METABOLISM, PHARMACOKINETICS, AND PROTEIN COVALENT BINDING OF RADIOLABELED MAXIPOST (BMS-204352) IN HUMANS


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ABSTRACT:

MaxiPost [3S]+-(5-chloro-2-methoxyphenyl)-1,3-dihydro-3-fluoro-6-(trifluoromethyl)-2H-indole-2-one; BMS-204352] is an investigational maxi-K channel opener to treat ischemic stroke. This study reports the disposition, metabolism, pharmacokinetics, and protein covalent binding of 14C-labeled MaxiPost in healthy male volunteers as well as in dogs and rats. After each human subject received a single dose of 10 mg 14C-labeled BMS-204352 (50 μCi) as a 5-ml intravenous infusion lasting 5 min, the plasma radioactivity concentrations showed a unique profile, wherein the concentration appeared to increase initially, followed by a terminal decline. The mean terminal t1/2 of plasma radioactivity (259 h) was prolonged compared with that of unchanged parent (37 h). Furthermore, the extractability of radioactivity in plasma decreased over time, reaching approximately 20% at 4 h after dosing. The unextractable radioactivity was covalently bound to plasma proteins through a des-fluoro-des-methyl BMS-204352 lysine adduct. Unchanged BMS-204352 and minor metabolites were identified in plasma extract following protein precipitation. The recovery of the radioactive dose in urine and feces was nearly complete in 14-day collections (approximately 37% in urine and 60% in feces). The N-glucuronide of the parent was the prominent metabolite in urine (16.5% of dose), whereas the parent was a major drug-related component in feces (11% of dose). Similar disposition, metabolism, pharmacokinetic, and protein covalent binding properties of 14C-labeled BMS-204352 were observed in humans, dogs, and rats.

Abbreviations: CL, total body clearance; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; LC/MS/MS, liquid chromatography/tandem mass spectrometry; NMR, nuclear magnetic resonance; PEG, polyethylene glycol; T1/2, terminal half-life; Tmax, time to reach the maximal drug concentration (at the Cmax); Vss, steady-state volume of distribution.

Stoke is a major cause of death and long-term disability, affecting more than 700,000 people in the United States annually (Williams et al., 1999). Acute ischemic stroke is the most common form, producing pathologically fatal levels of intracellular calcium (Ca2+) in neurons at risk. Maxi-K channels are large-conductance voltage- and Ca2+-activated K+ channel proteins (Chang et al., 1997). BMS-204352, chemically designated as (3S)+-(5-chloro-2-methoxyphenyl)-1,3-dihydro-3-fluoro-6-(trifluoromethyl)-2H-indole-2-one, is a maxi-K channel opener (Gribkoff et al., 2001). This compound has the potential to prevent and treat ischemic stroke. The fluoro-oxyindole, BMS-204352, provided significant levels of cortical neuroprotection in rat models of stroke by augmenting an endogenous mechanism for regulating Ca2+ entry and membrane potential to protect the neurons (Cheney et al., 2001; Gribkoff et al., 2001).

Metabolism appeared to be predominant in the disposition of BMS-204352 in rats and dogs (Krishna et al., 2002d). Following an intravenous infusion of [14C]BMS-204352 to rats (6 mg/kg) and dogs (2204352 in rats and dogs (Krishna et al., 2002d). Following an intravenous infusion of [14C]BMS-204352 to rats (6 mg/kg) and dogs (2 mg/kg), the AUC values of the unchanged BMS-204352 represented more than a very small fraction of the plasma radioactivity. Radioactivity was primarily excreted in the feces (more than 85% of administered dose over a 7-day collection period). Within an hour of dosing rats with [14C]BMS-204352, over two-thirds of the radioactivity in plasma is covalently bound to plasma proteins. The covalently bound metabolite was identified by acid hydrolysis of rat plasma proteins followed by isolation and characterization by NMR and mass spectral analyses as N-des-demethyl-des-fluoro BMS-204352 lysine adduct (Zhang et al., 2003). BMS-204352 exhibited linear pharmacokinetics at intravenous doses (0.4, 2.0, 5.0, and 10.0 mg/kg) to rats (Krishna et al., 2002b) and at intravenous (i.v.) doses (0.4, 0.9, and 2.0 mg/kg) to dogs (Krishna et al., 2002c). BMS-204352 also effectively penetrates into brain, the target site of action, resulting in brain-to-plasma ratios of greater than 7 in rats (Krishna et al., 2002a).

The present study investigated the mass balance, disposition, metabolism, pharmacokinetics, and protein covalent binding of [14C]BMS-204352 in humans after the intravenous administration of a 10-mg (50-μCi) dose. Comparative biotransformation in toxicological species (dogs and rats) is also presented. An efficient procedure for protein washing was developed to evaluate in vitro and in vivo protein covalent binding.

