Characterization of the In Vitro and In Vivo Metabolism and Disposition and Cytochrome P450 Inhibition/Induction Profile of Saxagliptin in Human\textsuperscript{S}

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ABSTRACT:

Saxagliptin is a potent dipeptidyl peptidase-4 inhibitor approved for the treatment of type 2 diabetes mellitus. The pharmacokinetics and disposition of \(^{14}C\)saxagliptin were investigated in healthy male subjects after a single 50-mg (91.5 \(\mu\)g) oral dose. Saxagliptin was rapidly absorbed (\(T_{\text{max}}\); 0.5 h). Unchanged saxagliptin and 5-hydroxy saxagliptin (M2), a major, active metabolite, were the prominent drug-related components in the plasma, together accounting for most of the circulating radioactivity. Approximately 97\% of the administered radioactivity was recovered in the excreta within 7 days postdose, of which 74.9\% was eliminated in the urine and 22.1\% was excreted in the feces. The parent compound and M2 represented 24.0 and 44.1\%, respectively, of the radioactivity recovered in the urine and feces combined. Taken together, the excretion data suggest that saxagliptin was well absorbed and was subsequently cleared by both urinary excretion and metabolism; the formation of M2 was the major metabolic pathway. Additional minor metabolic pathways included hydroxylation at other positions and glucuronide or sulfate conjugation. Cytochrome P450 (P450) enzymes CYP3A4 and CYP3A5 metabolized saxagliptin and formed M2. Kinetic experiments indicated that the catalytic efficiency (\(V_{\text{max}}/K_{\text{m}}\)) for CYP3A4 was approximately 4-fold higher than that for CYP3A5. Therefore, it is unlikely that variability in expression levels of CYP3A5 due to genetic polymorphism will impact clearance of saxagliptin. Saxagliptin and M2 each showed little potential to inhibit or induce important P450 enzymes, suggesting that saxagliptin is unlikely to affect the metabolic clearance of coadministered drugs that are substrates for these enzymes.

Introduction

The dipeptidyl peptidase-4 (DPP4) inhibitors are promising recent additions to the arsenal of therapies available for the treatment of type 2 diabetes mellitus (Scheen, 2012). The DPP4 enzyme is responsible for degrading and inactivating glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide, incretins that regulate blood glucose levels. GLP-1 is released postprandially and stimulates meal-induced insulin secretion and contributes to glucose homeostasis (Kieffer and Habener, 1999; Gorrell, 2005). By inhibiting the DPP4 enzyme, GLP-1 is sustained, thereby leading to increased activity and improved glycemic control in patients with type 2 diabetes (McIntosh et al., 2005). Because this mechanism results in a glucose-dependent release of insulin, DPP4 inhibitors are expected to offer important advantages over traditional diabetes treatments including low risk for hypoglycemia and weight gain (Gallwitz, 2008).

Saxagliptin (Onglyza; Bristol-Myers Squibb, Princeton NJ and AstraZeneca, Wilmington, DE) (Fig. 1) is an orally administered, small molecule, reversible DPP4 inhibitor approved for the treatment of type 2 diabetes mellitus. It was specifically designed for enhanced potency and selectivity and to provide extended inhibition of the DPP4 enzyme (Ageri et al., 2005). The ability of saxagliptin to affect reductions in glycosylated hemoglobin (HbA1c) and fasting plasma glucose in type 2 diabetes patients has been demonstrated in multiple

ABBREVIATIONS: DPP4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide-1; P450, cytochrome P450; HPLC, high-performance liquid chromatography; HLM, human liver microsomes; LSC, liquid scintillation counting; LC-MS/MS, liquid chromatography with tandem mass spectrometry; MRM, multiple reaction monitoring; AUC, area under plasma concentration-time curve; T-HALF, terminal phase half-life; CLR, renal clearance; DMSO, dimethyl sulfoxide; 3-MC, 3-methylcholanthrene; PB, phenobarbital; Rif, rifampicin; C\textsubscript{T}, threshold cycle.
Phase III clinical trials, both as a single agent and in combination regimens with metformin, a sulfonylurea or a thiazolidinedione (Kania et al., 2011). The most commonly used clinical dose of saxagliptin in adults is 5 mg, once daily (United States prescribing information for Onglyza, http://www.packageinserts.bms.com/pi/pi_onglyza.pdf).

In nonclinical pharmacokinetic studies, saxagliptin was rapidly absorbed and showed good oral bioavailability in rats (75%), dogs (76%), and monkeys (51%). A significant portion (33–60%) of the administered dose was excreted as unchanged drug in the urine in these species. Formation of 5-hydroxy saxagliptin (M2) was a major metabolic pathway, and this metabolite was a major circulating metabolite in all species (Fura et al., 2009). Metabolite M2 was pharmacologically active, with an in vitro DPP4 inhibitory activity approximately half that of saxagliptin (Auger et al., 2005; Fura et al., 2009).

The purpose of the current study was to investigate the in vivo disposition of saxagliptin and to determine its major metabolic pathways in healthy male subjects after administration of a single 50-mg (91.5 μCi) p.o. dose of [14C]saxagliptin. In addition, a series of in vitro studies were conducted to gain insight regarding possible cytochrome P450 (P450)-based drug-drug interactions between saxagliptin and potential concomitants. These included the identification of enzymes involved in the metabolism of saxagliptin and formation of M2 and the determination of the potential of saxagliptin and M2 to inhibit or induce P450 enzymes.

Materials and Methods

Chemicals. [14C]Saxagliptin (radiochemical purity 99.86%, specific activity 1.83 μCi/mg) with the C-14 label distributed between the carbonyl carbon and the adjacent carbon (Fig. 1) and stable-labeled 13C4,15N-saxagliptin, and 13C3,15N-5-hydroxy saxagliptin [internal standards for high-performance liquid chromatography (HPLC) analysis] were synthesized by the Radiochemistry Group of the Department of Chemical Synthesis, Bristol-Myers Squibb Research (Princeton, NJ) (Cao et al., 2007). Unlabeled saxagliptin (P, (S)-5R,5S,5S-2-((S)-2-amino-2-((3-hydroxyadamantan-1-yl)acetyl)-2-azabicyclo[3.1.0]hexane-3-carbonitrile); and reference standards for 5-hydroxy saxagliptin, (M2, (1R,3S,5S,5S)-2-((S)-2-amino-2-((1R,5S,5S)-3,5-dihydroxyadamantan-1-yl)acetyl)-2-azabicyclo[3.1.0]hexane-3-carbonitrile); degradant (D1, (1aS,3S,6aR,7aS)-4-((3-hydroxyadamantan-1-yl)-6-aminohexahydro-1H-cyclopenta[4,5]pyrrolo[1,2-a]pyrazin-3(1aH)-one); the S,R,S,S and S,S,S,R diastereomers of saxagliptin (Supplemental Fig. S1) were supplied by the Departments of Chemical Development or Chemical Synthesis (Bristol-Myers Squibb).

Selective chemical inhibitors of P450 enzymes for reaction phenotyping experiments were obtained from Sigma-Aldrich (St. Louis, MO), with the exception of montelukast, which was purchased from Sequoia Research Products (Pangbourne, UK), and benzylnirvanol (BD Biosciences, Woburn, MA). Chemical inducers, inhibitors, substrates, and metabolites of P450 enzymes and internal standards used in experiments to evaluate whether saxagliptin and M2 were inhibitors or inducers of P450 enzymes were procured by CellzDirect (Pittsboro, NC). All chemicals were of the highest purity available.

