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Characterization of the In Vitro and In Vivo Metabolism and Disposition and Cytochrome P450 Inhibition/Induction Profile of Saxagliptin in Human[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 μCi) oral dose. Saxagliptin was rapidly absorbed (T_{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_{max}/K_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 (Augeri et al., 2005). The ability of saxagliptin to affect reductions in glycosylated hemoglobin (HbA_{1C}) and fasting plasma glucose in type 2 diabetes patients has been demonstrated in multiple

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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_T, threshold cycle.

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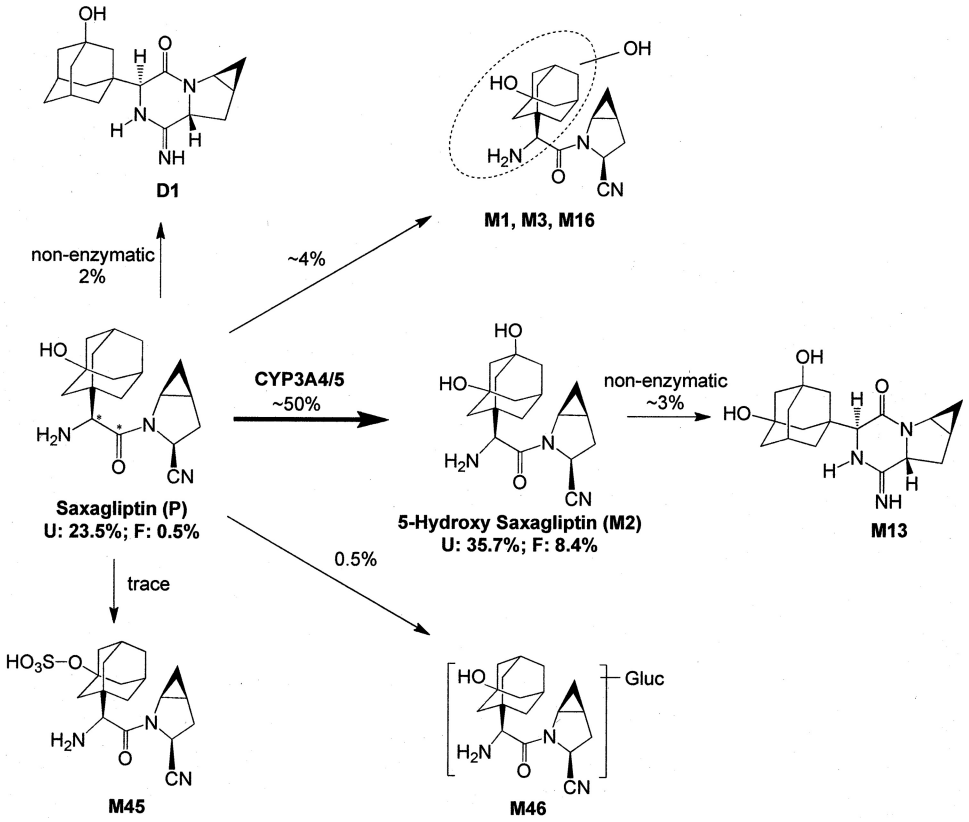

FIG. 1. Primary pathways for biotransformation of [^{14}C]saxagliptin in humans. The C-14 label was evenly distributed between the carbonyl carbon and the adjacent carbon, as indicated by asterisk (*) on the saxagliptin structure. The percentage of administered radioactivity recovered in urine and feces as saxagliptin and M2 is indicated underneath the structures. The estimated flux through each pathway (also reported as percentage of administered radioactivity) is indicated next to the corresponding arrow. D1, a degradant known to form in solution, was found in small quantities in all samples.