Materials and Methods

Chemicals. [14C]BMS-204352 (5 μCi/mg, radiochemical purity 99.5%), des-fluoro BMS-204352 lysine adduct (BMS-349821, 98% pure), and [13CD3]BMS-204352 (internal standard for LC/MS/MS analysis) were synthesized at Bristol-Myers Squibb (Dischino et al., 2003; Zhang et al., 2003). Ammonium acetate and PEG-300 were purchased from Aldrich Chemical...
Subjects, Drug Formulation, and Administration. This study was performed in accordance with the following codes and guidelines: Title 21, Part 56 CFR (Institutional Review Board Approval); Title 21, Part 50 CFR (Protection of Human Subjects); the principles of the Declaration of Helsinki and its amendments; and Good Clinical Practice. After being advised of the nature and risks associated with the study, all subjects were required to give informed and written consent prior to participation in the study. All subjects were in good health as determined by medical history, physical examination, and clinical laboratory tests conducted prior to the study based on inclusion and exclusion criteria. Eight subjects were healthy men of ages 25 to 42 with a body mass index between 18 and 30 kg/m², and were not found to have any medical reason to prevent participation. In addition, the effective radioactive dose-equivalent (i.e., dosimetry) for human male subjects after intravenous administration of 50 μCi of [14C]BMS-204352 was estimated to be only 2.7 mrem based on the results of the tissue distribution study in rats. This exposure is approximately the same as that from one chest X-ray or about 10% of that from the annual exposure to natural background radiation and is about 1000-fold less than the maximum annual effective radiation dose allowed by the Code of Federal Regulations. Therefore, the administration of 50 μCi of [14C]BMS-204352 to healthy male subjects was considered safe.

Each human subject received a single dose of 10 mg of [14C]BMS-204352 containing 50 μCi of radioactivity as a 5-ml i.v. infusion lasting 5 min on day 1. The [14C]BMS-204352 stock solution contained 10 mg/ml [14C]BMS-204352 (5 μCi/mg), 400 mg/ml polyethylene glycol, 10% ethyl alcohol (v/v), and 150 mg/ml polysorbate 80. The solution was provided in ampoules and diluted 1:5 (v/v) with D5W for use. The dosing solution was loaded into a 10-ml syringe for administration to the subject via an indwelling i.v. cannula. All subjects were dosed by manual push over a period of 5 min through the i.v. cannula. At the completion of dosing, the indwelling i.v. cannula was flushed with 10 ml of D5W.

Study Design and Safety Monitoring in Humans. This was an open-label study with a minimum of 14 days in-house. The subjects were dosed with [14C]BMS-204352 on day 1, and serial blood samples for pharmacokinetics (unchanged drug) and radioactivity analysis were collected through day 7. All of the urine and feces were collected over the 14 days, or until discharge, and analyzed for radioactivity. Plasma, urine, and feces were analyzed for bio-transformation profiles. Clinical safety monitoring included pre- and post-study vital signs measurements (temperature, respiratory rate, seated blood pressure, and heart rate), physical examinations, and clinicopathological laboratory tests such as hematology, serum chemistry, and urinalysis.

Blood, Urine, and Fecal Sample Collection from Human Subjects. Serial pharmacokinetic blood samples were drawn prior to dosing and postdose at 5, 15, 30, and 45 min and 1, 1.5, 2, 4, 8, 12, 24, 36, and 48 h and every 24 h through day 7. One milliliter of blood was collected at each pharmacokinetic sampling time to measure the radioactivity level and the parent compound in plasma. After collection in a prechilled 7-ml EDTA lavender-top Vacutainer tube, the pharmacokinetic blood samples were mixed and centrifuged for 10 min at 1300g at about 5°C to yield plasma. The plasma samples were frozen within 1 h of collection (at −70°C). Collection of blood (7 ml) for biotransformation profiling of BMS-204352 was performed at 15 min and 1, 4, 24, and 96 h postdose.

Urine and feces samples were collected continuously over 24 h of each day until the subjects were discharged from the clinic site. The total volume of urine and weights of feces collected each day were recorded. Representative pooled urine samples for biotransformation profiling (1% of each urine collection, 0–336 h) were prepared by combining portions of urine excreted during each collection interval. Fecal homogenate was prepared by mixing 3 g water/l g feces, followed by homogenization. Representative pooled fecal homogenates for biotransformation profiling from humans (0.5% of each fecal collection, 0–336 h) were prepared by combining portions of well mixed fecal homogenate during each collection interval.

Sample Collections from Dogs and Rats. The same formulation of [14C]BMS-204352 (10 mg/ml, 5 μCi/mg) used in human subjects was used for studies with rats and dogs. For rat samples, two groups of male Sprague-Dawley rats, group 1 (nine rats) and group 2 (three rats), were dosed by intravenous infusion over 3 min with [13CD3]BMS-204352 (6 mg/kg). Plasma (1, 4, and 24 h) was obtained from rats in group 1 (three rats per collection time) by terminal bleeding into tubes containing EDTA and centrifugation (10 min, 1300g at 4°C). Liver samples were also collected from three rats at 4 h after terminal bleeding, and liver homogenate was prepared by mixing with 3 g water/l g tissue followed by thorough homogenization. Urine and feces, collected at 24-h interval for 96 h, were obtained from rats in group 2. Fecal homogenate was prepared by mixing 3 g water/l g feces, followed by homogenizing with an Omni Mixor Homogenizer. For dog samples, plasma (1, 4, and 24 h), urine, and feces (at 24-h interval for 96 h) were obtained from three male beagle dogs following intravenous infusion over 15 min (2 mg/kg). Representative pooled samples were prepared for urine, fecal homogenate, and plasma for analysis.

