinsic clearance calculated from substrate depletion methodologies. Although sulfoxidation was not the only metabolic pathway observed in liver microsomes after incubations with CJ-13,610 (20 μ M) for metabolite profiling (Fig. 3), the observation that intrinsic clearance calculated by V_{max}/K_m for the sulfoxidation pathway was comparable to that calculated by substrate depletion methodologies (e.g., total metabolism) suggests that sulfoxidation is the predominant metabolic pathway at lower and more relevant concentrations (e.g., $\sim 1 \mu$ M). In fact, the sulfoxide has been reported to be the predominant circulating metabolite in plasma from humans after oral administration (Dalvie et al., 2009).

Experiments were also conducted in an effort to identify which liver microsomal enzymes were involved in the metabolism of CJ-13,610. When incubated with a battery of recombinantly expressed cytochrome P450 enzymes, it appeared that the CYP3A family (CYP3A4 and 3A5) was predominantly involved in the overall metabolism of CJ-13,610. In addition, when coincubated with the potent CYP3A inhibitor ketoconazole, sulfoxidation of CJ-13,610 was inhibited with an estimated IC_{50} of 7 nM, closely resembling the inhibitory potency of ketoconazole toward known selective probe substrates of CYP3A4 such as midazolam and testosterone (Racha et



FIG. 7. Simulated human pharmacokinetic profile of CJ-13,610 using various scaling methodologies compared with the clinical human pharmacokinetic profile after a single 30-mg oral dose to normal healthy subjects (n = 4). An absorption rate constant (k_a) of 0.83 h⁻¹ (estimated by modeling rat oral pharmacokinetic data) was used for human pharmacokinetic simulations, and clearance and Vd_{ss} parameters used in simulations are summarized in Table 4. All simulations were conducted using WinNonlin 5.2.

al., 2003). With CYP3A being the predominant metabolic pathway, as suggested by our in vitro studies, CJ-13,610 could be at risk for victim drug-drug interactions if coadministered with potent inhibitors of CYP3A4 such as azole antifungals or antimicrobials such as erythromycin. Clinical drug-drug interaction studies would have to be conducted to determine the magnitude of this potential interaction. hFMO3, a microsomal enzyme that typically oxidizes nucleophilic heteroatoms, did not appear to be involved in the sulfoxidation of CJ-13,610 (Fig. 5A), whereas benzydamine, a reported probe substrate for FMO3 (Störmer et al., 2000), was efficiently metabolized (data not shown). This result was somewhat surprising in consideration of the rapid oxidation of the sulfur by CYP3A4 and 3A5 and literature reports confirming that sulfur-containing drug molecules, including cimetidine, methimazole, and methyltolylsulfide, may be substrates for FMO3 (Cashman, 2000). It is unfortunate that detailed structure-activity relationship differences between cytochrome P450 and FMO enzymes have not been thoroughly investigated, so the chemical properties of CJ-13,610 that preclude a role for hFMO3 are not evident at this time. Recombinant human FMO1 and FMO5 were not tested for CJ-13,610 sulfoxidation activity, because hFMO3 is the primary isoform present in human liver.

The pharmacokinetic properties of CJ-13,610 were characterized in an early clinical program with healthy subjects after single-dose administration. Exposure was linear and proportional up to a dose of 300 mg (Fig. 6). With these data available, we chose to retrospectively evaluate various scaling methods to see which may be the most optimal for predicting the pharmacokinetic profile of CJ-13,610 in humans, information that would be tremendously valuable for any discovery programs working with this chemical class of compounds. First, based on the favorable in vitro-in vivo correlation for clearance observed with rat and dog (Table 1), we chose to scale clearance from human liver microsomes, and use volume of distribution from rat and dog single-species allometric scaling to compare simulations to the observed pharmacokinetic profile after a 30-mg single oral dose. This dose was arbitrarily selected for simulation, considering that dose linearity was observed with CJ-13,610 up to 300 mg. Because of a larger Vd_{ss} scaled from rat (6.7 l/kg), the half-life when HLM were used to predict clearance was longer (21.7 h) than observed (13.4 h), even though the AUC was closely predicted (Table 4). This resulted in a projected C_{max} (51 ng/ml) that was roughly one-third the observed C_{max} (144 ng/ml). However, when clearance from HLM was scaled to humans and Vd_{ss} was scaled from dog, the pharmacokinetic profile of CJ-13,610 was predicted within 10% for AUC, resulting in a favorable half-life projection of 10.4 h (Table 4). In this case, it seems that dog would be a preferred species for pharmacokinetic characterization and, more specifically, for predicting Vd_{ss} in humans. A report that was recently published by colleagues at Pfizer suggested that allometric

scaling from a single species [e.g., single-species allometric scaling (SSS)] may be just as successful as full allometric scaling using multiple species (Hosea et al., 2009). Therefore, these methods were also investigated for their predictive ability. In an attempt to predict the pharmacokinetic profile of CJ-13,610 using rat SSS, the AUC and $C_{\rm max}$ were underpredicted, because of the higher projected clearance and Vd_{ss}, although the projected half-life closely predicted the observed half-life (Table 4). When the same single-species approach was taken using dog, the pharmacokinetic profile for CJ-13,610 was predicted with comparable success compared with use of in vitro HLM data, attributable to similar scaled clearance (3.2 ml/min/kg from dog SSS).

In summary, CJ-13,610 seems to be metabolized in vitro exclusively by the cytochrome P450 family of drug-metabolizing enzymes and specifically the CYP3A subfamily. Oxidation of the sulfur heteroatom was the predominant metabolic pathway and was consistently observed across the species tested. Overall, good in vitro-in vivo correlation was observed in rat and dog and the human pharmacokinetic profile of CJ-13,610 observed in the clinic after a single oral dose was closely predicted using traditional scaling methods from human liver microsomes. These data provide confidence moving forward that scaling in vitro intrinsic clearance data from HLM may be a successful approach for predicting clearance in humans for this chemical series. It is anticipated that structurally related analogs within the same chemical class will probably possess similar metabolic clearance routes, and thus the use of predicted hepatic clearance derived from human liver microsomes may result in a high probability of successful pharmacokinetic predictions in humans and serve as an efficient method for selection of additional drug candidates with suitable pharmacokinetic properties.

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