Human liver microsomes (HLM; 19 donors male/female) and individual human cDNA-expressed cytochrome P450 enzymes were purchased from BD Biosciences. Reaction Phenotyping kit (version 7) from XenoTech, LLC (Lenexa, KS). Monoclonal antibodies with inhibitory activity for specific P450 enzymes were purchased as a selective chemical inhibitors of P450 enzymes for reaction phenotyping kit (version 7). Deionized water was prepared using a MilliQ ultrapure water system (Millipore Corporation, Billerica, MA). All organic solvents were HPLC grade, and other reagents were reagent grade or better.

Clinical Study Design, Dosing, and Sample Collection. The clinical phase of the study was conducted at the Bristol-Myers Squibb Clinical Research Center (Hamilton, NJ). This was an open-label, nonrandomized single dose study. Six healthy males participated in the study. The mean age was 29 years.
standards (13C4, 15N) LC-MS/MS methods. In brief, after the addition of stable-labeled internal standards (14C)saxagliptin containing 91.5 µCi of radioactivity, immediately followed by 240 ml of water on day 1. Blood samples were collected at selected time points via an indwelling catheter or direct venipuncture into Vacutainers (BD Biosciences Medical Supplies, Franklin Lakes, NJ) containing K3EDTA and were centrifuged to obtain plasma for pharmacokinetic and biotransformation analysis. The total urine and fecal output was collected for the duration of the study (0–168 h). On the morning of day 6, a single 30-ml oral dose of Milk of Magnesia was administered to each subject to facilitate defecation before release from the clinical facility. All subjects were released on the morning of day 8.

Blood samples (6 ml total per time point) for the plasma pharmacokinetic analysis of saxagliptin, M2, and radioactivity were collected predose and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120, 144, and 168 h postdose. Additional aliquots (10 ml per time point) for metabolite profiling were collected in conjunction with the pharmacokinetic samples at predose and at 1, 2, 4, 8, 12, 24, 48, 96, 144, and 168 h postdose. Cumulative urine was collected predose and over 0 to 12 h, 12 to 24 h, and thereafter in 24-h intervals through 168 h for determining saxagliptin, M2, and radioactivity concentrations and for metabolite profiling. Feces were collected predose and over 24-h intervals postdose for the measurement of radioactivity concentrations and for metabolite profiling. All samples were stored at −20°C or below until analysis.

Radioactivity Analysis. Radioactivity in plasma, urine, and feces was measured by liquid scintillation counting (LSC) on a Model LS 6500 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Plasma and urine were mixed with Emulsifier-Safe scintillation fluid (PerkinElmer Life and Analytical Sciences) and counted directly. Water was added to each fecal sample to form an approximately 20% (w/w) feces mixture, which was homogenized using a probe-type homogenizer (Kinematica Polytron model no. PT 45-80; Brinkman Instruments, Westbury, NY). Aliquots of fecal homogenate were then combusted using a sample oxidizer before counting by LSC, as described previously (Christopher et al., 2008).

Quantification of Saxagliptin and M2 in Plasma and Urine Samples. The concentrations of saxagliptin and M2 in individual plasma and urine were determined with validated liquid chromatography/tandem mass spectrometry (LC-MS/MS) methods. In brief, after the addition of stable-labeled internal standards (13C14, 15N-saxagliptin and 13C14, 15N-5-hydroxy saxagliptin) to each plasma or urine sample, the analytes were isolated by solid-phase extraction (Waters Oasis HLB, 10 mg; Waters, Milford, MA). The eluates were evaporated to dryness, reconstituted in mobile phase, and then applied to an Atlantis dC18, 2.1 × 50 mm, 5-µm HPLC column (Waters). The LC-MS system used for plasma samples consisted of LC10AD delivery pumps (Shimadzu Corporation, Columbia, MD) and a Series 200 Autosampler (PerkinElmer Life and Analytical Sciences). The HPLC system was interfaced to either a Quattro Premier mass spectrometer (Waters Corporation, Manchester, UK) for the plasma method or an API1000 mass spectrometer (AB Sciex, Foster City, CA) for the urine method. The mass spectrometers were operated in positive ion electrospray mode, and analytes were monitored by multiple reaction monitoring (MRM) with transitions that were characteristic for each analyte. For saxagliptin and M2, the standard curve ranges were 5 to 1000 and 10 to 2000 ng/ml, respectively, for the plasma method and 25 to 5000 and 50 to 10,000 ng/ml, respectively, for the urine method.

Pharmacokinetic Analysis of Saxagliptin, M2, and Total Radioactivity. The noncompartmental pharmacokinetic parameters of saxagliptin, M2, and total radioactivity were determined from plasma concentration versus time profiles and urine concentrations with cumulative urinary excretion volumes using noncompartmental methods with Kinetica 4.2 in eToolbox (Thermo Fisher Scientific, Waltham, MA). The single-dose pharmacokinetic parameters determined included the following: maximum observed concentration (Cmax); time of maximum concentration (Tmax); area under the plasma concentration-time curve (AUC) between time 0 and the last quantifiable concentration [AUC(0–T)]; and AUC between time 0 to infinity [AUC(INF)], terminal phase half-life (T1/2), renal clearance (CLR), and percentage of urinary excretion. The percentage of fecal excretion was determined for total radioactivity only, and the calculation was based on cumulative fecal weights and total radioactivity concentrations. For the percentage of dose excreted in urine and feces, the actual dose of saxagliptin administered to each subject was determined by subtracting the weight of the dosing syringe (in grams) after dosing from the weight of the dosing syringe (in grams) before dosing and multiplying by the density of the dosing solution (1.0 g/ml) and the concentration of the dosing solution (5 mg/ml).

Preparation of Samples for Biotransformation Profiling and Identification of Metabolites. Representative pools of plasma, urine, and feces were prepared for metabolite profiling and identification experiments. Plasma samples were segregated by collection time (i.e., 1, 2, 4, and 8 h), and equal volumes from all subjects were combined. Plasma samples collected after 8 h were not analyzed because the radioactivity in these samples was too low to produce meaningful profiles. Urine and fecal homogenate pools (0–168 h) were prepared across all subjects by combining a percentage of the volume (urine) or weight (fecal homogenate) proportional to the total amount excreted over each interval.

Pooled plasma and fecal homogenate samples were each extracted with three volumes of methanol/acetonitrile 50:50 (v/v). After centrifugation at 2500g for 40 min, the pellets were extracted an additional two times with methanol/acetonitrile/water (25:25:50, v/v/v). The supernatants from each extraction step were combined and evaporated to dryness under nitrogen. The dried residues were reconstituted in methanol/acetonitrile/water (~10:20:70, v/v/v), and the resulting supernatants were analyzed by HPLC with offline radioactivity detection or LC-MS/MS. The recovery of radioactivity from extracted plasma and fecal samples was approximately 100%. Pooled urine samples were centrifuged at 11,000 g to remove any particulates and analyzed without additional processing.