Quantitation of BMS-204352 in Human Plasma. BMS-204352 was quantified in human plasma using a sensitive and selective LC/MS/MS method. Briefly, to 0.5 ml of human plasma were added the internal standard, [13CD3]BMS-204352, and 5 mM ammonium acetate buffer solution before performing a liquid-liquid extraction using toluene as the solvent. The organic layer was separated, evaporated to dryness, reconstituted, and injected into the LC/MS/MS system equipped with a negative ion electrospray detector (Micromass Quattro LC; Waters) and with a PerkinElmer Series 200 LC autosampler (PerkinElmer Life and Analytical Sciences, Boston, MA). Selected reaction monitoring included m/z 358 to m/z 338 for BMS-204352 and m/z 362 to m/z 342 for the internal standard. Isocratic chromatographic separation was achieved using a YMC basic S 5 × 50 mm 5-μm column (YMC, Inc., Wilmington, NC). The mobile phase was composed of methanol and water (715:285, v/v) with 5 mM ammonium acetate and was delivered using a Shimadzu LC-10AD pump (Shimadzu Scientific Instruments, Inc., Columbia, MD) at a flow rate of 0.2 ml/min. The standard curve range was 0.05 to 25 ng/ml (lower limit of quantitation, 0.05 ng/ml) and was fitted to a 1/x quadratic regression model. Intra- and interassay precisions were within 4% relative standard deviation. The deviations from nominal concentration values were within 20% for the lower limit of quantitation and 15% for other quality controls. Stability of the processed samples, performed at three different nominal concentrations ranging from 0.15 to 20 ng/ml, indicated that the measured concentrations deviated by no greater than 8.3% when samples were placed at room temperature for 48 h. In addition, BMS-204352 was stable in human plasma at room temperature for 7 days, deviating no greater than 7% at two nominal concentrations. Appropriately spiked quality control samples were also assayed with the study samples. The values of precision (% CV) of the analytical quality control samples were ±6.6%. The mean percentage deviations from the nominal concentrations were ±10.1%. The analytical data demonstrated excellent reproducibility, specificity, sensitivity, and precision for measurement of BMS-204352.

Determination of Radioactivity in Plasma, Urine, and Feces. Blood (0.1 ml) was accurately taken into a 7-ml scintillation vial containing 0.5 ml of tissue solubilizer (Scintigest; Fisher Scientific Co., Pittsburgh, PA), mixed thoroughly, and allowed to stand for 24 to 48 h at room temperature. Solution (prepared by mixing 1% sodium pyruvate solution in methanol, glacial acetic acid, and methanol at the ratio of 4:3:1, v/v, 0.1 ml) was then added, followed by 5 ml of the scintillation fluid (ScintiVerse II; Fisher Scientific Co.). The vials were capped and solution was mixed before counting. Plasma samples (0.1 or 0.2 ml) and urine samples (1.0 ml) were accurately taken into 20-ml scintillation vials, and 15 ml of the scintillation fluid was added. The vials were capped and mixed thoroughly before counting.

All fecal samples for each day were pooled together from each subject and the total weight was determined. A sufficient but accurately measured volume of water (about 2–5 times by weight) was added to the specimen and the mixture was homogenized in a blender to give a free flowing slurry. The total volume was then recorded, an aliquot of about 50 g was saved, and the rest discarded. The fecal homogenate (0.2 ml) was solubilized and counted as that for blood samples.

The sample vials were counted in the liquid scintillation counter (Beckman model LS 9000 CE) for 30 min or when the counting error reached 1%.
whichever came first. Quench was monitored using the H factor for each sample. The correction for quenching was done using a previously determined quench correction curve (percent age efficiency versus H factor) using quenched standards. The samples were analyzed in duplicates and the average results were reported. If the deviation of any sample was greater than 10% from the mean value, the samples were reanalyzed in duplicates.

HPLC and LC/MS Procedures for Biotransformation Analyses. HPLC for biotransformation analyses was performed on a Shimadzu Class VP system equipped with two pumps (model LC-10AT), an autoinjector (SIL 10AD), and a diode array detector (SPD-M10A) (Shimadzu, Kyoto, Japan). A Zorbax RX C-18 column (4.6 mm x 250 mm, 5 µm) (Agilent Technologies, Palo Alto, CA) equipped with a guard column was used. All HPLC analyses were performed by enclosing the column in an Eppendorf CH-30 column heater (Eppendorf-5 Prime, Inc., Boulder, CO) maintained at 35°C. The mobile phase flow rate was 1 ml/min. The retention time of the parent compound was confirmed before each run by radioactivity or UV detection. HPLC effluent was collected at 0.25-min intervals after sample injection into 96-deep-well Lumaplates with a Gilson model 202 fraction collector (Gilson Medical Electronics, Middleton, WI). The effluent in the plates was dried with a Speed-Vac (Savant Instruments, Holbrook, NY), and the plates were counted for 10 min per well with a TopCount scintillation analyzer (PerkinElmer Life and Analytical Sciences).

Solvent System I. A gradient of two solvent systems, A, and B, was used for HPLC profiling of plasma, urine, and feces samples. Solvent A was water containing 15 mM ammonium acetate, pH 4.3. Solvent B consisted of methanol/solvent A (95:5, v/v). Solvent B was started at 0 and then increased in a linear manner as follows: 45% (15 min), 56% (30 min), 64% (50 min), 80% (70 min), and 90% (75 min).

