Disposition and Metabolism of LY2452473, a Selective Androgen Receptor Modulator, in Humans

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ABSTRACT:

The disposition and metabolism of isopropyl N-[(2S)-7-cyano-4-(2-pyridylmethyl)-2,3-dihydro-1H-cyclopenta[b]indol-2-yl]carbamate (LY2452473), a selective androgen receptor modulator in humans was characterized after a single 15-mg (100 µCi) oral dose of [14C]LY2452473 to six healthy male subjects. LY2452473 was absorbed rapidly (time to reach maximum plasma concentration for both LY2452473 and total radioactivity was 2–3 h) and cleared slowly (plasma terminal t1/2 of 27 h for LY2452473 and 51 h for the total radioactivity). LY2452473 and metabolites S5 (acetylamide) and S12 (hydroxylation on the cyclopentene) were minor circulating entities in plasma, accounting for approximately 42, 21, and 35% of the total radioactivity exposure, respectively, as calculated from relative area under the concentration versus time curves from zero to 48 h derived from the plasma radiochromatograms. The radioactive dose was almost completely recovered after 312 h with 47.9% of the dose eliminated in urine and 46.6% in feces. Minimal LY2452473 was detected in excreta, indicating that metabolic clearance was the main route of elimination. Multiple metabolic pathways were observed with no single metabolic pathway accounting for more than 30% of the dose in excreta. Metabolite S10 (a diol across the cyclopenta-indole linkage) was the largest excretory metabolite (approximately 14% of the dose). S10 displayed interesting chemical and chromatographic properties, undergoing conversion to the corresponding epoxide under acidic conditions and conversion back to the diol under neutral conditions. An in vitro phenotyping approach indicated that CYP3A4 was the largest contributor to LY2452473 depletion.

Introduction

Selective androgen receptor modulators are classified as androgen receptor ligands (Gao and Dalton 2007). Isopropyl N-[(2S)-7-cyano-4-(2-pyridylmethyl)-2,3-dihydro-1H-cyclopenta[b]indol-2-yl]carbamate (LY2452473) is a selective androgen receptor modulator being developed for the treatment of disorders related to hypogonadism in men. Data from clinical studies of anabolic anabolic steroids (for example, testosterone or methyltestosterone) suggest that androgens play an important role in bone formation (Falafati-Nini et al., 2000; Leder et al., 2003). Other studies have demonstrated an increase in muscle mass and strength, which may correlate with a decrease in the frequency of patient falls (Wolfson et al., 1995; Schroeder et al., 2003; Orwell et al., 2006). It has also been suggested that the reason some men with erectile dysfunction do not obtain satisfactory outcomes from currently available phosphodiesterase 5 inhibitors (PDE5i) may be impaired endothelial-derived factors in the penile arteries and underlying endothelial dysfunction itself (Sullivan et al., 2001), resulting in insufficient nitric oxide release. Androgen supplementation is thought to improve erectile function by increasing nitric-oxide synthase expression, which increases penile nitric-oxide synthase synergy with the PDE5i (Shabsigh et al., 2004; Rosenthal et al., 2006; Buvat et al., 2009). Unfortunately, androgen use is limited because individuals may develop adverse reactions including sleep apnea, acne, weight gain, hirsutism, voice changes, emotional changes, alopecia, adverse lipid changes, increased hematocrit levels, and abnormal liver function test results (Gelfand and Wiita 1997; Basaria and Dobs 1999; Bachmann et al., 2002).

LY2452473 has the potential advantage of eliciting androgen receptor specificity and tissue selectivity to produce the beneficial effects of testosterone on bone, muscle, and improvements in erectile function over a PDE5i alone, with minimal steroid-related adverse effects. The objective of this work was to characterize the disposition

ABBREVIATIONS: LY2452473, isopropyl N-[(2S)-7-cyano-4-(2-pyridylmethyl)-2,3-dihydro-1H-cyclopenta[b]indol-2-yl]carbamate; PDE5i, phosphodiesterase inhibitor; LSN2519879, S4, (2S)-2-amino-4-(2-pyridylmethyl)-2,3-dihydro-1H-cyclopenta[b]indole-7-carbonitrile; LSN2588307, S5, N-(2S)-7-cyano-4-(2-pyridylmethyl)-2,3-dihydro-1H-cyclopenta[b]indol-2-ylacetamide; LSN3040390, S43, [1S]-2-hydroxy-1-methyl-ethyl-N-(2S)-7-cyano-4-(2-pyridylmethyl)-2,3-dihydro-1H-cyclopenta[b]indol-2-yl)carbamate; LSN3040393, S60, [1R]-2-hydroxy-1-methyl-ethyl-N-(2S)-7-cyano-4-(2-pyridylmethyl)-2,3-dihydro-1H-cyclopenta[b]indol-2-yl)carbamate; LSN2892448, isopropyl N-[(2S)-7-cyano-4-[dideuterio(3,4,5,6-tetradeterio-2-pyridyl)methyl]-2,3-dihydro-1H-cyclopenta[b]indol-2-yl]carbamate; LSN2533520, 2-amino-4-[[3-fluorophenyl)methyl]-2,3-dihydro-1H-cyclopenta[b]indol-7-carbonitrile hydrochloride; LSN3049319, S12, isopropyl N-[(2S,3S)-7-cyano-3-hydroxy-4-(2-pyridylmethyl)-2,3-dihydro-1H-cyclopenta[b]indol-2-yl]carbamate; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS/MS, tandem mass spectrometry; %ROI, percentage region of interest; MS, mass spectrometry; ESI, electrospray ionization; HLM, human liver microsomes; P450, cytochrome P450; rP450, recombinant P450; AUC, area under the concentration versus time curve.
and metabolism of LY2452473 in humans to help guide future clinical development.

Materials and Methods

LY2452473, isopropyl N-[(2S)-7-[(14C)cyano-4-(2-pyridylmethyl)-2,3-dihydro-1H-cyclopenta[b]indole-2-yl]carbamate (14C)LY2452473] (Fig. 1), (2S)-2-amino-4-(2-pyridylmethyl)-2,3-dihydro-1H-cyclopenta[b]indole-7-carbonitride (LSN2519879, S4), (2S)-7-cyano-4-(2-pyridylmethyl)-2,3-dihydro-1H-cyclopenta[b]indole-2-yl]acetamide (LSN2588307, S5), [(1S)-2-hydroxy-1-methyl-ethyl] N-[(2S)-7-cyano-4-(2-pyridylmethyl)-2,3-dihydro-1H-cyclopenta[b]indole-2-yl]carbamate (LSN3040390, S43), [(1R)-2-hydroxy-1-methyl-ethyl] N-[(2S)-7-cyano-4-(2-pyridylmethyl)-2,3-dihydro-1H-cyclopenta[b]indole-2-yl]carbamate (LSN2892448), and 2-amino-4-[(3-fluorophenyl)methyl]-2,3-dihydro-1H-cyclopenta[b]indole-7-carbonitride hydrochloride (LSN2533520) were synthesized at Eli Lilly and Company (Indianapolis, IN). Isopropyl N-[(2S,3S)-7-cyano-3-hydroxy-4-(2-pyridylmethyl)-2,3-dihydro-1H-cyclopenta[b]indole-2-yl]carbamate (LSN3049319, S12) was synthesized at Adesix, Inc. (New Castle, DE). The radiochemical purity of [14C]LY2452473 was approximately 98.7% as determined by HPLC. The 14C-labeled LY2452473 was blended with the nonlabeled LY2452473 to give a specific activity of 6.35 μCi/ml for each study. The 96-well Scintiplates were purchased from Wallac (Turku, Finland).