LC-Radiochromatographic and LC-MS/MS Methods for Metabolite Profiling of In Vivo Samples and Identification of Metabolites. Samples for metabolite profiling were analyzed on a Shimadzu LC-10AD HPLC system (Shimadzu Corporation), equipped with two 10AD VP pumps, a S-L10AD autoinjector, a model SCL-10A system controller, and an SPD-M10A photo-diode array detector. A Zorbax 4.6 × 250 mm, 5-µm RX-C8 column (Agilent Technologies, Santa Clara, CA) maintained at 30°C was used to separate drug-related components. The mobile phase consisted of two solvents: 1) mobile phase (A) 0.1% formic acid and 1% acetonitrile in water and 2) mobile phase (B) 0.1% formic acid in acetonitrile. The mobile phase flow rate was 0.5 ml/min. The gradient program used for sample elution was as follows: hold isocratic at 0% B (0–5 min); linear gradient from 0 to 20% B (5–35 min); hold isocratic at 20% B (35–42 min); linear gradient from 20 to 30% B (42–45 min); hold isocratic at 30% B (45–50 min); linear gradient from 30 to 40% B (50–52 min); linear gradient from 40 to 80% B (52–55 min); hold isocratic at 80% B (55–60 min); return to 0% B (60–62 min); re-equilibrate at 0% B for 10 min before the next injection.

For quantification of metabolites by radioactivity, the HPLC eluate was collected in a 0.25-min intervals on Wallac ScintiPlate-96-well plates with a Gilson Model FC 204 fraction collector (Gilson, Middleton, WI). The plates were evaporated to dryness on a Savant Speed-Vac (Savant Instruments Inc., Holbrook, NY) and counted for 10 min/well with a PerkinElmer 1450 MicroBeta Wallac TRILUX Liquid Scintillation and Luminescence Counter (PerkinElmer Life Sciences, Turku, Finland) to quantify radioactivity. Radioprofiles were prepared by plotting the net counts per minute values obtained from the MicroBeta versus time after injection using Microsoft Excel (Microsoft Corporation, Redmond, WA). The metabolites were quantified based on the percentage of total radioactivity in each peak relative to the entire radiochromatogram.

Mass spectral analysis was performed on a Finnigan LCQ Deca XP ion trap mass spectrometer equipped with an electrospray ionization probe (Thermo Fisher Scientific). Analyses were performed in the positive ion mode. Samples were introduced into the mass-spectrometer after chromatographic separation, using the same HPLC method used for radioprofiling. High purity nitrogen was used as the sheath and the auxiliary gas with levels at 60 and 10 (relative flow rate), respectively. The capillary temperature was 350°C. The nitrogen gas
with pooled HLM (0.25 mg/ml), 1 to 2 mM NADPH, and 2 mM MgCl2 in 0.1 M, pH 7.4. Incubations (final volume, 0.5 ml) were conducted by incubating saxagliptin, at concentrations of 1 and 10 μM, in singlicate with individual lots of HLM from 16 different donors. For each lot, the activities of CYP1A2, -2A6, -2B6, -2C8, -2C9, -2C19, -2D6, -3A4/5, and -4A11 had been determined by the vendor using marker substrates specific for each enzyme (Technical Information for Reaction Phenotyping Kit version 7, ref: 0510189; Xenotech, LLC). The reaction mixtures contained 0.25 mg/ml of HLM, 1 or 10 μM saxagliptin, 1 mM NADPH, and 0.1 M phosphate buffer with 2 mM MgCl2 (pH 7.4). Incubations (final volume, 0.5 ml) were conducted for 30 min at 37°C in a shaking water bath. Reactions were stopped with the addition of ice-cold acetonitrile. The concentration of M2 in each sample was then determined by analyzing an aliquot of the resulting supernatant by LC-MS/MS with multiple reaction monitoring as described below. Plots of M2 versus marker substrate activity were prepared, and r values were calculated with Microsoft Excel (Office 2003; Microsoft Corporation).

The metabolism of saxagliptin to M2 by specific P450 enzymes was also investigated with HLM in the presence of specific chemical or monoclonal antibody inhibitors of P450 enzymes. Chemical inhibitors included direct inhibitors, tranylcypromine (2 μM, CYP2A6), montelukast (3 μM, CYP2C8), sulfaphenazole (10 μM, CYP2C9), benzylvinilarn (1 μM, CYP2C19), quindidine (1 μM, CYP2D6), ketoconazole (1 μM, CYP3A4/5) and time-dependent inhibitors, furafylline (10 μM, CYP1A2), orphenadrine (50 μM, CYP2B6), diethylidithiocarbamate (50 μM, CYP2E1), troleandomycin (20 μM, CYP3A4/5), and 1-aminoantzenotrazole (1000 μM, all P450s). Anti-P450 monoclonal antibodies included anti-CYP1A2, anti-CYP2B6, anti-CYP2C8, anti-CYP2C19, anti-CYP2D6, and anti-CYP3A4/5. A final concentration of 5 to 7 μl of antibody mixture was used per incubation. The antibody solutions were used as received; the concentration of each of the antibodies was not provided.

Saxagliptin at concentrations of 1 and 10 μM, was incubated in triplicate with pooled HLM (0.25 mg/ml), 1 to 2 mM NADPH, and 2 mM MgCl2 in 0.1 M phosphate buffer (pH 7.4), in the presence or absence of chemical or anti-P450 monoclonal antibody inhibitors. The final volume of the reaction mixtures was 1 ml for the chemical inhibitor experiments and 0.25 ml for the anti-P450 antibody experiments. For incubations with direct chemical inhibitors, all ingredients except NADPH were added to the incubation tubes, and the samples were equilibrated at 37°C for 2 to 3 min before incubation. Then, NADPH was added to initiate the reactions. For incubations with time-dependent chemical inhibitors, all ingredients except saxagliptin were added to the incubation tubes. The samples were equilibrated at 37°C for 2 to 3 min, and then 1 mM NADPH was added to initiate a 15-min preincubation with the inhibitors. After the preincubation period, saxagliptin and an additional 1 mM NADPH were added to initiate the reactions. For incubations with anti-P450 antibodies, the antibodies were preincubated with HLM in phosphate buffer on ice for 20 min, and then warmed at 37°C for 10 min. Saxagliptin and NADPH were added to the incubation mixtures to initiate the reactions. To establish the initial rate of metabolism of saxagliptin to M2 in HLM, incubations without chemical inhibitors or with antibodies against egg lysozyme (Hy-Hei-9) rather than anti-P450 antibodies were conducted. The rate of M2 formation in other incubations was normalized to the appropriate control incubation. Negative control incubations were carried out in the same manner, but they either lacked NADPH or contained heat-inactivated microsomes (boiled for 5 min).

After the appropriate equilibrations and preincubation periods, HLM incubations were carried out for 30 min at 37°C. An equivalent volume of ice-cold acetonitrile was added to stop each reaction. Samples were vortex mixed and centrifuged to precipitate proteins. The concentration of M2 in each sample was then determined by analyzing an aliquot of the resulting supernatant by LC-MS/MS.

Concentration-Dependent Metabolism of Saxagliptin to M2. The kinetic for the formation of M2 were determined in pooled HLM and expressed as NADPH and CYP3A4 and CYP3A5. Incubations (0.25 ml total volume, in triplicate) contained 1 mM NADPH, 2 mM MgCl2, 0.1 mM phosphate buffer (pH 7.4), saxagliptin, and HLM (0.25 mg protein/ml), or expressed CYP3A4 or CYP3A5 (10 pmol P450 enzyme/ml). Twelve concentrations of saxagliptin from 1 to 800 μM were evaluated. The HLM incubations were conducted at 37°C. After the designated incubation period (30 min for HLM, 10 min for CYP3A4 and CYP3A5), reactions were quenched by adding an equal volume of ice-cold acetonitrile (0.25 ml). The quenched reaction mixtures were vortexed to mix and centrifuged. The concentration of M2 in each sample was then determined by analyzing an aliquot of the resulting supernatant by LC-MS/MS.