Solvent System II. A gradient of two solvent systems, A, and B, was used for HPLC profiling of HCl hydrolyses of plasma proteins. Solvent A was water containing 15 mM ammonium acetate, pH 4.3. Solvent B consisted of acetonitrile/solvent A (95:5, v/v). Solvent B was started at 25% and then increased as follows (linear between steps): 50% (35 min), 80% (40 min), and then maintained at 80% for 2 min before it was decreased to 25% in 3 min.

LC/MS analysis was performed on a Finnigan LCQ mass spectrometer with an ESI probe (Thermo Finnigan, San Jose, CA). Samples were analyzed in the negative ion mode. Half the HPLC eluent was directed to the Finnigan LCQ mass spectrometer through a divert valve set to divert the flow from 0 to 5 min. From 5 min until the end of the HPLC run, the eluent flow was directed to the mass spectrometer. The capillary temperature used for analysis was set at 220°C. The nitrogen gas flow rate, spray current, and voltages were adjusted to give maximum sensitivity. The other half of the HPLC eluent was directed to a fraction collector to 96-well plates.

Sample Preparation for Biotransformation Analysis. Plasma. Plasma was pooled by combining an equal volume from each subject and extracted by addition of 2 ml of acetonitrile to 1 ml of plasma, vortexed for 1 min, and centrifuged at 110°C for 30 min. The supernatant was removed and saved. The extraction was repeated three times and all supernatants were combined. Aliquots of 0.5 ml of this solution were mixed with Ecolite cocktail and counted for radioactivity. The solution was then evaporated to dryness under a stream of nitrogen. The residue was resuspended in a 1/10 volume of solvent system II (30% A and 70% B) and centrifuged at 3000g for 10 min before injecting onto the HPLC column.

Urine. A portion of 15 ml of the human urine (0–336 h) was passed through a 10-ml C18 cartridge. The radioactive material was eluted with methanol and concentrated under a stream of nitrogen. The residue was redissolved in 150 µl of solvent system II (30% A and 70% B) and centrifuged at 3000g for 10 min before injecting onto the HPLC column.

Acid Hydrolysis of Plasma Proteins. A 2-ml portion of each pooled plasma (1, 4, and 24 h) of humans, dogs, and rats was extracted with 6 ml of acetonitrile three times, and the protein pellet was washed twice with 6 ml of acetonitrile. The protein residues from three time points (1, 4, and 24 h) were combined in a 15-ml Pyrex tube (9826) and dried under a nitrogen stream. Hydrochloric acid (6 N, 8 ml) was added to the protein pellet. After vortexing and complete dissolution, the solution was refluxed in a closed vessel for 48 h at 110°C. The hydrolysate was neutralized with 6 N NaOH solution containing 1 M sodium phosphate and passed through a 10-ml C18 cartridge. The radioactive material was eluted with methanol and concentrated under a stream of nitrogen. BMS-349821 (1 mg, the des-fluoro lysine adduct) was incubated with 6 N HCl separately under identical conditions. The residue was dried under a N2 stream and redissolved in 250 µl of solvent system II (30% A and 70% B), and centrifuged at 3000g for 10 min before injecting onto the HPLC column.

Extensive Extraction of Human Fecal Homogenate. Portions of pooled human fecal homogenate (0–336 h) were acidified with formic acid to 0.25% (v/v) and extracted with several solvents including acetonitrile, methanol, chloroform, 1-butanol, ethyl acetate, and r-butyl methyl ether. Soxhlet extraction in acetone was also evaluated. General procedures included mixing fecal homogenate with organic solvent by vortexing, sonication, and centrifugation. The supernatant was removed and saved. The extraction was repeated once. Radioactivity was determined in the combined supernatant. Solvent was evaporated under a stream of nitrogen. The residue was resuspended in a 1/10 volume (of original fecal homogenate) in 30% water and 70% methanol. After centrifugation for 10 min at 3000g, radioactivity was determined in the samples.

For comparison, [14C]BMS-204352 was incubated with fresh fecal homogenate (n = 2). A pooled fresh feces homogenate (prepared by homogenizing a mixture of 300 g of feces and 900 ml of water) was spiked with [14C]BMS-204352 (5 µCi/mg, 14,000 dpm/ml, equivalent to the radioactivity level in the 0- to 336-h pooled human fecal homogenate). The mixture was incubated for 24 h at 37°C and then extracted three times with 3 volumes of acetone. The radioactivity in the combined supernatant was determined. After evaporation to dryness, the extracted material was dissolved in solvent system I (30% A and 70% B), and analyzed by HPLC.

Pharmacokinetic Analysis. The plasma concentration versus time data for radioactivity and unchanged BMS-204352 were analyzed by a noncompartmental method (Gibaldi and Perrier, 1982). The peak plasma concentration, Cmax, and the time to reach peak concentration, Tmax, were recorded directly from experimental observations. The AUC was calculated by a combination of the trapezoidal and log-trapezoidal methods. The AUC was calculated from time 0 to the time, T, of last measurable concentration (AUC0, T). The first order rate constant of decline of radioactivity concentrations and unchanged BMS-204352, expressed as equivalents of BMS-204352, in the terminal phase of each plasma concentration versus time profile, K, was estimated by log-linear regression (using no weighting factor) of at least three data points, which yielded a minimum mean square error. The absolute value of K was used to estimate the apparent terminal elimination half-life, t1/2. The last measurable concentration and the rate constant, K, were used to extrapolate the AUC0,T up to infinity to estimate AUC∞.