Subjects and Dosing.

This study was conducted at Covance Clinical Research Unit Inc. (Madison, WI). A total of six healthy male volunteers between the ages of 22 and 48 years and body weights of 75.5 to 91.3 kg participated in this study. All the subjects provided written, informed consent before participation in the study. The study protocol and consent documents were approved by the institutional review board. The study was conducted in accordance with applicable laws and regulations, Good Clinical Practice, and the Declaration of Helsinki. Subjects fasted from at least 10 h before dosing until 4 h after dosing. Each subject received 15 mg of LY2452473 (considered to be in the therapeutic dose range) with approximately 100 μCi of radioactivity, which was prepared by dissolving 14C-labeled and nonlabeled LY2452473 in 5 ml of ethanol, and then suspended with 10 ml of Ora-Plus and 190 ml of purified water. The dosing container was rinsed twice with vehicle, and the dose was administered to each subject. There were no deaths or other serious adverse effects observed during the study. Thus, no subject discontinued the study because of an adverse event.

Sample Collection.

Venous blood samples were collected in heparin-containing tubes at 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 48, 72, 96, 120, and 168 h postdose. After removal of a portion sample for measurement of radioactivity in whole blood, plasma was prepared by centrifugation. Urine samples were collected before dosing (as a control sample) and at 0 to 6, 6 to 12, 12 to 24, and every 24 h thereafter until the specified release criterion was met. Feces samples were collected before dosing (as a control sample), and every 24 h postdose until the specified release criterion was met. The specified release criterion was that at least 90% of the administered radioactivity was recovered or the radioactive dose in urine and feces samples together was less than 1.0% of the total administered radioactivity during a 24-h collection period for two consecutive collection periods. The last urine and feces samples for all the subjects were collected up to 312 h postdose. All samples were stored frozen at approximately −70°C and put on dry ice for shipment.

Measurement of Total Radioactivity.

All measurements of total radioactivity were performed by liquid scintillation counting in a Packard 2900TR liquid scintillation counter (Packard Instruments, Wallach, MA). Aliquots of plasma (approximately 0.2 g) and urine (approximately 3 to 5 times the volume of the corresponding whole blood) were directly mixed with Ultima Gold XR scintillation cocktail (PerkinElmer Life and Analytical Sciences) and counted. Radioactivity in feces and whole blood was measured after combustion. Before combustion, fecal samples were homogenized with addition of ethanol-water (50/50; v/v) at approximately 3 to 5 times original fecal weight. Aliquots of fecal homogenates (approximately 0.5 g) and whole blood (approximately 0.2 g) were combusted in a Packard Sample Oxidizer (model 307). The resulting 14CO2 was trapped in a mixture of Carbo-Sorb and Permafluor and assayed by liquid scintillation counting.

Pharmacokinetic Analysis.

Plasma concentrations of LY2452473 for each subject were determined using a validated LC-MS/MS method at Advion, Inc. (Ithaca, NY). The assay was validated for a concentration range of 0.1 to 25 ng/ml for LY2452473 (a 4000-fold dilution was validated, extending the range to 100,000 ng/ml). Samples above the limit of quantification were diluted and reanalyzed to yield results within the calibration range. Pharmacokinetic parameters for LY2452473 and total radioactivity in plasma were calculated by standard noncompartmental methods using WinNonlin Professional (version 5.2; Pharsight, Mountain View, CA). Plasma concentration values below the limit of quantification were excluded from the analysis except for those before the first quantifiable concentration postdose, which were set to a value of 0 for the noncompartmental analysis of individual subjects. Mean concentrations at a given time point were calculated only if ≥2/3 of samples were quantifiable.

Sample Preparation for Metabolite Profiling and Identification.

Plasma samples collected at 1, 2, 4, 8, 24, and 48 h from each subject were extracted individually using a protein precipitation method. For each volume of plasma, 2 volumes of acetonitrile were added, the mixture was vortexed and centrifuged, and the supernatant was collected. This process was repeated 2 more times for each sample. The supernatants for each sample were combined, dried down, and reconstituted in appropriate volumes of acetonitrile and 10 mM ammonium acetate (2:8, v/v) for analysis. The average extraction recovery was 96.4 ± 12.8% (S.D.). Fecal homogenates with radioactivity >5% of the radioactive dose from each subject were extracted individually three times with approximately 1.5 (w/v) acetonitrile-10 mM ammonium acetate. The extracts were dried down and reconstituted in appropriate volumes of 1.2 (v/v) of methanol-10 mM ammonium acetate. The average extraction recovery was 94.4 ± 7.0% (S.D.). Urine samples collected from 0 to 6, 6 to 12, 12 to 24, and 24 to 48 h were pooled, as were samples collected from 48 to 72, 72 to 96, and 96 to 120 h, based on percentage of urine weight for each subject. Pooled urine samples were extracted with C18 SPE cartridges (Bond Elute, Varian, Inc., Palo Alto, CA). The cartridges were conditioned with methanol followed by 10 mM ammonium acetate. After loading, samples were washed with 10 mM ammonium acetate and eluted with 50:50 (v/v) methanol-10 mM ammonium acetate followed by methanol. The collected samples were dried down and reconstituted in appropriate volumes of methanol-10 mM ammonium acetate (1.3, v/v) for analysis. The average extraction recovery was 98.0 ± 4.7% (S.D.).

Metabolite Radio Profiling by HPLC.