LC-MS/MS Method for Quantification of M2 in In Vitro Samples. Internal standard, 13C15N3-hydroxy saxagliptin, was added to the quenched reaction mixtures from the in vitro incubations before LC-MS/MS analysis. The LC/MS system used for quantification of M2 in in vitro samples consisted of 10 AD-VP pumps, a model SCL system controller and a degasser (Shimadzu Corporation), a LEAP HTC PAL autosampler equipped with a cooling stack maintained at 10°C (CTC Analytics, Carrboro, NC), and a Micromass Quattro Ultima triple quadrupole mass spectrometer (Waters). Masslynx software (version 4.0 or 4.1; Waters) was used to control the instrumental and acquire data. Chromatographic separation of M2 from other mono-hydroxylated metabolites was achieved on an Agilent Zorbax SB-C8 column (4.6 × 75 mm, 3.5-μm particle size) (Agilent, Wilmington, DE) maintained at ambient temperature. The mobile phase consisted of two solvents: mobile phase (A), 0.1% formic acid in water; and mobile phase (B), 0.1% formic acid in acetonitrile. The mobile phase flow rate was 0.3 ml/min. The linear gradient program used for elution of the sample components was as follows: hold isotropic at 15% B (0–1 min); from 15 to 30% B (0.1–3 min); from 30% to 35% B (3–4 min); from 35 to 40% B (4–5 min); from 40 to 80% B (5–5.5 min); hold isotropic at 80% B (5.5–6.5 min); return to 15% B (6.5–7 min); re-equilibrate at 0% B for 5 min before the next injection.

The Micromass Quattro Ultima mass spectrometer was operated in positive electrospray ionization mode. Ultra-high purity nitrogen was used for the nebulizing and desolvation gases at flow rates of approximately 85 and 1000 l/h, respectively. The capillary voltage was 3.5 kV, the cone voltage was 36V, and the collision energy was 45 eV. The desolvation temperature was 300°C, and the source temperature was 150°C. Detection of 5-hydroxy saxagliptin and its internal standard were achieved through MRM. The individual selected reaction monitoring transitions were 332 → 196 for M2 and 335 → 196 for the internal standard.

Assessment of Potential of Saxagliptin and M2 to Inhibit P450 Enzymes. The potential for saxagliptin and M2 to inhibit P450 enzymes in a direct or time-dependent manner was assessed with HLM (n = 15 donors, mixed gender pool; CellDirect, Durham, NC). IC50 values for nine enzymes were determined using probe substrates specific for each of the enzymes evaluated. The metabolic reactions monitored and probe substrate concentrations used were phenacetin O-deethylation (50 μM, CYP1A2), coumarin 7-hydroxylation (1 μM, CYP2A6), buprofen hydroxylation (20 μM, CYP2B6), paclitaxel 6-hydroxylation (5 μM, CYP2C8), tolbutamide hydroxylation (140 μM, CYP2C9), 5-menthyenonin 4′-hydroxylation (50 μM, CYP2C19), bufuralol 1′-hydroxylation (40 μM, CYP2D6), chlorozoxanone 6-hydroxylation (50 μM, CYP2E1), midazolam 1′-hydroxylation and testosterone 6β-hydroxylation (5 and 50 μM, respectively, CYP3A). The final concentration of each probe substrate was near the experimentally determined Km value for the indicated enzyme.
To evaluate whether saxagliptin or M2 were competitive inhibitors of P450 enzymes, saxagliptin (at concentrations of 0, 0.1, 1, 5, 20, and 50 μM), M2 (at concentrations of 0, 0.1, 1, 10, 50, and 200 μM), or prototypical P450 inhibitors (positive controls) were mixed with HLM, and the probe substrates in 100 mM phosphate buffer (pH 7.4) in a total volume of approximately 0.5 mL. After a 3-min equilibration at 37°C, 1 mM NADPH was added to initiate the reactions. The reactions were carried out using previously established conditions to ensure linearity with respect to protein concentration and incubation time. Incubations were stopped with addition of organic solvents. To assess the time-dependent inhibition, saxagliptin, M2, or positive control inhibitors were preincubated for 15 min at 37°C with pooled human liver microsomes in the presence and absence of 1 mM NADPH. After the preincubation, P450-specific probe substrates were added to the incubation mixtures at the same concentrations used above. Metabolite formation in incubations with test compounds and control inhibitors was assessed with validated LC-MS/MS methods for each of the reaction products as described in Supplemental Table S1. Then, the percentage remaining was calculated. If inhibition reached significant levels (i.e., the percentage remaining vs. uninhibited incubations containing NADPH but without test compounds or control inhibitors) was assessed as described in Supplemental Table S1. Then, the percentage remaining activity was determined by comparison of probe substrate metabolism in incubations containing NADPH but without test compounds or control inhibitors. If inhibition reached significant levels (i.e., the percentage remaining activity was <50%), IC50 values were reported.

Assessment of Potential of Saxagliptin and M2 to Induce P450 Enzymes. The potential of saxagliptin and M2 to induce the expression of mRNA levels and/or P450 enzyme activity of CYP1A2, -2B6, and -3A4 was investigated in primary cultures of freshly isolated human hepatocytes, as described previously (Hong et al., 2011). Human hepatocytes isolated from three different individual donors (lots Hu 211, Hu 223, and Hu 224; CellzDirect) were used. Donor information is provided in Supplemental Table S3. The cultured human hepatocytes were treated once daily for three consecutive days with either saxagliptin (0.2, 1, 5, and 25 μM), M2 (0.2, 1, 10, and 100 μM), solvent control [0.1% dimethyl sulfoxide (DMSO)] or known prototypical inducers, 3-methylcholanthrene [(3-MC) 2 μM], a prototypical CYP1A2 inducer, rifampicin [(RIF) 10 μM], a prototypical CYP3A4 inducer.

At the end of the treatment period, microsomes were isolated from a subset of the various hepatocyte incubations. Enzyme activity was determined by incubating microsomal samples with probe substrates specific for each P450 enzyme and then measuring the formation of marker metabolites by LC-MS/MS, as described in Supplemental Table S1. The probe substrate concentration and quantity of microsomal protein in each assay were as follows: 100 μM phenacetin and 0.02 mg/ml protein for CYP1A2; 250 μM buproprion and 0.02 mg/ml protein for CYP2B6; and 200 μM testosterone and 0.01 mg/ml protein for CYP3A4. The relative fold induction in enzymatic activity was calculated by comparing the rate of metabolite formation for treatment groups to that of the negative control group (0.1% DMSO).

RESULTS

Excretion of the Radioactive Dose. After administration of a single 50-mg, 91.5 μCi oral dose of [14C]saxagliptin to healthy male subjects, the mean cumulative recovery of radioactivity over the study duration (168 h) was ∼97% (Table 1). The majority of the radioactivity (mean value, ∼75%) was excreted in the urine. Approximately 22% was recovered in the feces.