The total body clearance (CLT) and steady-state volume of distribution (Vss) of the radioactivity and unchanged BMS-204352 were calculated as follows: CLT = Dose/AUC and Vss = mean residence time x CLT.

The volume of urine and weight of feces collected over each interval and the concentrations of radioactivity in the corresponding excreta samples were used to calculate the cumulative percentage of the administered dose recovered in the urine and feces for the estimation of urinary (%Urine) and fecal (%Feces) excretion of radioactivity (expressed in ng/ml or ng/g of BMS-204352 equivalents).

Microsomal Incubation. Human liver microsomal preparations (pooled from 12 subjects) were purchased from BD Gentest (Woburn, MA). [14C]BMS-204352 (25 µM) was incubated with microsomal proteins (1 mg/ml), NADPH (1.25 mM) in 0.5 ml of 60 mM phosphate buffer, pH 7.4, in triplicates. The incubations were done in 15-ml glass test tubes at 37°C in a shaking water bath. Each assay was started by addition of NADPH followed by incubation for 45 min. A control incubation without NADPH was done with each set of experiments. Four volumes of acetonitrile/methanol (3:1, v/v) were
added to stop the reactions and to precipitate proteins by centrifugation for 1 h at 3000g and 4°C.

Protein Washing for Determination of Covalent Binding of Radioactivity. The protein pellet from microsomal incubations, plasma protein precipitation, or protein precipitation from the liver homogenate was washed in four steps. The first step used 5 volumes of methanol (or acetone), sonication for 10 min, and centrifugation for 30 min. The second step used 5 volumes of acetonitrile/methanol (3:1, v/v). The mixture was frozen at −20°C for 1 h before sonication for 10 min and centrifuged for 1 h. In the third step, a half-volume of 6 M urea solution for microsomal proteins and 1 volume of 10 M urea solution for protein precipitated from plasma or liver homogenate was added to dissolve the protein pellet before mixing with 10 volumes of acetonitrile/methanol (3:1, v/v), followed by centrifugation at 3000g for 1.5 h. Aliquots of the supernatant were checked for radioactivity, and no more washing was done if the radioactivity level was within 2-fold of that from the control samples. Otherwise, in the forth step, 5 volumes of acetonitrile/ethanol (3:1, v/v) was used as in the second step. The protein pellet was dissolved in 6 or 10 M urea. Aliquots were counted for radioactivity by scintillation counting. Aliquots of the protein solution were diluted with water to 4 M urea before determination of protein concentration by the protein kit from Bio-Rad (Hercules, CA). This extensive protein washing procedure gave approximately 80% of the protein recovery from microsomal incubations, plasma, and liver homogenate.

Results

Safety in Humans. A single dose of 10 mg (50-μCi radioactivity) of [14C]BMS-204352 was well tolerated in humans. There were no serious adverse events. The most frequent adverse event, headache, was reported by five subjects. The only other adverse events reported were back pain (n = 4), lightheadedness (n = 2), and taste disturbance (n = 2). One subject discontinued early for a moderate taste disturbance after 2 min of a scheduled 5-min i.v. infusion, which caused the subject to have a moderate panic reaction. The physical examinations, vital signs measurements, electrocardiograms, and clinical laboratory tests showed no significant changes.

Pharmacokinetic Results. The pharmacokinetic parameters for the unchanged drug and radioactivity are presented in Table 1. The human plasma concentration-time profiles for BMS-204352 and radioactivity are presented in Fig. 1. Following intravenous administration, the radioactivity concentration in human plasma increased initially with time for up to 24 h and then decreased slowly. The plasma Cmax of radioactivity (BMS-204352 equivalents) was twice that of the unchanged BMS-204352. The Tmax of radioactivity was much delayed compared with that of unchanged BMS-204352. The mean terminal elimination t1/2 of plasma radioactivity was prolonged compared with that of the unchanged BMS-204352. Comparison of the plasma AUC values for the unchanged BMS-204352 and for radioactivity indicated that less than 0.4% of the plasma radioactivity was accounted for by the unchanged drug. Similarly, dog and rat plasma radioactivity Tmax values were delayed, and the mean terminal elimination t1/2 values were prolonged following i.v. administration of [14C]BMS-204352 (Table 1). The AUC values of the unchanged BMS-204352 represented less than 3% of the plasma radioactivity in dogs and rats.

The plasma clearance value (CLT) of the unchanged drug after i.v. administration to humans was approximately comparable to the liver blood flows (18.5 l/h for a 10-kg dog and 0.83 l/h for a 0.25-kg rat; Davies and Morris, 1993). This is in contrast to the liver blood flow of 87 l/h for a 70-kg man (Davies and Morris, 1993), which was more than 275-fold the clearance for plasma radioactivity.