Metabolites in various matrices were separated on a reverse-phase HPLC column (Discovery C18, 4.6 × 150 mm, 5-μm particle size; Supelco, Bellefonte, PA). Mobile phase A was 10 mM aqueous ammonium acetate and mobile phase B was methanol with the following gradient: 0 to 3 min, held at 10% of B; 3 to 80 min, linear gradient from 10 to 50% B; 80 to 95 min, linear gradient from 50% to 70% B; 95 to 95.1 min, ramping from 70% to 90% B; 95.1 to 100 min, isocratic at 90% B; 100 to 100.1 min, ramping from 90% to 10% B; and 100.1 to 105 min, isocratic at 10% B for column re-equilibration. The total HPLC flow rate was 1 ml/min, which was split postcolumn so that approximately 0.2 ml/min was introduced to the ion source of the mass spectrometer for metabolite identification and 0.8 ml/min was collected into 96-well Scintiplates for radio-profiling. HPLC frac-
tions were collected at 15-s intervals from 0 to 96 min. The plates were dried in a vacuum centrifuge dryer and counted for radioactivity (counts per minute) in a MicroBeta counter (Trilux; PerkinElmer Life and Analytical Sciences). The radiochromatograms were reconstructed, and radioactive peaks were integrated using ProFSA (PerkinElmer Life and Analytical Sciences) on the basis of counts per minute of each fraction versus time. A peak with a peak height <10 cpm was not integrated and defined as below the quantification level. Each integrated peak was given a percentage region of interest (%ROI) value, and the %ROI values for all integrated peaks on a radiochromatogram together were 100%. The amount of each metabolite and parent compound in plasma was expressed as the percentage of the total radioactivity in a given plasma sample, which was calculated by the %ROI of a given peak multiplied by the extraction recovery. The amount of each metabolite and parent compound in excreta was expressed as a percentage of the dose, which was calculated by the %ROI of a given peak multiplied with extraction recovery and percentage of the dose excreted in a given sample.

**LC-MS and LC-MS/MS Analysis.** The same HPLC conditions as described above for metabolite radioprofileing were used for LC-MS and LC-MS/MS analyses. In addition, a smaller diameter HPLC column (Discovery C18, 2.1 x 150 mm, 5-µm particle size; Supelco) with the same mobile phases and same gradient at flow rate 0.25 ml/min (no split) was applied for reanalysis of isolated S10 and degradation products. LC-MS and LC-MS/MS analyses for nominal mass measurement were conducted on an ion trap mass spectrometer (Finnigan LCQ Advantage; Thermo Finnigan, Waltham, MA). The mass spectrometer was set to positive electrospray ionization (ESI) mode with capillary temperature at 350°C and spray voltage at 5.0 kV. The full-scan MS spectra were obtained from m/z 120 to m/z 1000. The MS/MS spectra were obtained with the collision energy at 35%. LC-MS and LC-MS/MS analyses for accurate mass measurements were conducted on a Finnigan LTQ Orbitrap XL mass spectrometer or on a SYNAPT G2 mass spectrometer (Waters, Manchester, UK). The MS and MS/MS data from the LTQ Orbitrap instrument were generated in positive ESI mode with a capillary temperature at 350°C and spray voltage at 5.0 kV. The full-scan MS spectra were obtained from m/z 120 to m/z 1000. The MS/MS spectra were obtained with the collision energy at 45% using the HCD collision cell. The MS and MS/MS data from SYNAPT G2 were generated at both positive and negative ESI modes with source temperature at 120°C, desolvation temperature at 600°C, capillary voltage at 2.75 kV, and cone voltage at 18 V. The full-scan MS and MS/MS spectra were obtained from m/z 50 to m/z 1000. The MS/MS spectra were obtained with collision energy ramp from 20 to 30 eV.

**Nanospray MS* Analysis.** To obtain further mass spectrometry data for structure elucidation, MS* (n = 2–5) spectra were generated using nanospray mass spectrometry. HPLC fractions containing the metabolite(s) of interest collected during metabolite radioprofileing were reconstituted in an appropriate volume of methanol-water-acetic acid (50:50:1, v/v/v). The reconstituted samples were infused using nanospray (NanoMate; Advion) into the ion source of an ion trap mass spectrometer (LCQ Advantage). The NanoMate was operated with gas pressure at 0.3 psi and spray voltage at 1.5 kV. The LCQ Advantage instrument was operated at positive ESI with capillary temperature at 200°C and collision energy at 35%.

**Metabolite S10 Generation in Human Liver Microsomes.** To fully characterize the structure of metabolite S10, an incubation was conducted to obtain this metabolite in sufficient amount. The incubation was performed in 100 ml of phosphate buffer (100 mM, pH 7.4) containing HLMs (mixed gender pool of 10 subjects) (1 mg/ml protein), LY2452473 (50 μM), and NADPH-regenerating cofactors at 37°C for 4 h. After incubation, the protein was precipitated with an equal volume of acetonitrile. After centrifugation, the supernatant was concentrated 3- to 4-fold in a 40°C water bath with nitrogen purge for metabolite isolation by preparative HPLC. The semipreparative HPLC was performed with an HPLC column (Discovery C18, 10 x 250 mm, 5-µm particle size; Supelco) with mobile phase A (0.2% formic acid) and mobile phase B (acetonitrile) at the following gradient: 0 to 5 min, held at 20% of B; 5 to 25 min, linear gradient from 20 to 35% B; 25 to 30 min, linear gradient from 35 to 90% B; 30 to 38 min, isocratic at 90% B; 38 to 38.1 min, ramping to 20% B; and 38.1 to 45 min, isocratic at 20% B for column re-equilibration. Flow rate was 4 ml/min. Fractions were collected at 15-s intervals. Fractions containing metabolite S10 were combined and freeze-dried under vacuum.

**H/D Exchange Experiments.** Synthetic standards or metabolites isolated from in vitro or in vivo matrices were dissolved in acetoni-terile-2H₅ formic acid in D₂O (50:50, v/v). Samples were introduced through a syringe pump into the ion source of the mass spectrometer (Waters SYNAPT G2), which was set at positive ESI with capillary voltage at 2.75 kV, sample cone voltage at 18 V, source temperature at 120°C, desolvation temperature at 450°C, cone gas at 30 l/h, and desolvation gas at 450 l/h. TiCl₃ Reaction of Metabolite S10. Metabolite S10 fractions collected during fecal radioprofileing were reconstituted in approximately 200 μl of methanol, mixed with 6 μl of TiCl₃, placed on ice for approximately 90 min, and neutralized with 100 μl of 5 N KOH. The resulting sample was centrifuged at 10,000 rpm for 10 min, and the supernatant was blown to dryness in a 40°C water bath with nitrogen purge and reconstituted in 60 μl of methanol-water (50:50) for LC-MS analysis.

**NMR Analysis of Metabolite S10.** LY2452473 and the isolated S10 sample were dissolved in approximately 150 μl of acetoni-terile-d₅ and transferred to a Wilmad 328 NMR tube. Data were acquired on a AVANCE 600-MHz NMR spectrometer (Bruker, Newark, DE) used with a gradient, triple-resonance cold probe. The suite of experiments included standard proton, two-dimensional homonuclear gradient correlated spectroscopy, hetero-nuclear single quantum coherence, and long-range heteronuclear multiple bond correlation. The data were acquired at 25°C and referenced to the solvent.

**Identification of Human P450s Involved in the Depletion of LY2452473.** HLMs (pool of 200 donors) were from Xenotech, LLC (Lexena, KS). Recombinant P450 enzymes (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, and CYP3A5) were obtained from Cypex, Ltd. (Dundee, Scotland, UK). CYP2B6, CYP2C8, CYP2C9, CYP2E1, CYP3A4, and CYP3A5 were expressed with cytochrome b₅. The other P450s (CYP1A2, CYP2C19, CYP2D6, and CYP2J2) were not expressed with cytochrome b₅. Incubations, LC-MS/MS analysis, and data handling were performed by Xenotech, LLC.