For one subject (subject 6), the total radioactivity recovered (∼55%) was substantially lower than the recovery for the other subjects, which ranged from 84 to 106%. Although the amount of radioactivity recovered in the feces for this subject (21.5%) was similar to the other subjects (∼9–33%), the urinary recovery of radioactivity was only ∼34%, compared with ∼70 to 81% for the other subjects. This difference was evident from the first, 0–12 h urine collection, where the recovery for subject 6 was only ∼28%, compared with ∼65 to 72% for the other subjects. Pharmacokinetic analysis of plasma exposures of saxagliptin and M2 indicated that this subject had plasma exposures that were similar to the other five subjects (data not shown). These data are consistent with a possible sampling error for an early urine collection for subject 6. The data from subject 6 were therefore excluded from the calculation of the mean and S.D. values for urinary excretion reported in Table 1. However, the data from this subject were included in the calculations of fecal recovery and pharmacokinetic parameters (Table 2).

Pharmacokinetic Parameters. The mean plasma concentration versus time profiles for saxagliptin, M2, a summation of saxagliptin and M2, and total radioactivity, after administration of [14C]saxagliptin, are shown in Fig. 2. The mean pharmacokinetic parameters are presented in Table 2. Saxagliptin was rapidly absorbed with a Tmax of ∼0.5 h postdose. Tmax values for M2 and total radioactivity occurred approximately 3-4 h after saxagliptin.

TABLE 1

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Time (h)</th>
<th>% Recovered Per Collection Interval</th>
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<th>Mean ± S.D.*</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>48–168</td>
<td>2.13</td>
<td>0.99</td>
<td>1.83</td>
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<tr>
<td>Feces</td>
<td>0–168</td>
<td>70.93</td>
<td>72.71</td>
<td>70.21</td>
</tr>
<tr>
<td></td>
<td>24–48</td>
<td>&lt;LOQ</td>
<td>30.64</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>48–72</td>
<td>&lt;LOQ</td>
<td>32.06</td>
<td>16.26</td>
</tr>
<tr>
<td></td>
<td>72–96</td>
<td>5.61</td>
<td>0.06</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>96–144</td>
<td>7.96</td>
<td>&lt;LOQ</td>
<td>5.60</td>
</tr>
<tr>
<td></td>
<td>0–14</td>
<td>13.57</td>
<td>33.01</td>
<td>31.85</td>
</tr>
<tr>
<td>Total</td>
<td>0–16</td>
<td>86.50</td>
<td>105.72</td>
<td>102.06</td>
</tr>
</tbody>
</table>

NS, no sample; <LOQ, below the limit of quantification.

* n = 5, the values for subject 6 were not included in the calculation of mean urinary excretion or total excretion; mean fecal excretion values include data from all six subjects.
Mean pharmacokinetic parameters for saxagliptin, M2, and radioactivity after administration of a single oral dose of [14C]saxagliptin

<table>
<thead>
<tr>
<th>PK Parameter</th>
<th>Saxagliptin</th>
<th>M2*</th>
<th>Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_max, ng/ml</td>
<td>279</td>
<td>606</td>
<td>779</td>
</tr>
<tr>
<td>Mean, %CV</td>
<td>(13)</td>
<td>(11)</td>
<td>(10)</td>
</tr>
<tr>
<td>AUC(INF), ng · h/ml</td>
<td>845</td>
<td>2943</td>
<td>3874</td>
</tr>
<tr>
<td>Mean, %CV</td>
<td>(17)</td>
<td>(7)</td>
<td>(9)</td>
</tr>
<tr>
<td>t_max, h</td>
<td>0.50</td>
<td>1.50</td>
<td>1.50</td>
</tr>
<tr>
<td>Median (min, max)</td>
<td>(0.25, 0.50)</td>
<td>(1.00, 2.00)</td>
<td>(1.00, 1.50)</td>
</tr>
<tr>
<td>CLR, ml/min</td>
<td>234</td>
<td>102</td>
<td>165</td>
</tr>
<tr>
<td>Mean (S.D.)</td>
<td>(0.31)</td>
<td>(0.31)</td>
<td>(0.22)</td>
</tr>
<tr>
<td>%Urinary excretion</td>
<td>23.5</td>
<td>35.7</td>
<td>74.9</td>
</tr>
<tr>
<td>Mean (S.D.)</td>
<td>(23)</td>
<td>(10)</td>
<td>(14)</td>
</tr>
<tr>
<td>%Fecal excretion</td>
<td>0.5</td>
<td>8.4</td>
<td>22.1</td>
</tr>
<tr>
<td>Mean (S.D.)</td>
<td>(N.A.)</td>
<td>(N.A.)</td>
<td>(9.5)</td>
</tr>
</tbody>
</table>

%CV, percentage coefficient of variation; N.A., not applicable.
* Values reported for M2 may include small amounts of other hydroxylated metabolite isomers.
† n = 5; CLR and urinary excretion values do not include data for subject 6.
* Determined via biotransformation profiling of pooled 0- to 168-h urine or fecal samples; therefore, S.D. was not determined.

TABLE 2

at approximately 1.5 h postdose. Thereafter, the concentrations of saxagliptin, M2, and total radioactivity all declined rapidly with elimination half-life values of <3 h. The AUC(INF) values indicated saxagliptin and M2 together comprised almost all of the circulating radioactivity; based on their molar ratios, they represented approximately 22 and 72%, respectively, of the AUC of total radioactivity. On the basis of measurements with the validated LC-MS/MS assay, approximately 23.4 and 35.7% of the administered radioactivity was excreted in the urine as unchanged saxagliptin and M2, respectively.

Subsequent to the analysis for this study, it was determined that the validated LC-MS/MS method used for quantitation was not completely specific for the measurement of M2. Other mono-hydroxylated metabolites with the same MRM transition (i.e., M1, M3, and M13) eluted in close proximity to M2 under the chromatographic conditions used and may have been included in the M2 plasma measurements. Therefore, the reported values for M2 may be overestimated by up to 9% (based on radioprofiling data from plasma and urine samples) due to interference from other hydroxylated metabolites.

Identification of Metabolites. The structures of the metabolites of saxagliptin were characterized by LC-MS/MS analysis on a LCQ Deca XP ion trap mass spectrometer. Similarities in HPLC retention time and mass spectrometric fragmentation patterns with authentic standards facilitated the identification of saxagliptin, M2, and the cyclic amide degradant (D1) in the human samples. The structures of other metabolites were proposed based on their mass spectrometric fragmentation patterns relative to saxagliptin or the other available reference standards. The mass spectrometric data supporting the structural elucidation of the metabolites are summarized in Table 3.

Saxagliptin had a protonated molecule [M+H]⁺ of m/z 316 and one major MS² fragment at m/z 180 resulting from cleavage of the carbon-carbon bond between the carbonyl carbon and the carbon of the methanamine group. The metabolites generally underwent similar fragmentation, enabling the localization of structural modifications on either the amino-methyl-adamantyl group or the formyl-methanoprolineneitrile moiety.

Metabolite M2 had a protonated molecule [M+H]⁺ of m/z 332 (16 Da higher than saxagliptin). The product ion mass spectrum showed a major MS² fragment at m/z 196, consistent with hydroxylation on the amino-methyl-adamantyl group. Like M2, metabolites M1, M3, and M16 each had a protonated molecular ion [M+H]⁺ of m/z 332 (16 Da higher than saxagliptin) and a major MS² fragment at m/z 196. These MS data suggested that these metabolites were positional isomers of mono-hydroxylated saxagliptin with the site of the hydroxylation for all three metabolites on the amino-methyl-adamantyl group. Metabolite M16 had an additional MS² major fragment at m/z 314 (loss of −18 Da from the protonated molecular ion), indicative of a loss of water. This fragment was not observed as a significant fragment in the MS² spectra for either M1, M2, or M3.