Cumulative urinary and fecal excretion of radioactivity from humans is presented in Fig. 2. Radioactivity was primarily excreted in feces (60% of administered dose). Radioactivity in urine accounted for approximately 37% of the administered dose. The overall mean

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**TABLE 1**

Mean pharmacokinetic parameters of BMS-204352 in humans, dogs, and rats following intravenous administration with [14C]BMS-204352

<table>
<thead>
<tr>
<th>Species (n)</th>
<th>Dose</th>
<th>Analyte</th>
<th>Cmax (S.D.)</th>
<th>Tmax (Range)</th>
<th>AUC (S.D.)</th>
<th>t1/2 (S.D.)</th>
<th>CLT (S.D.)</th>
<th>Vss (S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (8)</td>
<td>10 mg</td>
<td>BMS-204352</td>
<td>121 (57)</td>
<td>0.1 (0.1)</td>
<td>184 (46)</td>
<td>37 (14)</td>
<td>64 (29)</td>
<td>714 (527)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Radioactivity</td>
<td>208 (62)</td>
<td>(16, 24)</td>
<td>(18,182)</td>
<td>(115)</td>
<td>(0.11)</td>
<td>(5)</td>
</tr>
<tr>
<td>Dog (3)</td>
<td>2 mg/kg</td>
<td>BMS-204352</td>
<td>2155 (411)</td>
<td>0.1 (0.1)</td>
<td>9811 (881)</td>
<td>16 (0.7)</td>
<td>21 (2.0)</td>
<td>41 (5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Radioactivity</td>
<td>7026 (933)</td>
<td>(24, 32)</td>
<td>444,812 (54,764)</td>
<td>158 (15)</td>
<td>0.46 (0.01)</td>
<td>(5)</td>
</tr>
<tr>
<td>Rat (3)</td>
<td>6 mg/kg</td>
<td>BMS-204352</td>
<td>1430 (498)</td>
<td>0.1 (0.1)</td>
<td>1066 (206)</td>
<td>2.3 (0.3)</td>
<td>0.65 (0.12)</td>
<td>1.6 (0.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Radioactivity</td>
<td>2621 (57)</td>
<td>3.0</td>
<td>48,804 (44447)</td>
<td>22 (5.5)</td>
<td>0.014 (0.003)</td>
<td>(3)</td>
</tr>
</tbody>
</table>

a Units for Cmax: BMS-204352, ng/ml and radioactivity, ng-Eq/ml. Units for AUC: BMS-204352, ng/ml and radioactivity, ng-Eq/ml.

b Portions of pharmacokinetics data in dogs and rats have been presented previously (Krishna et al., 2002d).
recovery of radioactivity in the urine and feces, collected over 14 days postdose, was approximately 97% of the administered dose. Of the total amount recovered, >90% was recovered in 8 days and almost 80% of administered dose was recovered in the urine and feces in a relatively short period of time (3 days). During a 4-day collection from rats and dogs, approximately 5% of the dose was excreted in urine and 70 to 78% excreted in feces following i.v. administration of [14C]BMS-204352 (data not shown).

Protein Covalent Binding and Biotransformation Profile in Plasma. Table 2 shows extraction of radioactivity in plasma of humans, dogs, and rats. Figure 3 shows the radioactivity profile in plasma extracts after protein precipitation. In human plasma, the acetonitrile-extractable radioactivity decreased from 85% at 0.5 h and 55.3% at 1 h to approximately 20% after 4 h. The parent compound was the major component in plasma extracts at all time points and was the only radioactive peak observed in 24-h plasma extract. One major metabolite peak was observed in 1- and 4-h human plasma. This metabolite decreased to undetectable levels by 24 h. This metabolite had a molecular ion at m/z 534 and a major fragment ion at m/z 358 (loss of 176, a glucuronic acid), consistent with the N-glucuronide of BMS-204352, which had the same retention time and mass spectral properties as the major metabolite in human urine and dog bile (Zhang et al., 2004). Similarly, the parent compound was also the major component and N-glucuronide of BMS-204352 was a minor component in dog and rat plasma extract samples (Fig. 3).

The percentage of the unextractable radioactivity in plasma increased with time up to 81% at 120 h in humans (Table 2). Based on a plasma volume of 3000 ml for humans (Davies and Morris, 1993), the percentage of the radioactive dose covalently bound to plasma proteins was calculated to be about 0.5 to 5% in humans. Similarly, the unextractable radioactivity in plasma proteins represented up to 7.2% of the dose at 24 h in dogs and 5.8% of the dose at 4 h in rats. Assuming albumin comprised 80% rat plasma proteins, approximately 1.5% of plasma albumin molecules were labeled by 14C at 4 h in rats after dosing. Current and previous studies (Zhang et al., 2003) suggest that the unextractable plasma radioactivity increases with time to a maximum at 24 h for dogs and at 4 h for rats (the latest time point examined in dogs).

Figure 4 shows the comparative radioactivity profile of acid hydrolysate of plasma proteins of humans, dogs, and rats following i.v. administration of [14C]BMS-204352. Upon acid hydrolysis, most of the radioactivity in combined 1-, 4-, and 24-h plasma proteins was recovered in a single radioactive peak (peak 2) at 21.5 min. The profile of acid hydrolysate of des-fluoro BMS-204352 lysine adduct (BMS-349821) is also shown for comparison. LC/MS analysis of the peak at 21.5 min showed a molecular ion at m/z 470 and a prominent MS2 fragment ion at m/z 324, suggesting a structure of des-fluoro-des-methyl BMS-204352 lysine adduct. This adduct has the same structure as the one isolated from acid hydrolysis of rat plasma
TABLE 2