**Conditions for P450 Reaction Phenotyping.** Incubation mixtures (0.2 ml) contained potassium phosphate buffer (50 mM, pH 7.4), MgCl₂ (3 mM), and EDTA (1 mM, pH 7.4) with an NADPH-generating system. The NADPH-generating system consisted of NADP (1 mM, pH 7.4), glucose 6-phosphate (5 mM, pH 7.4), and glucose-6-phosphate dehydrogenase (1 unit/ml). LY2452473 was dissolved in acetone and added directly to the incubation mixtures with a final acetoni-terile concentration of 1% (v/v). For screening, LY2452473 (1 μM) was incubated with a panel of recombinant human P450s (0.05 pmol/μl) for 30 min. According to the screening results, several P450s showing notable substrate depletion were selected for further optimization of incubation conditions. The optimized incubations were conducted with the same LY2452473 concentration (0.1 μM) but with different rP450 enzyme concentrations (0.05 pmol/μl for CYP2C9 and CYP2D6 and 0.025 pmol/μl for CYP2C19, CYP2J2, and CYP3A4) and different incubation times (30 min for CYP2C9 and CYP2J2 and 10 min for CYP2D6, CYP2J2, and CYP3A4). Incubations of LY2452473 with control Bactosomes and membranes containing human NADPH-cytochrome P450 oxidoreductase but not human cytochrome P450 (reductase control) served as negative controls.

Reactions were initiated by the addition of the NADPH-generating system and were terminated by the addition of 175 μl of stop reagent (acetoni-terile) containing two internal standards (LSN2892448 and LSN2533520, at final concentrations of 2.5 ng/ml). After reactions were terminated, samples were normalized to the volume of 0.4 ml by addition of 25 μl of blank (50:50, acetoni-terile-water). Samples were centrifuged (920g for 10 min at 10°C), and supernatant fractions were analyzed by LC-MS/MS.

**Intrinsic clearance (CLᵢᵣᵣ) of substrate depletion was calculated as**

\[
CLᵢᵣᵣ = kᵢᵣᵣ × \text{Incubation volume} \]

\[
\text{pmol} \times \text{rCYP} \]

where \(kᵢᵣᵣ\) is the substrate depletion rate constant determined by calculating the slope from time (minutes) and natural log (ln) percentage of control remaining data using the LINEST function in Excel. For estimation of relative contribution of each individual P450 to LY2452473 depletion, CLᵢᵣᵣ values were normalized by multiplying CLᵢᵣᵣ by reported hepatic P450 content (Rodrigues, 1999, Lee et al., 2010) and summed, and the fraction metabolized (fᵢᵣᵣ) was determined. To assess the effects of known P450-selective inhibitors on
LY2452473 depletion, LY2452473 (2 μM) was incubated in duplicate with HLMs (0.2 mg of protein/ml) for 30 min in the presence of the chemical inhibitors: 0.1, 1, and 10 μM sulfaphenazole (CYP2C9 inhibitor); 1, 10, and 50 μM modafinil (CYP2C19 inhibitor); 0.1, 1, and 10 μM quinidine (CYP2D6 inhibitor); and 0.01, 0.1, 0.5, and 1 μM ketoconazole (CYP3A4/5 inhibitor).

Results

Excretion of the Radioactive Dose and Plasma Pharmacokinetics. The radioactivity excretion profiles were similar among subjects (Fig. 2). The mean recovery of radioactivity in urine and feces over 0 to 312 h postdose was approximately 94.5 ± 3.03% with 47.9 ± 8.35% radioactivity recovered in urine and 46.6 ± 8.17% in feces. The majority (80%) of the administered radioactivity was recovered in the first 120 h postdose. The individual plasma concentration versus time profiles of LY2452473 and total radioactivity were similar in all subjects (data not shown). The mean concentration versus time curves for LY2452473 in plasma and for total radioactivity in plasma and in whole blood are illustrated in Fig. 3. The peak concentrations occurred between 2 and 3 h postdose (Table 1) for both LY2452473 and the total radioactivity. The mean \( C_{\text{max}} \) of LY2452473 was approximately 51% of that of the total radioactivity and plasma exposure (AUC\( _{0-\infty} \)) of LY2452473 accounted for approximately 17% of that of the total radioactivity exposure. The concentrations of LY2452473 and the total radioactivity showed a biphasic decline with a mean terminal half-life of approximately 27 h for LY2452473 and 51 h for the total radioactivity.

Radioprofiles of LY2452473 and Metabolites in Plasma and Excreta. The representative radioprofiles of plasma, urine, and feces are shown in Figs. 4, 5, and 6, respectively. Amounts of LY2452473 and metabolites in plasma and excreta are presented in Tables 2 and 3, respectively. LY2452473 was a predominant circulating entity in plasma during early time points, but the relative percentages of metabolites increased over time, reflecting a slower clearance of metabolites than parent from plasma. Major circulating metabolites included S5 (resulting from the amide hydrolysis to S4 with subsequent acetylation) and S12 (aliphatic hydroxylation at the cyclopentene ring). To assess the relative exposure of major circulating entities, the concentration (nanogram-equivalents per milliliter) values at 1, 2, 4, 8, 24, and 48 h for LY2452473 and the major circulating metabolites were calculated on the basis of relative peak areas on the radiochromatogram from a given plasma sample and the total nanogram-equivalents per milliliter value of the original sample. The resulting concentration values were used to calculate the AUC\( _{0-48} \). These values are to be considered as semiquantitative only and could be different from those generated from the validated quantitative LC-MS/MS method used in the pharmacokinetic analysis. The exposures (AUC\( _{0-\infty} \)) calculated in this way for LY2452473, S5, and S12 relative to the total radioactivity exposure were 42, 21, and 35%, respectively. A few additional metabolites were also detected in plasma, but they accounted for a minor amount of radioactivity.

Pooled 0 to 48 and 48 to 120 h urine samples from all six subjects were profiled. More than 25 metabolites were identified, whereas the parent was not detected in urine. Together, identified urinary metabolites accounted for 33% of the administered dose in the 0 to 120 h urine, and none of these metabolites alone accounted for more than 4% of the dose.

Fecal homogenates with radioactivity >5% of the administered dose in each subject were selected for radioprofiling. The selected fecal samples ranged from 0 to 192 h postdose and covered approximately 94% of the total dose eliminated in feces. LY2452473 and 10 metabolites were identified in feces. LY2452473 accounted for 2% of

![Fig. 2](image-url) Arithmetic mean (± S.D.) cumulative excretion of total radioactivity in urine, feces, and overall, after oral administration of a single 15-mg (100 μCi) dose of \(^{14}C\)LY2452473c in healthy male human subjects.