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 (Augeri 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 [^{14}C]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 comedications. 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. [^{14}C]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 $^{13}C_4$,^{15}N-saxagliptin, and $^{13}C_4$,^{15}N-5-hydroxy saxagliptin [internal standards for high-performance liquid chromatography (HPLC) analysis] were synthesized by the Radiochemistry Group of the NJ) (Cao et al., 2007). Unlabeled saxagliptin (P, (1*S*,3*S*,5*S*)-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, (1*S*,3*S*,5*S*)-2-((*S*)-2-amino-2-((1r,3*R*,5*S*,7*S*)-3,5-dihydroxyadamantan-1-yl)acetyl)-2-azabicyclo[3.1.0]hexane-3-carbonitrile); degradant (D1, (1a*S*,4*S*,6a*R*,7a*S*)-4-(-3-hydroxyadamantan-1-yl)-6-iminohexahydro-1*H*-cyclopropa[4,5]pyrrolo[1,2-a]pyrazin-3(1a*H*)-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. Individual lots (n = 16) of HLM, for which the vendor had determined the activities of various P450 enzymes, were purchased as a Reaction Phenotyping kit (version 7) from XenoTech, LLC (Lenexa, KS). Monoclonal antibodies with inhibitory activity for specific P450 enzymes were obtained from Kristopher W. Krausz at the Laboratory of Metabolism, National Institutes of Health (Bethesda, MD).

Ecolite scintillation cocktail was obtained from MP Biomedicals (Irvine, CA), and Emulsifier-Safe and Permofluor E+ scintillation fluid were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Deionized water was prepared using a MilliQ ultrapure water system (Millipore Corporation, Billerica, MA). All organic solvents were HPLC grade, and other regents 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

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(range, 25 to 37 years), and the mean body mass index was 23.6 kg/m^2 (range, 19.7 to 28.1 kg/m^2). The study was conducted in accordance with the Declaration of Helsinki and guidelines on Good Clinical Practice. Before study initiation, the study protocol and informed consent documents were approved by the Institutional Review Board of the New England Institutional Review Board (Wellesley, MA). All study participants provided written informed consent before the initiation of study-specific procedures.

After a 10-h fast, each subject received a single 50-mg p.o. solution dose of [^{14}C]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 K_3EDTA 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, 2.0, 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 ($^{13}C_4$,^{15}N-saxagliptin and $^{13}C_4$,^{15}N-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 API3000 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 (C_{max}); 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 (T-HALF), 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 fecal 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 2500*g* 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 SIL-10AD autoinjector, a model SCL-10A system controller, and an SPD-M10A photodiode 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 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

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flow rate, spray current, and voltages were adjusted as required to achieve maximum sensitivity or optimal fragmentation of drug-related components. Reference standards, available for saxagliptin, M2, D1, M13, and the *S,R,S,S* and *S,S,S,R* epimers of saxagliptin were used to confirm the retention time and mass-spectral fragmentation patterns of these analytes.

Identification of Enzymes Involved in the Metabolism of Saxagliptin and in the Formation of M2. [^{14}C]Saxagliptin (10 μM) was incubated at 37°C with pooled HLM (1 mg/ml protein) and individually expressed human P450 enzymes (500 pmol/ml each, including CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5) in 100 mM phosphate buffer (pH 7.4), fortified with 1 mM NADPH to evaluate which enzymes were capable of metabolizing the compound and forming the major metabolite, M2. After the incubation period (30 min for expressed enzymes; 60 min for HLM), the reactions were terminated by adding 1 to 2 volumes of ice-cold acetonitrile. The samples were then centrifuged, and the resulting supernatants were analyzed by LC-MS/MS and off-line radioanalysis. The analytical methodology was similar to that described for biotransformation profiling and metabolite identification of in vivo samples, with the exception that drug-related components were separated on a YMC ODS-AQ *S*-3 120A column, maintained at 30°C, and the mobile phase gradient was modified, with a shorter run time (48 min).

A correlation analysis between the formation of M2 and P450 activity was 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, -2E1, -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 $MgCl_2$ (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), benzylnirvanol (1 μM, CYP2C19), quinidine (1 μM, CYP2D6), ketoconazole (1 μM, CYP3A4/5) and time-dependent inhibitors, furafylline (10 μM, CYP1A2), orphenadrinie (50 μM, CYP2B6), diethyldithiocarbamate (50 μM, CYP2E1), troleandomycin (20 μM, CYP3A4/5), and 1-aminobenzotriazole (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 $MgCl_2$ 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 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 kinetics for the formation of M2 were determined in pooled HLM and expressed CYP3A4 and CYP3A5. Incubations (0.25 ml total volume, in triplicate) contained 1 mM NADPH, 2 mM $MgCl_2$, 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, $^{13}C_4$,^{15}N-5-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 quantitation of M2 in in vitro samples consisted of two 10AD-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 instrumentation 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 isocratic at 15% B (0–0.1 min); from 15 to 30% B (0.1–3 min); from 30 to 38% B (3–4 min); from 38 to 40% B (4–5 min); from 40 to 80% B (5–5.5 min); hold isocratic 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. Ultrahigh 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-hydroxysaxagliptin 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; CellzDirect, Durham, NC). IC_{50} 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), bupropion hydroxylation (20 μM, CYP2B6), paclitaxel 6-hydroxylation (5 μM, CYP2C8), tolbutamide hydroxylation (140 μM, CYP2C9), *S*-mephenytoin 4′-hydroxylation (50 μM, CYP2C19), bufuralol 1′-hydroxylation (40 μM, CYP2D6), chlorzoxazone 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