Degradant D1, an isomer of saxagliptin with a keto-iminopiperazine ring structure, is a known impurity that forms as a degradation product in solution (Jones et al., 2011). D1 had a protonated molecule [M+H]⁺ of m/z 316, identical to the mass of saxagliptin. A normalized collision energy of 23%, which produced a large MS² fragment at m/z 180 for saxagliptin, resulted in little or no fragmentation of D1 (data not shown). However, at a higher normalized collision energy of 30%, two major MS² fragment ions of D1 were observed at m/z 288 and 180. The fragment ion at m/z 288 (28 Da less than the molecular ion) corresponded to loss of the carbonyl group, whereas the ion at m/z 180 most likely resulted from an additional cleavage to form a fragment similar to that observed for saxagliptin.

M13 had a protonated molecule [M+H]⁺ of m/z 332 (16 Da higher than saxagliptin), suggesting that it was a hydroxylated metabolite. Like D1, M13 did not show fragmentation when subjected to a normalized collision energy of 23% in the mass spectrometer, but at 30%, two major MS² fragment ions were observed at m/z 196 (M + 16) and 304 (loss of carbonyl group). Based on the MS data, and comparison of LC-MS/MS data to a subsequently synthesized reference standard, M13 is proposed to be a cyclized product of M2, having a keto-iminopiperazine ring structure.

M45 had a protonated molecule [M+H]⁺ of m/z 396, 80 Da higher than saxagliptin, which is characteristic of a sulfate conjugate. The MS² spectra for M45 showed major fragment ions at m/z 298 and 260. The fragment at m/z 298 (neutral loss of −98 Da from the protonated molecular ion) was indicative of loss of H₂SO₄, whereas the fragment at m/z 260 resulted from cleavage of the carbon-carbon bond between the carbonyl carbon and the carbon of the methanamine group. Based on the MS data, M45 was identified as a sulfate conjugate of saxagliptin with the site of sulfation most likely on the hydroxyl group of the adamantyl ring.

FIG. 2. Mean concentration versus time profiles of total radioactivity, saxagliptin, M2, and the summed contribution of saxagliptin and M2, in plasma from healthy male subjects after oral administration of 50 mg (91.5 μCi) of [14C]saxagliptin. *, the concentration values reported for M2 may contain small amounts of other hydroxylated metabolites; based on radioprofiling results of plasma samples, the contribution of these metabolites is expected to be <9% of the reported M2 concentration at each time point.
Metabolites M46 had a protonated molecule $[M + H]^+$ of $m/z$ 492, 176 Da higher than saxagliptin. The MS$^2$ spectra showed a major fragment ion at $m/z$ 316, corresponding to a loss of glucuronic acid. These data suggested that M46 is a direct glucuronide conjugate of the parent compound; however, the exact site of glucuronidation could not be localized based on the MS fragmentation data.

**Metabolite Profiles in Plasma.** Metabolite profiles from pooled plasma collected at 4 h postdose are shown in Fig. 3A. Similar profiles were obtained at 1, 2, and 8 h postdose. Consistent with the pharmacokinetic data, parent drug and the M2 metabolite were the major circulating components, representing 20 to 30% and 60 to 70% of the plasma radioactivity at the 1- to 8-h time points, respectively. Additional minor circulating metabolites that each represented 4% of the plasma radioactivity at each time point included the following: hydroxylated metabolites (M1, M3, M13), a sulfate conjugate (M45), and a glucuronide conjugate (M46). A small amount of degradant D1 (2%) was also detected in the plasma at each time point.

**Potential for Chiral Interconversion of Saxagliptin.** To determine whether any of the chiral centers of saxagliptin were susceptible to chiral inversion, the metabolite profiles of in vivo samples were examined for the presence of diastereomers of saxagliptin. Other than saxagliptin and D1, no additional peaks corresponding to the protonated molecule $[M + H]^+$ of $m/z$ 316 were observed. In addition, there were no peaks eluting at the retention time of authentic reference standards for the $S,R,S,S$ and $S,S,S,R$ epimers of saxagliptin. These two epimers were chromatographically resolved from saxagliptin on the chromatographic method used for metabolite profiling (data not shown). Of all the saxagliptin diastereomers (Supplemental Fig. S1), formation of these two would be the most probable, because they are the product of inversion at only one of each of the two independent stereogenic sites.

**Identification of Cytochrome P450 Enzymes Involved in the Metabolism of Saxagliptin.** The biotransformation of $[^{14}C]$saxagliptin was investigated in HLM and a panel of recombinant P450 enzymes (Fig. 4, A–C). At a substrate concentration of 10 $\mu$M, the turnover of saxagliptin in HLM was ~32% after a 1-h incubation. The M2 metabolite was the primary metabolite formed, along with several additional minor mono-hydroxylated metabolites. The saxagliptin metabolite profiles with CYP3A4 and CYP3A5 after a 30-min incubation were similar to that observed with the HLM, indicating that these enzymes were capable of catalyzing the formation of M2 and other minor metabolites. There was no significant turnover of saxagliptin with any of the other P450 enzymes evaluated. Consistent with these observations, the rate of formation of M2 in a panel of 16 lots of HLM from individual donors showed good correlation with the vendor-reported CYP3A activity, which was based on rates of testosterone metabolism.

**Table 3**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$[M + H]^+$</th>
<th>Major Fragment Ions</th>
<th>Metabolic Pathway(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saxagliptin</td>
<td>316</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>332</td>
<td>196</td>
<td>Hydroxylation</td>
</tr>
<tr>
<td>5-Hydroxy saxagliptin (M2)</td>
<td>332</td>
<td>196</td>
<td>Hydroxylation</td>
</tr>
<tr>
<td>M3</td>
<td>332</td>
<td>196</td>
<td>Hydroxylation</td>
</tr>
<tr>
<td>M13</td>
<td>332</td>
<td>304, 196</td>
<td>Hydroxylation, cyclization</td>
</tr>
<tr>
<td>M16</td>
<td>332</td>
<td>314, 196</td>
<td>Hydroxylation</td>
</tr>
<tr>
<td>M45</td>
<td>396</td>
<td>298, 260</td>
<td>Sulfation</td>
</tr>
<tr>
<td>M46</td>
<td>492</td>
<td>474, 316, 298</td>
<td>Glucuronidation</td>
</tr>
<tr>
<td>D1</td>
<td>316</td>
<td>288, 180</td>
<td>Cyclization</td>
</tr>
</tbody>
</table>
absence of NADPH, M2 formation was diminished by inhibitor 1-aminobenzotriazole (a general P450 inhibitor), or in the presence of NADPH to assess their potential to inhibit P450 enzymes. For all P450 enzymes investigated, the IC_{50} values for direct inhibition (no preincubation) were greater than the highest concentrations tested. >50 μM for saxagliptin and >200 μM for its M2 metabolite. When saxagliptin and M2 were preincubated with HLM in the presence of NADPH for 15 min, before the reporting phase of the incubation, the IC_{50} values were also greater than the highest concentrations tested, >50 and >200 μM, respectively (Supplemental Table S2). These data suggest that at concentrations exceeding 100-fold of their clinically relevant concentrations, saxagliptin and its M2 metabolite were not direct or time-dependent inhibitors of P450 enzymes.