![Fig. 3](image-url) Arithmetic mean (± S.D.) concentrations of LY2452473 in plasma and total radioactivity in plasma and in whole blood after oral administration of a single 15-mg (100 μCi) dose of \(^{14}C\)LY2452473 in healthy male human subjects (top panel, Cartesian plot; bottom panel, semilogarithmic plot).
AUC0–t, which maximum drug concentration reached, extra-vascular administration; N.C., not calculated.

After oral administration of a single 15-mg (100 μCi) dose of [14C]LY2452473 in healthy male human subjects, the largest radioactive peaks in feces were S3 (parent + O), S10 (parent + O + H2O), and S13 (parent + O), accounting for 8, 14, and 10% of the administered dose in the fecal samples analyzed. Each of the other seven metabolites accounted for less than 4% of the administered dose in the fecal samples analyzed.

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TABLE 1
Plasma mean pharmacokinetic parameters of LY2452473 and total radioactivity in healthy male human subjects after oral administration of a single 15-mg (100 μCi) dose of [14C]LY2452473

<table>
<thead>
<tr>
<th>Geometric Mean (CV%) (n = 6)</th>
<th>LY2452473</th>
<th>Total Radioactivity (Radioequivalents)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax ng/ml</td>
<td>91.5 (21)</td>
<td>179 (17)</td>
</tr>
<tr>
<td>tmax h</td>
<td>2 (2–3)</td>
<td>3 (2–3)</td>
</tr>
<tr>
<td>t1/2, h</td>
<td>27 (20–39)</td>
<td>51 (37–63)</td>
</tr>
<tr>
<td>Vz/F, liters</td>
<td>444 (22)</td>
<td>N.C.</td>
</tr>
<tr>
<td>CV%, coefficient of variation</td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
</tbody>
</table>

Structural Elucidation of LY2452473 and Major Metabolites by LC-MS and LC-MS/MS. LC-MS and LC-MS/MS with nominal mass and accurate mass measurements were used for identification of LY2452473 and its metabolites. The molecular ions and characteristic product ions for LY2452473 and metabolites found in human plasma, urine, and feces are listed in Table 4. The proposed metabolic pathways are shown in Fig. 7.

LY2452473. The protonated molecular ion of LY2452473 was m/z 375. The proposed fragmentation pathway is shown in Fig. 8. Loss of C7H15O2 gave the product ion m/z 333. Loss of C7H15O2 gave the product ion m/z 289. Loss of C7H15O2 and NH3 led to the product ion m/z 272 (base peak in the product ion spectrum). Loss of C7H15O2, NH3, and C7H15 gave product ion m/z 246. Loss of C7H15O2 and the pyridinylmethyl radical gave product ion m/z 197. Loss of C7H15O2, the pyridinylmethyl radical, and NH3 gave product ion m/z 180. Other product ions included the cyano-indole-methyl cation m/z 155, the protonated methyl pyridine ion m/z 94, the methyl pyridine radical ion m/z 93, the H2O adduct of the pyridinyl methyl cation m/z 110, and the protonated pyridine ion m/z 80. Metabolites showed similar fragmentation pathways, which allowed the elucidation and assignment of metabolite structures.

S3. The protonated molecular ion of S3 was m/z 391. The accurate mass of the M + H+[O] of S3 was 391.1765, which is consistent with the chemical formula C22H23O3N4 (parent + O). MS2 of S3 gave the product ions at m/z 288 (corresponding to the ion m/z 272 observed in parent + O), m/z 262 (corresponding to the ion m/z 246 observed in parent + O), m/z 213 (corresponding to the ion m/z 197 observed in parent + O), m/z 196 (loss of NH3 from the ion m/z 213), m/z 171 (corresponding to the ion m/z 155 observed in parent + O), and m/z 93 (the same ion as seen in the parent). The fragment ion m/z 171 suggests oxidation on the indole ring. A deuterium exchange experiment indicated that S3
had two exchangeable protons (one more exchangeable proton than the parent drug), confirming the addition of a hydroxyl group. No water loss was observed, suggesting that an aromatic hydroxylation had occurred.

S4. The protonated molecular ion of S4 was m/z 289. The accurate mass of the M + H⁺ of S4 was 289.1445, which is consistent with the chemical formula C₁₀H₁₂O₃N₄ (parent – C₃H₆CO₂). The MS² of S4 gave the product ions at m/z 272, m/z 246, m/z 197, m/z 180, m/z 155, and m/z 93 (the same ions as observed in the parent). Comparison of the HPLC retention time and characteristic product ions of S4 with those of an authentic synthetic standard LSN3049319 confirmed the identification of S4.

S5. The protonated molecular ion of S5 was m/z 331. The accurate mass of the M + H⁺ of S5 was 331.1546, which is consistent with the chemical formula C₂₀H₁₉ON₄ (acetylation of S4). The MS² of S5 gave the product ions at m/z 272, m/z 180, m/z 155, and m/z 93 (the same ions as observed in the parent). Comparison of the HPLC retention time and characteristic product ions of S5 with those of an authentic synthetic standard, LSN2588307, confirmed the identification of S5.

S10. The full-scan MS spectra of S10 gave a [M + H⁺] at m/z 391. The accurate mass of the M + H⁺ of S10 was 391.1756, which is consistent with the chemical formula C₂₂H₂₃O₃N₄ (parent + O). MS² of S10 gave the product ions at m/z 287 (loss of C₃H₆CO₂ and H₂O), m/z 270 (base peak) (loss of NH₃ from ion m/z 287), m/z 262 (the ion m/z 246 observed in parent + O), m/z 195 (the ion m/z 197 observed in the parent + O – H₂O), m/z 194 (the counterpart cation of m/z 195), and m/z 93 and m/z 80 (the same ions as seen in the parent). The fragment ion m/z 287 indicated no change on the isopropyl. The ions m/z 110 and m/z 93 indicated no change on the pyridine ring. Significant loss of water suggested an aliphatic hydroxylation. A deuterium exchange experiment indicated that S10 had one exchangeable proton (same number of exchangeable protons as the parent drug), whereas all the other monohydroxylated metabolites showed one more exchangeable proton than the parent. When S10 was incubated with TiCl₃, it did not undergo reduction back to the parent, suggesting that S10 was not likely to be an N-oxide. To further elucidate the structure of S10, an incubation with HLMs was conducted to generate a sufficient amount of S10 for NMR analysis (see NMR analysis below for the definitive structure of S10).

S12. The protonated molecular ion of S12 was m/z 391. The accurate mass of the M + H⁺ of S12 was 391.1759, which is consistent with the chemical formula C₂₂H₂₃O₃N₄ (parent + O). MS² of S12 gave the product ions at m/z 287 (loss of C₃H₆CO₂ and H₂O), m/z 270 (base peak) (loss of NH₃ from ion m/z 287), m/z 195 (corresponding to the ion m/z 197 observed in the parent + O – H₂O), and m/z 93 (the same ion as seen in the parent). The fragment ion m/z 287 indicated no change on the isopropyl. The existence of the ions m/z 110 and m/z 93 indicated no change on the pyridine ring. Significant loss of water suggested an aliphatic hydroxylation. Comparison of the HPLC retention time and characteristic product ions of S12 with those of an authentic synthetic standard LSN3049319 confirmed the identification of S12.