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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 time-dependent 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 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%), IC_{50} 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 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], phenobarbital [(PB) 1000 μM, a prototypical CYP2B6 inducer], and 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 bupropion 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).

Another subset of the various hepatocyte incubations were used for mRNA determination. After cell lysis, total mRNA was isolated using the RNeasy Mini or Midi Kit (QIAGEN, Valencia, CA) cDNA, according to the manufacturer's instructions. Total RNA was quantified using the RiboGreen RNA Quantitation reagent and kit (Invitrogen, Carlsbad, CA). Samples were subsequently diluted to the desired RNA concentration (10 ng/μl). TaqMan real-time quantitative polymerase chain reaction was then used to determine the levels of CYP1A2, CYP2B6, and CYP3A4 present in each sample using primers and probes specific for each enzyme (Applied Biosystems, Foster City, CA). The reactions were characterized during the point in the polymerase chain reaction cycle at which the amplification product was first detected [threshold cycle (C_T)]. C_T values, baseline, and threshold levels were automatically calculated by the ABI 7500 Sequencer software (Applied Biosystems). A difference in one C_T was considered equivalent to a 2-fold difference in gene expression (i.e., an exponential relationship, $2^{\Delta CT}$). Relative-fold mRNA content was determined for each treatment group relative to the endogenous control gene expression and 0.1% DMSO vehicle control for each sample.

Results

Excretion of the Radioactive Dose. After administration of a single 50-mg, 91.5 μCi oral dose of [^{14}C]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- to 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 [^{14}C]saxagliptin, are shown in Fig. 2. The mean pharmacokinetic parameters are presented in Table 2. Saxagliptin was rapidly absorbed with a T_{max} of ~0.5 h postdose. T_{max} values for M2 and total radioactivity occurred

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TABLE 1

Excretion of radioactivity in human urine and feces after administration of a single oral dose of [^{14}C]saxagliptin

Values in italics represent total excretion values over the entire study period.

Matrix	Time (h)	% Recovered Per Collection Interval						
		Subject Number						Mean ± S.D.[a]
		1	2	3	4	5	6	
Urine	0–12	65.88	66.70	64.89	71.64	71.63	27.65	68.15 ± 3.25
	12–24	0.63	4.27	1.65	5.26	4.47	1.02	3.26 ± 2.00
	24–48	2.29	0.75	1.84	2.53	2.07	4.02	1.90 ± 0.69
	48–168	2.13	0.99	1.83	1.60	1.43	1.26	1.60 ± 0.43
	0–168	*70.93*	*72.71*	*70.21*	*81.03*	*79.60*	*33.95*	*74.89 ± 5.05*
Feces	0–24	<LOQ	30.64	0.04	0.07	11.14	1.40	7.22 ± 12.27
	24–48	NS	2.06	16.26	9.02	8.51	14.88	8.46 ± 6.56
	48–72	<LOQ	0.25	9.95	0.24	1.79	4.90	2.86 ± 3.94
	72–96	5.61	0.06	NS	NS	1.11	NS	1.13 ± 2.24
	96–144	7.96	<LOQ	5.60	<LOQ	0.46	0.33	2.39 ± 3.48
	0–144	*13.57*	*33.01*	*31.85*	*9.33*	*23.01*	*21.51*	*22.05 ± 9.50*
Total	0–168	*84.50*	*105.72*	*102.06*	*90.36*	*102.61*	*55.46*	*97.05 ± 9.13*

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