<table>
<thead>
<tr>
<th>Species</th>
<th>Time (h)</th>
<th>Radioactivity</th>
<th>CH₃CN-Extracted</th>
<th>Covalently Bound</th>
<th>Percentage Covalent Binding</th>
<th>Percentage Dose</th>
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<tbody>
<tr>
<td>Human</td>
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<td>1038</td>
<td>180</td>
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<td></td>
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<td>1315</td>
<td>646</td>
<td>588</td>
<td>44.7</td>
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<tr>
<td></td>
<td>4</td>
<td>2085</td>
<td>474</td>
<td>1628</td>
<td>78.1</td>
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<tr>
<td></td>
<td>24</td>
<td>2550</td>
<td>393</td>
<td>2056</td>
<td>80.8</td>
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<tr>
<td></td>
<td>120</td>
<td>1620</td>
<td>126</td>
<td>1324</td>
<td>81.7</td>
<td>3.5</td>
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<td>1.0</td>
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<td></td>
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<td>6374</td>
<td>53.8</td>
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<td>3040</td>
<td>15,420</td>
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<td>75,860</td>
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<td>4</td>
<td>143,160</td>
<td>18,240</td>
<td>124,920</td>
<td>87.3</td>
<td>5.8</td>
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<tr>
<td></td>
<td>24</td>
<td>62,000</td>
<td>7600</td>
<td>54,400</td>
<td>87.7</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* Radioactivity covalently bound to human plasma protein was directly determined by redissolving the protein precipitate followed by liquid scintillation counting. Radioactivity covalently bound to rats and dog plasma proteins was calculated as the difference between total and extractable radioactivity.

* Plasma volumes of 3000, 515, and 7.8 ml were assumed for humans, dogs, and rats, respectively (Davies and Morris, 1993).

**Fig. 3.** Biotransformation profiles of plasma extracts following intravenous administration of [14C]BMS-204352 to humans at 1, 4, and 24 h, to dogs at 4 h, and to rats at 4 h. M represents N-glucuronide of BMS-204352. P represents the parent compound. The percentage extraction of radioactivity is shown in parentheses. The samples were analyzed by HPLC under solvent system I conditions as described under Materials and Methods.
proteins following i.v. administration of [14C]BMS-204352 (Zhang et al., 2003).

**Biotransformation Profiles in Urine and Feces.** Figure 5 shows the radioactivity profiles in urine and in acetone extracts of the pooled fecal homogenate of humans. The parent peak (P) accounted for 5% of the radioactivity in human urine. The prominent radioactive metabolite M at 38 min accounted for 45% of the radioactivity in human urine, or 16.5% of the radioactive dose. Minor metabolites each accounted for 2 to 5% of the radioactivity. The major metabolite was the N-glucuronide of BMS-204352, as identified previously (Zhang et al., 2004). In human fecal homogenate, about 47% of the radioactivity was extractable into acetone. The parent compound (P), the most prominent peak, accounted for 17% of the radioactivity in the sample, which could result from hydrolysis of the biliary N-glucuronide of BMS-204352. The remaining radioactivity in the extract of human fecal homogenate was distributed among many minor metabolites. No metabolite peak accounted for more than 5% of the radioactivity in the feces. Human fecal homogenate was exhaustively extracted with several solvent systems. In every case, less than 50% of the radioactivity was extracted. Therefore, the nature of unextractable radioactivity in feces was not determined. To study metabolizing effects of intestinal microorganisms on [14C]BMS-204352, the parent compound was incubated with a fresh fecal homogenate. Radioactivity was recovered quantitatively as parent compound after extraction and HPLC analysis. Since no effort was made to ensure anaerobicity of the fecal incubation, the results suggest that BMS-204352 might be stable in the incubations with fresh feces.

Radioactivity was primarily excreted in feces (70–80% of the administered dose) in dogs and rats. Radioactivity in urine accounted for less than 5% of the administered dose in both species, suggesting...
that nonrenal elimination plays a predominant role in the disposition of BMS-204352 in dogs and rats. The major radioactivity component in feces was the parent compound in dogs and rats following i.v. administration of [14C]BMS-204352. However, the glucuronide of the parent compound was the major radioactive component (33% of dose for dogs and 11% of dose for rats) in bile of bile duct-cannulated dogs and rats (Zhang et al., 2004). These results suggested that BMS-204352 was eliminated mainly through glucuronidation of the parent compound from livers, and the glucuronide was hydrolyzed in intestines in vivo. 

Figure 6 summarizes the proposed pathways for biotransformation of [14C]BMS-204352 in humans.

**Evaluation of in Vitro and in Vivo Protein Covalent Binding of [14C]BMS-204352.** Table 3 shows protein covalent binding of [14C]BMS-204352 in liver microsomal incubations and plasma of humans, dogs, and rats following i.v. administration as well as in rat liver homogenate. Among the three species tested, [14C]BMS-204352 showed the highest level (1215 pmol/mg · h) of protein covalent binding in human liver microsomes and the lowest level (16.5 pmol/ mg) in human plasma. There was a more than 70-fold difference between microsomal incubations and plasma of humans. For the dog, the covalent binding in liver microsomal incubations (302 pmol/mg · h) was more than 5-fold higher than in plasma (55.3 pmol/mg). For rat, in vivo covalent binding (447.3 pmol/mg in plasma and 633.4 pmol/mg in liver homogenate) was similar to the value of in vitro microsomal incubations (703.6 pmol/mg · h).