Assessment of the Potential of Saxagliptin and M2 to Inhibit P450 Enzymes. The enzyme activities and mRNA levels of CYP1A2, CYP2B6, and CYP3A4 were determined in primary cultured human hepatocytes obtained from three individual donors after treatment with either saxagliptin (0.2, 1.0, 5.0, and 25 μM), M2 (0.2, 1.0, 10, and 100 μM), or prototypical inducers 3-MC (positive control inducer for CYP1A2), PB (positive control inducer for CYP2B6), and RIF (positive control inducer for CYP3A4). The mean enzyme activity and mRNA data from three donors are summarized in Fig. 6, and the individual data for each donor are provided in Supplemental Tables S4 and S5. At the concentrations tested, saxagliptin and M2 were not cytotoxic as indicated by morphological integrity and lactate dehydrogenase activity assays (data not shown). Treatment with saxagliptin and M2 at concentrations up to 25 or 100 μM, respectively, did not result in meaningful increases of CYP1A2, CYP2B6, or CYP3A4 enzyme activity or mRNA content, suggesting that these compounds are not inducers of these P450 enzymes at clinically relevant concentrations.

Discussion

After administration of a single, oral 50-mg dose of [^{14}C]saxagliptin (91.5 μCi) to healthy human subjects, saxagliptin was rapidly absorbed into the systemic circulation. The T_{max} value was 0.5 h for the parent compound and 1.5 h for both the M2 metabolite and total radioactivity. After reaching T_{max}, the concentrations for all three analytes declined rapidly, each with elimination half-life values of <3 h (Table 2). Consistent with the short half-life, nearly 80% of the administered radioactivity was recovered within the first 24 h after dosing. By the end of the 7-day study, a total of 97% of the administered dose was recovered, of which 74.9% was eliminated in the urine and 22.1% was excreted in the feces (Table 1). In urine and feces, respectively, approximately 23.5 and 0.5% of the drug-related radioactivity was recovered as saxagliptin and 35.7 and 8.4% was recovered as M2 (Table 2; Fig. 1). These data indicate that saxagliptin was well absorbed (≥97%), and that the formation of M2 representing the major metabolic pathway. Minor metabolic pathways included hydroxylation at other positions and glucuronide or sulfate conjugation. In addition, small amounts of both saxagliptin and M2 underwent intramolecular cyclization to form compounds with a keto-iminopiperazine ring structure, D1 and M13, respectively (~2–3% of the dose each; Fig. 1). These cyclic amidine products are known degradation products of this chemotype, and their formation is favored under aqueous, alkaline conditions (Jones et al., 2011). In vitro, the formation of

6β-hydroxylation (r = 0.985; Fig. 4D) or midazolam 1’-hydroxylation (r = 0.949; Fig. 4E).

In HLM incubations conducted in the presence of the chemical inhibitor 1-aminobenzotriazole (a general P450 inhibitor), or in the absence of NADPH, M2 formation was diminished by >99%, indicating that the metabolism of saxagliptin to M2 was a P450-mediated process. The CYP3A inhibitors ketoconazole and troleandomycin both inhibited the formation of M2 from saxagliptin (1 μM) in human liver microsomes by ≥97%, and an anti-CYP3A4/5 antibody inhibited M2 formation by >89% (Supplemental Fig. S2).

Kinetic experiments indicated that the formation of M2 from saxagliptin in HLM and expressed CYP3A4 and CYP3A5 followed Michaelis-Menten kinetics (Fig. 5, A–C). The K_m values for the formation of M2 were 94.8 μM in HLM and 81.7 and 252 μM in CYP3A4 and CYP3A5 incubations, respectively (Fig. 5D). The V_{max} values were 496 pmol M2/mg protein/min in HLM, 31.7 pmol M2/pmol P450/min in CYP3A4, and 24.0 pmol M2/pmol P450/min in CYP3A5. The catalytic efficiency (V_{max}/K_m) for CYP3A4 (0.388 μl/pmol P450/min) was approximately 4-fold higher than for CYP3A5 (0.095 μl/pmol P450/min), suggesting that CYP3A4 was the major P450 enzyme involved in the formation of M2.

Assessment of the Potential of Saxagliptin and M2 to Induce P450 Enzymes. The enzyme activities and mRNA levels of CYP1A2, CYP2B6, and CYP3A4 were determined in primary cultured human hepatocytes obtained from three individual donors after treatment with either saxagliptin (0.2, 1.0, 5.0, and 25 μM), M2 (0.2, 1.0, 10, and 100 μM), or prototypical inducers 3-MC (positive control inducer for CYP1A2), PB (positive control inducer for CYP2B6), and RIF (positive control inducer for CYP3A4). The mean enzyme activity and mRNA data from three donors are summarized in Fig. 6, and the individual data for each donor are provided in Supplemental Tables S4 and S5. At the concentrations tested, saxagliptin and M2 were not cytotoxic as indicated by morphological integrity and lactate dehydrogenase activity assays (data not shown). Treatment with saxagliptin and M2 at concentrations up to 25 or 100 μM, respectively, did not result in meaningful increases of CYP1A2, CYP2B6, or CYP3A4 enzyme activity or mRNA content, suggesting that these compounds are not inducers of these P450 enzymes at clinically relevant concentrations.

Discussion

After administration of a single, oral 50-mg dose of [^{14}C]saxagliptin (91.5 μCi) to healthy human subjects, saxagliptin was rapidly absorbed into the systemic circulation. The T_{max} value was 0.5 h for the parent compound and 1.5 h for both the M2 metabolite and total radioactivity. After reaching T_{max}, the concentrations for all three analytes declined rapidly, each with elimination half-life values of <3 h (Table 2). Consistent with the short half-life, nearly 80% of the administered radioactivity was recovered within the first 24 h after dosing. By the end of the 7-day study, a total of 97% of the administered dose was recovered, of which 74.9% was eliminated in the urine and 22.1% was excreted in the feces (Table 1). In urine and feces, respectively, approximately 23.5 and 0.5% of the drug-related radioactivity was recovered as saxagliptin and 35.7 and 8.4% was recovered as M2 (Table 2; Fig. 1). These data indicate that saxagliptin was well absorbed (≥97%), and that the formation of M2 representing the major metabolic pathway. Minor metabolic pathways included hydroxylation at other positions and glucuronide or sulfate conjugation. In addition, small amounts of both saxagliptin and M2 underwent intramolecular cyclization to form compounds with a keto-iminopiperazine ring structure, D1 and M13, respectively (~2–3% of the dose each; Fig. 1). These cyclic amidine products are known degradation products of this chemotype, and their formation is favored under aqueous, alkaline conditions (Jones et al., 2011). In vitro, the formation of
D1 or M13 in HLM incubations with saxagliptin or M2, respectively, was low (approximately 1%) and was similar to negative controls containing only compound and buffer (data not shown). Therefore, we concluded that these cyclization reactions are non-enzymatic processes. Based on the structures proposed from LC-MS/MS analyses (Table 3), all of the metabolites and degradants retained both of the C-14 labels, thus confirming the suitability of the labeling scheme for [14C]saxagliptin used in this study.