S13. The protonated molecular ion of S13 was m/z 391. The accurate mass of the M + H⁺ of S13 was 391.1756, which is consistent with the chemical formula C₂₃H₂₅O₃N₄ (parent – C₃H₆CO₂). The MS² of S13 gave the product ions at m/z 287 (loss of C₃H₆CO₂ and H₂O), m/z 305 (loss of C₆H₅NO from ion m/z 305), m/z 288 (loss of NH₃ from m/z 305), m/z 262 (the ion m/z 246 observed in the parent + O), m/z 198 (loss of C₆H₅NO from ion m/z 305), m/z 181 (loss of NH₃ from m/z 198), m/z 194 (the counterpart cation of m/z 195), and m/z 93 and m/z 80 (the same ions as seen in the parent). The fragment ion m/z 287 indicated no change on the isopropyl. The existence of the ions m/z 110 and m/z 93 indicated no change on the pyridine ring. Significant loss of water suggested an aliphatic hydroxylation. Comparison of the HPLC retention time and characteristic product ions of S13 with those of an authentic synthetic standard LSN3049319 confirmed the identification of S13.

FIG. 6. HPLC radiochromatograms of feces from healthy male human subjects after oral administration of a single 15-mg (100 μCi) dose of [¹⁴C]LY2452473.

The AUC₀–₄₈ values were calculated using the software Watson (version 7.1) based on nanogram-equivalents per milliliter of 1-, 2-, 4-, 8-, 24-, and 48-h plasma. The nanogram-equivalents per milliliter value for LY2452473 or each metabolite was calculated by multiplying the percentage of total radioactivity value for each compound with nanogram-equivalents per milliliter of the total radioactivity at a given time point. These values are to be considered semiquantitative only.

### TABLE 2

Mean relative distribution of LY2452473 and metabolites in plasma from healthy male human subjects after oral administration of a single 15-mg (100 μCi) dose of [¹⁴C]LY2452473

<table>
<thead>
<tr>
<th>Peak</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>24 h</th>
<th>48 h</th>
<th>Relative Exposure (AUC₀–₄₈)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td>0.00</td>
<td>0.00</td>
<td>0.92</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>N.C.</td>
</tr>
<tr>
<td>S5</td>
<td>0.00</td>
<td>0.00</td>
<td>1.74</td>
<td>10.48</td>
<td>28.48</td>
<td>38.68</td>
<td>20.6</td>
</tr>
<tr>
<td>S12</td>
<td>21.90</td>
<td>22.58</td>
<td>24.24</td>
<td>36.62</td>
<td>37.11</td>
<td>30.05</td>
<td>34.7</td>
</tr>
<tr>
<td>S43</td>
<td>0.00</td>
<td>0.00</td>
<td>0.97</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>N.C.</td>
</tr>
<tr>
<td>S60</td>
<td>0.00</td>
<td>0.89</td>
<td>2.20</td>
<td>6.94</td>
<td>0.00</td>
<td>0.00</td>
<td>2.3</td>
</tr>
<tr>
<td>Parent</td>
<td>74.50</td>
<td>72.93</td>
<td>66.33</td>
<td>42.36</td>
<td>28.81</td>
<td>27.67</td>
<td>42.2</td>
</tr>
<tr>
<td>Total</td>
<td>96.40</td>
<td>96.40</td>
<td>96.40</td>
<td>94.41</td>
<td>96.40</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

N.C., not calculated.
TABLE 3

Mean (n = 6) percentage of dose excreted in urine and feces identified as LY2452473 and metabolites from healthy male human subjects after oral administration of a single 15-mg (100 μCi) dose of [14C]LY2452473

<table>
<thead>
<tr>
<th>Peak</th>
<th>Urine</th>
<th>Feces</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY2452473</td>
<td>N.D.</td>
<td>1.84 ± 2.43</td>
<td>1.84 ± 2.43</td>
</tr>
<tr>
<td>S3</td>
<td>N.D.</td>
<td>8.06 ± 2.57</td>
<td>8.06 ± 2.57</td>
</tr>
<tr>
<td>S4</td>
<td>3.39 ± 1.78</td>
<td>N.D.</td>
<td>3.39 ± 1.78</td>
</tr>
<tr>
<td>S5 + S40⁴</td>
<td>2.78 ± 1.06</td>
<td>0.95 ± 0.64</td>
<td>3.73 ± 1.22</td>
</tr>
<tr>
<td>S10 + S60⁵</td>
<td>0.89 ± 0.12</td>
<td>13.69 ± 4.91</td>
<td>14.58 ± 4.97</td>
</tr>
<tr>
<td>S13</td>
<td>N.D.</td>
<td>9.71 ± 2.01</td>
<td>9.71 ± 2.01</td>
</tr>
<tr>
<td>S38</td>
<td>N.D.</td>
<td>3.62 ± 2.18</td>
<td>3.62 ± 2.18</td>
</tr>
<tr>
<td>S53</td>
<td>3.57 ± 0.86</td>
<td>0.11 ± 0.28</td>
<td>3.69 ± 1.04</td>
</tr>
<tr>
<td>Other minor metabolites together⁶</td>
<td>22.48 ± 5.28</td>
<td>0.36 ± 1.73</td>
<td>25.12 ± 5.95</td>
</tr>
<tr>
<td>Total identified</td>
<td>33.11 ± 5.17</td>
<td>40.63 ± 8.19</td>
<td>73.73 ± 5.88</td>
</tr>
<tr>
<td>Total dose eliminated</td>
<td>43.75 ± 9.32</td>
<td>43.89 ± 7.74</td>
<td>87.64 ± 6.57</td>
</tr>
</tbody>
</table>

N.D., not detected, which was set at zero for calculation.
⁴ S5 and S40 were coeluted in urine and summed, and S5 was not detected in feces.
⁵ S10 and S60 were coeluted in urine, and S60 was not detected in feces.
⁶ An additional 23 minor metabolites were observed in urine and/or feces, each of which accounted for less than 3% of the administered dose. They are summed and presented here.

m/z 126 (the ion m/z 110 seen in the parent + O), and m/z 108 (C₈H₁₇NO, loss of H₂O from m/z 126). The fragment ion m/z 287 indicated no change on the isopropyl. The existence of the ions m/z 198, m/z 126, and m/z 108 indicated an oxidation on the pyridine ring. A deuterium exchange experiment showed that S13 had two exchangeable protons (one more exchangeable proton than the parent drug), confirming S13 as a hydroxylated metabolite.