Both pharmacokinetic data and biotransformation profiling results (Figs. 2 and 3A) indicated that unchanged saxagliptin and M2 were the most prominent drug-related components in the plasma; together, the two compounds accounted for almost all of the circulating radioactivity; based on their molar ratios, they represented approximately 22 and 72%, respectively, of the AUC of total radioactivity. Minor drug-related species in plasma included other hydroxylated metabolites, glucuronide- and sulfate-conjugated metabolites, and rearrangement products, D1 and M13. The M2 is pharmacologically active, with an in vitro potency for DDP4 inhibition that was approximately half that of saxagliptin (Fura et al., 2009). The serum protein binding of saxagliptin and its M2 metabolite are both negligible (Fura et al., 2009); therefore, their relative in vivo potencies are expected to be similar to the values determined in vitro. Because of its potency and high plasma concentrations, M2 is expected to significantly contribute to the observed pharmacological effect of saxagliptin. Other metabolites and degradants, due to their low abundance in plasma and/or significant structural modifications, are not expected to contribute to the pharmacological activity of saxagliptin. All human circulating metabolites were represented in one or more of the preclinical species (rat, dog, and monkey), with no disproportionate human metabolites observed (H. Su, personal communication). Metabolite M2 was the predominant metabolite found in all species (Fura et al., 2009), and each of the toxicology species provided 2-fold exposure multiples for this metabolite, relative to a 5-mg human dose, which was sufficient for toxicological evaluation.

Saxagliptin has four chiral centers. Although chiral inversion of saxagliptin was not expected to occur through traditional metabolic mechanisms, i.e., oxidation of a secondary alcohol or conjugation of a carboxylic acid with acetyl CoA (Wsol et al., 2004), chiral inversion might occur via a chemical mechanism, either in vivo or ex vivo, during sample storage or processing. Therefore, we examined the in vivo samples for the presence of diastereomers of saxagliptin. No peaks with the same protonated molecule as saxagliptin and D1 were observed in the LC-MS/MS chromatograms of the plasma, urine, or feces samples. Furthermore, there were no
peaks corresponding to the retention time of authentic reference standards for the \( S,R,S,S \) and \( S,S,S,R \) epimers in the pooled urine, feces, or plasma samples. Of all the saxagliptin diastereomers, the formation of these two were considered most likely, because they were each the product of inversion at only one of the two independent stereogenic sites; the other two chiral centers were conformationally locked within the cyclopropyl ring system. These data suggest that saxagliptin does not undergo measurable chiral inversion either in vivo, after administration to humans, or during subsequent sample processing.

Although the dose used in the clinical portion of the current study was higher than the one ultimately selected as the prescription dose (5 mg), saxagliptin showed predictable, dose-proportional, multiple-dose pharmacokinetics over the range of 5 to 400 mg with minimal accumulation when dosed once daily (Boulton and Geraldes, 2007). It is therefore expected that the pharmacokinetic, metabolism, and excretion results from the current study will be applicable to lower doses within this range.

Consistent with the in vivo data, M2 was the predominant metabolite of saxagliptin formed in human liver microsomes (Fig. 4A). P450 enzymes CYP3A4 and CYP3A5 were both capable of metabolizing saxagliptin to M2 (Fig. 4, B and C); little or no turnover was observed with other P450 enzymes. Experiments with human liver microsomes and chemical and monoclonal antibody inhibitors for specific P450 enzymes indicated that catalytic efficiency (\( V_{\text{max}}/K_{\text{m}} \)) for M2 formation by CYP3A4 was approximately 4-fold higher than for CYP3A5 (Fig. 5), suggesting that the formation of M2 will be predominantly mediated by CYP3A4, even in subjects with high expression levels of CYP3A5. The \( CYP3A5^{*3} \) variant results in functionally defective CYP3A5 enzyme; whites are known to have a higher frequency (\( \sim 90\% \)) of the \( CYP3A5^{*3} \) allele, compared with blacks (\( \sim 32\% \)), Hispanics (\( \sim 63\% \)), and Asians (\( \sim 73\% \)) (Xie et al., 2004). Population analysis of data from Phase II/III clinical trials suggested that race (whites, blacks, Hispanic, or Asian) had no impact on exposures of either saxagliptin or M2 (D. Boulton, personal communication), confirming the minimal contribution of CYP3A5 in the metabolism of saxagliptin.

When saxagliptin (100 mg) was coadministered with ketoconazole (200 mg, once every 12 h) in a clinical drug-drug interaction study, the AUC of saxagliptin increased by 145% and the AUC of M2 decreased by 88% (Patel et al., 2011). In addition, when saxagliptin (5 mg) was coadministered with rifampin at steady state (600 mg, once daily for 6 days), the AUC of saxagliptin decreased by 76% (Upreti et al., 2011). Thus, the role of CYP3A enzymes in the metabolism of saxagliptin was confirmed in these two clinical studies.

Saxagliptin and M2 each demonstrated little potential to inhibit or induce P450 enzymes in primary cultures of human hepatocytes, suggesting that saxagliptin is unlikely to affect the metabolic clearance of coadministered drugs that are substrates for P450 enzymes. The lack of an inhibitory effect of saxagliptin on CYP3A was con-
firmed in a clinical drug-drug interaction study between saxagliptin and simvastatin, where little or no increase in the AUC values of simvastatin and simvastatin acid (4 and 16%, respectively) was observed when 40 mg of simvastatin was administered together with a 10-mg dose of saxagliptin (Patel et al., 2011).

In a separate clinical study, the absolute bioavailability of saxagliptin in healthy subjects was 50% after administration of a single 5-mg dose (Xu et al., 2011). The current in vivo and in vitro experimental data support this result. Although at least 74.9% of the saxagliptin dose was absorbed after oral administration, metabolite profiling results suggested that a substantial portion of the administered dose was metabolized to M2.

In summary, after a 50-mg (91.5 µCi) p.o. dose to healthy male volunteers, [14C]saxagliptin was rapidly absorbed, and recovery of the administered radioactivity was good (97%). At least 74.9% of the dose was absorbed based on the amount of radioactivity recovered in urine. Saxagliptin and its active M2 metabolite were the predominant circulating components, together comprising almost all of the plasma radioactivity. Elimination of saxagliptin occurred via both renal excretion and metabolism, with the formation of M2 by CYP3A4 representing the major metabolic pathway. Neither saxagliptin nor M2 inhibited or induced P450 enzymes in vitro, suggesting that saxagliptin is unlikely to alter the clearance of concomitants that are metabolized by these enzymes.

Acknowledgments
We acknowledge Dr. Tong Li and colleagues at the Bristol-Myers Squibb Clinical Pharmacology Unit (Hamilton, NJ) for overseeing the clinical portion of the study and Christopher Black and the staff at CellzDirect for conducting the in vitro studies on the inhibition and induction potential of saxagliptin.

Authorship Contributions
Participated in research design: Su, Boulton, Iyer, Humphreys, and Christopher.
Conducted experiments: Su, Barros Jr., Wang, and Christopher.
Contributed new reagents or analytic tools: Cao and Bonacorsi Jr.
Performed data analysis: Su, Boulton, Iyer, and Christopher.
Wrote or contributed to the writing of the manuscript: Su, Boulton, Iyer, Humphreys, and Christopher.

References

FIG. 6. Evaluation of the potential induction effects of saxagliptin and M2 on P450 enzymes in primary cultures of human hepatocytes. A, fold increase in CYP1A2, CYP2B6, and CYP3A4 enzyme activity relative to solvent control. B, fold increase in CYP1A2, CYP2B6, and CYP3A4 mRNA content relative to solvent control. Positive control inducers were 3-MC, PB, and Rif for CYP1A2, CYP2B6, and CYP3A4, respectively. The results presented represent the average ± S.D. from three individual preparations of human hepatocyte donors. *, where indicated, mRNA could not be obtained from one of the donor hepatocytes; therefore, these values represent the average of the remaining two donors. #, the CYP1A2 mRNA levels were not determined in hepatocyte preparations treated with either PB or Rif.


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