Additional structural characterization of metabolite S10. To definitively identify the structure of metabolite S10, this metabolite was generated through HLM incubation as described under Materials and Methods. The 100-mL incubation with 50 μM LY2452473 gave an approximate yield of 10% S10. S10 was isolated using a semipreparative HPLC system with acidic mobile phase as described under Materials and Methods. After isolation, the collected S10 was reanalyzed by LC-MS in positive ESI mode with the same HPLC system used for S10 isolation) for 2 to 5 h and analyzed by LC-MS. It was found that all the material was converted back to S10. When LY2452473 was dissolved in 0.1 N HCl-acetonitrile (50:50, v/v) or in water-acetonitrile (50:50, v/v) containing 0.3% H₂O₂, an epoxide across the cyclo pentene-indole linkage (degradation product A) and the corresponding diol (degradation product G), respectively, were formed. The NMR data of the new species derived from S10 matched that of the degradation product A. For comparison, S10 and degradation products A and G were analyzed in parallel using the same LC-MS conditions. The HPLC retention time and MS and MS/MS spectra of S10 were in agreement with those for degradation product G and the HPLC retention time and MS and MS/MS spectra of the new species derived from S10 were in agreement with those for degradation product A. Therefore, S10 was eventually identified as a diol across the cyclo pentene-indole linkage. During the isolation of S10 using an acidic mobile phase, it was assumed that S10 was converted to the corresponding epoxide. To verify the hypothesis, degradation product G was mixed with 0.2% formic acid (the mobile phase used for S10 isolation) for 2 to 5 h and analyzed by LC-MS. It was found that the degradation product G was indeed converted to the degradation product A. To further confirm S10 as a diol, S10 was analyzed using negative ESI mode, giving an M – H m/z 407 (parent + O + H₂O, a diol). This result obviously indicated that S10 encountered source-induced loss of H₂O, which gave S10 a false molecular ion M + H⁺ at m/z 391 (parent + O) instead of m/z 409 (parent + O + H₂O) in positive ESI mode. The conversion of the diol to an epoxide can also explain the result of the H/D exchange experiment whereby the reconstitution of S10 in 0.2% formic acid/methanol resulted in conversion of S10 to the epoxide, which did not show an additional exchangeable proton.

Identification of P450s Involved in the Depletion of LY2452473.

In the initial screening, LY2452473 was incubated with a panel of recombinant P450 enzymes including 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 2J2, 3A4, and 3A5 to evaluate substrate depletion. CYP1A2, 2B6, 2C8, 2E1, and 3A5 did not show any substrate depletion and were excluded from further evaluation under optimal conditions.
Table 6 presents the substrate depletion CL\(_{int}\) by selected P450s under optimized conditions. CL\(_{int}\) for CYP3A4 was 0.75 \(\mu\)l/min/1 pmol. After normalization for hepatic content of the contributing P450s, CYP3A4 was the largest contributor (68%) to substrate depletion, suggesting a major role of CYP3A4 in the hepatic clearance of LY2452473. CYP2J2 showed the second largest contribution to substrate depletion (15%) after normalization for hepatic content.

Known direct-acting chemical inhibitors were used to evaluate the roles of P450s in the metabolism of LY2452473 in a second system, human liver microsomes. Inhibitor concentration dependence and \(\leq 15\%\) inhibition were the criteria for identifying inhibition. Ketoconazole (a selective inhibitor of CYP3A4) showed 100% inhibition of substrate depletion, consistent with a major role for CYP3A4, whereas sulfaphenazole (a selective CYP2C9 inhibitor), modafinil (a selective CYP2C19 inhibitor), and quinidine (a selective CYP2D6 inhibitor) showed approximately 20 to 40% inhibition of substrate depletion, suggesting minor contributions of CYP2C9, CYP2C19, and CYP2D6.

**Discussion**

The objective of this study was to determine the excretion and metabolism of LY2452473 after oral administration of 15 mg (100 \(\mu\)Ci) of \([^{14}\text{C}]\)LY2452473 to six healthy male human subjects. The mean total recovery of radioactivity in urine and feces was high: 94.5% after a 13-day period. Fecal and renal elimination of \([^{14}\text{C}]\)LY2452473 were approximately equal. Most of the administered radioactivity was recovered within the first 120 h postdose (mean of 80.1%). Metabolic profiling of feces and urine revealed that LY2452473 was extensively metabolized in humans with \(\leq 2\%\) \(<\) of the dosed radioactivity eliminated as unchanged parent. The excretion profile was similar across all six subjects (Fig. 2).
After oral administration, $^{14}$C-LY2452473 was rapidly absorbed into the circulation with peak plasma concentrations of total radioactivity and parent compound occurring within 2 to 3 h. As a result of first-pass metabolism, the $C_{\text{max}}$ for total radioactivity was higher than the $C_{\text{max}}$ for LY2452473 (Table 1). Furthermore, a comparison of AUC values of total radioactivity and LY2452473 showed that the parent represented approximately 17% drug-related material based on $\text{AUC}_{0-\infty}$. The major metabolic pathways in humans are shown in Fig. 7. In addition to the parent compound, metabolites S5 and S12 were the major circulating entities. Metabolite S5 resulted from the N-acetylation of the amine metabolite S4, and metabolite S12 is a monohydroxy metabolite. The structures of S4, S5, and S12 were confirmed by comparison of the LC-MS, LC-MS/MS, and HPLC retention times with those of authentic synthetic standards. LY2452473 was the predominant circulating entity at early time points. However, the ratios of metabolites to parent increased over time, indicating slower elimination of metabolites, which is consistent with the pharmacokinetic data that the mean terminal half-life for total radioactivity was longer than the mean terminal half-life of LY2452473 in plasma. According to the radioprofiling data, the plasma exposures of parent, S5, and S12 accounted for 42, 21, and 35% of the total radioactivity on the basis of $\text{AUC}_{0-48}$ calculated from total plasma radioactivity and relative peak areas in the plasma radiochromatographic profiles (Table 2). The relative percentage of plasma exposure of the parent calculated from radioprofiles ($\text{AUC}_{0-48}$) was larger than that from pharmacokinetic analysis ($\text{AUC}_{0-\infty}$) (42% versus 17%). This discrepancy provided additional evidence that metabolites had slower elimination and longer residence time in plasma, which resulted in decreased contribution of the parent to the total radioactivity exposure when a longer time interval ($0-\infty$) was taken into account. In clinical development, it must be demonstrated that animals in toxicology testing have been exposed appropriately to major circulating human metabolites. Metabolites S5 and S12 were considered major circulating metabolites, e.g., >10% of drug-related radioactivity.

**Fig. 8.** Fragmentation pathways of LY2452473 generated by the LTQ Orbitrap mass spectrometer with positive ESI and HCD collision energy at 45%.

**Fig. 9.** Extracted ion chromatograms showing interconversions of the diol and epoxide of LY2452473 (data generated by the SYNAPT G2 mass spectrometer with positive ESI). A, S10 (diol) was converted to an epoxide after isolation with the acidic mobile phase. B, epoxide from S10 was converted back to the diol after reconstituted in a neutral solvent (50:50, methanol/water). C, degradation product A (epoxide) with partial conversion to the diol. D and E, degradation product G (diol) and conversion to epoxide in acid solution.
exposure according to the current International Conference on Harmonization M3(R2); Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals (2009; http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Multidisciplinary/M3_R2/Step4/).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( \text{CL}_{\text{int}} )</th>
<th>Specific Content</th>
<th>Normalized ( \text{CL}_{\text{int}} )</th>
<th>( f_m ) Relative to Total Normalized ( \text{CL}_{\text{int}} ) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C9</td>
<td>0.10</td>
<td>96</td>
<td>10</td>
<td>8.5</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>0.30</td>
<td>19</td>
<td>5.7</td>
<td>4.9</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>0.30</td>
<td>10</td>
<td>3.0</td>
<td>2.6</td>
</tr>
<tr>
<td>CYP2J2</td>
<td>1.79</td>
<td>10</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>0.75</td>
<td>108</td>
<td>80</td>
<td>68</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>117</td>
<td>100</td>
</tr>
</tbody>
</table>

\( \text{CL}_{\text{int}} \) of substrate depletion was calculated by \( k_{\text{depl}} \times \text{incubation volume per picomole of rP450}. \) Normalized \( \text{CL}_{\text{int}} \) was calculated by multiplying \( \text{CL}_{\text{int}} \) by reported hepatic specific content from Rodrigues (1999) and Lee et al. (2010). \( f_m \) was calculated by summing relevant normalized \( \text{CL}_{\text{int}} \) and dividing normalized \( \text{CL}_{\text{int}} \) for an individual rP450 by the relevant total (\( \times 100 \)).

Whereas all urinary metabolites were minor (each <4% of the dose), metabolites S3 (parent + O, oxidation on the indole aromatic ring), S10 (parent + O + H\(_2\)O, diol across the cyclopenta-indole linkage), and S13 (parent + O, oxidation on the pyridine) were identified as three major fecal metabolites, accounting for a mean of 8, 14, and 10% of the dose. Each fecal metabolite of an additional seven accounted for less than 4% of the dose and was considered relatively minor. The parent accounted for 2% of the dose in the fecal samples analyzed.

Of interest, preliminary LC-MS and LC-MS/MS data generated in positive mode suggested that S10 was an aliphatic hydroxylated metabolite or an N-oxide on the cyclopenta[indole ring moiety. However, the H/D exchange results showed that S10 had the same number of exchangeable protons as the parent compound, whereas, as expected, the monohydroxylated metabolites showed an additional exchangeable proton. These data suggested that S10 was not a hydroxylated metabolite. TiCl\(_3\) can selectively reduce N-oxide to amine (Kulanthaivel et al., 2004). Incubation of S10 with TiCl\(_3\) did not result in the reduction of S10 back to the parent, signifying that S10 was not an N-oxide. To definitively identify S10, the metabolite was generated from human liver microsomal incubation and isolated for NMR analysis. After isolation, the majority of S10 was unexpectedly converted to a new chemical entity under acidic conditions, which was assigned as the monohydroxylated metabolite or an N-oxide.
condition. S10 and the new entity were at equilibrium. NMR results from the converted new chemical entity suggested that it was a stable epoxide metabolite formed by oxidation across the cyclopentene double bond on the indole (Adam et al., 1994). When the material was reconstituted in the solvent with neutral pH, the equilibrium shifted toward S10. This phenomenon was also observed for a pair of stressed degradation products A (epoxide) and G (diol) (Fig. 9). LC-MS, LC-MS/MS, and NMR data comparison between S10 and degradation products A and G eventually led to the definitive structure for S10 as a diol formed at the double bond of the cyclopentene ring.

Overall, LY2452473 was eliminated in humans by extensive metabolic pathways. Oxidative hydrolysis of the carbamate ester to the amine S4 (LSN2519879) with subsequent acetylation led to the formation of the major circulating metabolite S5 (LSN2588307). This pathway was also involved in the formation of several excretory metabolites including S4, S5, S38, S53, S55, S56, S57, S58, S59, and S61 (see Fig. 7 for structures), together accounting for approximately 18% of the dose in excreta. This pathway has also been demonstrated with other carbamate esters (Yumibe et al., 1996; Schmidt et al., 2006; Ghosal et al., 2009). Oxidation of the cyclopentene ring formed the major circulating metabolite S12 (LSN3049319, presented in plasma only) as well as the major fecal metabolite S10. Summation of all the metabolites associated with oxidation of the cyclopentene ring (S10, S44, S46, S48, S49, S50, S51, S52, S53, S55, S56, and S58) accounted for approximately 29% of the dose in excreta. Oxidation of the phenyl ring contributed to the formation of the major fecal metabolite S3. The total of S3 plus two additional phenyl ring oxidation metabolites S2 and S38 accounted for approximately 14% of the dose in excreta. Oxidation of the pyridine ring was another metabolic pathway, leading to the formation of major fecal metabolite S13. All metabolites associated with oxidation of the pyridine ring (S13, S26, S41, S42, S47, S52, S57, and S61) accounted for approximately 16% of the dose in excreta. Oxidation of the isopropyl was involved in the formation of several metabolites (S39, S40, S41, S42, S43, S45, S47, S48, S54, and S60) that accounted for approximately 13% of the dose in excreta. N-Oxidation, phase II glucuronidation, and sulfation were also observed.

Because multiple metabolic pathways are involved in the clearance of LY2452473, and no single metabolite accounts for >15% of the administered dose, a substrate depletion assay provides a comprehensive evaluation of the involvement of enzyme(s) in the overall clearance of LY2452473 without requiring monitoring of specific metabolite(s). LY2452473 depletion in HLMs required NADPH (preliminary experiments, data not shown), indicating P450 involvement. Thus, LY2452473 depletion by ketoconazole was identified to conduct individual P450s responsible for the clearance of LY2452473.

The results from the recombinant P450s indicated that CYP3A4 was responsible for 68% of hepatic LY2452473 depletion. Inhibition of LY2452473 depletion by ketoconazole in human liver microsomes verified the major role of CYP3A4 in LY2452473 hepatic clearance. Complete inhibition of LY2452473 by ketoconazole may reflect the fact that higher concentrations of ketoconazole could potentially inhibit other P450s (Khojasteh et al., 2011). CYP2J2 showed the second largest contribution to LY2452473 depletion. CYP2J2 has emerged as a contributor to drug metabolism (Lee et al., 2010, 2012), but its overall impact is yet to be determined. Finally, CYP2C9, CYP2C19, and CYP2D6 showed minor contributions to hepatic CLint of LY2452473 liver metabolism.

Overall, the results of the in vitro reaction phenotyping suggest that a clinical drug-drug interaction study to determine the effects of a CYP3A4 inhibitor, such as ketoconazole, on the pharmacokinetics of LY2452473 is warranted.