**Discussion**

Metabolic and pharmacokinetic profiles of [14C]BMS-204352 in humans, dogs, and rats were similar. Plasma concentration versus time profiles of unchanged drug and radioactivity were markedly different. Following i.v. administration of [14C]BMS-204352 to humans, dogs, and rats, the radioactivity level in plasma increased initially with T_{max} values of 24 h for humans, 32 h for dogs, and 3 h for rats, and C_{max} values were twice as high as those of the unchanged BMS-204352. This unusual observation suggested that the drug bound to tissue(s) upon dosing, followed by a relatively slower release of radioactivity into the plasma compartment. Volume of distribution for all three species was approximately 7 to 17 times larger than the respective total body water volumes, suggesting extensive extravascular distribution of the drug and, presumably, binding to tissue proteins. In rats, a tissue to plasma ratio of 3.2 for the liver was higher than that of all 25 other organs tested at an apparent T_{max} of 0.25 h after i.v. administration (unpublished data). The results suggested that the radioactivity preferably partitioned to the rat liver. Similar AUC values of unchanged BMS-204352 in plasma and in rat urine were observed following i.v. and oral administration, although an extensive hepatic metabolism in rats was observed. This further suggested that the compound may initially partition into the rat liver, even after i.v. administration. Pharmacokinetic profiles of [14C]BMS-204352 in humans were similar to the reported pharmacokinetic profiles of [14C]BMS-204352 in dogs and rats (Krishna et al., 2002d). It is likely that the compound may also partition into livers of humans and dogs upon i.v. dosing. The radioactivity binding to rat liver appeared to be rapid (within 15 min) given that 1) the concentrations of radioactivity and the unchanged BMS-204352 were similar in the earliest plasma sample taken at 15 min, 2) unextractable radioactivity was less than 30% at the 15-min plasma sampling time of all three species, and 3) the radioactivity as well as the percentage of unextractable radioactivity increased at later time points (Zhang et al., 2003).

Following i.v. administration of [14C]BMS-204352 to humans, dogs, and rats, the plasma clearance value of the unchanged drug was generally comparable to that of the respective liver blood flows; they were >275-, 45-, and 45-fold higher than the clearance of plasma radioactivity in humans, dogs, and rats, respectively. These results suggested that BMS-204352 was a high extraction drug, which was converted to a protein covalently bound metabolite that is eliminated slowly from plasma. The plasma AUC of unchanged BMS-204352 represented less than 3% of total of radioactivity for humans, dogs, and rats, suggesting an extensive metabolism of BMS-204352 after i.v. dosing. Up to 80% of the radioactivity in plasma of humans, dogs, and rats was covalently bound to plasma proteins. The unextractable radioactivity was covalently bound to plasma proteins through a lysine adduct as characterized by acid hydrolysis, HPLC separation, and LC/MS analysis. Recently, the covalently bound adduct of BMS-204352 in rat plasma proteins was shown to result from O-demethylation and replacement of the ring-fluorine atom of [14C]BMS-204352 by the α-amino group of a lysine residue in proteins (Zhang et al., 2003). In addition, metabolic activation of BMS-204352 appears to proceed through an ortho-quinone methide, which is formed by cytochrome P450-mediated O-demethylation and spontaneous loss of hydrogen fluoride. In vitro incubations in human microsomes with selective inhibitors indicated that the protein covalent binding was prevented by CYP3A4 inhibitors, suggesting that the bioactivation was mediated by CYP3A4 (Zhang et al., 2003).

[14C]BMS-204352 seemed to partition into the liver after i.v. dosing and was bioactivated by cytochrome P450 to form a relatively stable reactive species. The reactive species then penetrated into tissues and covalently bound to plasma proteins. Most of the unextractable radioactivity covalently bound to albumin in rats (Zhang et al., 2003). Presumably, the unextractable radioactivity in human and dog plasma was also mostly covalently bound to albumin, as observed in rats. In this context, the elimination of covalently bound radioactivity would depend mostly on the me-
The metabolism of tethered albumin molecules. Actually, the half-lives of radioactivity (11 days, 7 days, and 1 day for humans, dogs, and rats, respectively) are somewhat similar to the half-lives of albumin in humans, dogs, and rats (19, 6.8, and 2.6 days for humans, dogs, and rats, respectively) (Schreiber et al., 1970; Morris and Preddy, 1986; Reed et al., 1988). The percentage of covalent binding of radioactivity in plasma proteins reached the plateau at approximately T\text{max} for plasma radioactivity. Since the delayed T\text{max} for radioactivity (compared with the unchanged BMS-204352) was probably caused by protein covalent binding, the T\text{max} values of plasma radioactivity may correlate to the efficiency of bioactivation/covalent binding and subsequent release of bound protein molecules from the liver.

There does not appear to be a direct correlation between T\text{max} of human plasma radioactivity and microsomal bioactivation/protein covalent binding. However, compared with rats, the lower bioactivation activity in dog liver microsomes did predict a delayed T\text{max} for the plasma radioactivity in the dog.

In general, there are reversible and irreversible types of protein covalent binding. Covalent binding through disulfide bonds of cysteine residues of proteins with sulfhydryl-containing drugs, such as captopril and matrix metalloproteinase inhibitor BMS-275291, are examples of reversible-type covalent binding (Zhang et al., 2000). The drug material(s) can be released from the covalently bound adduct(s) through replacement by free thiols in

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**TABLE 3**

**In vitro and in vivo protein covalent binding of [14C]BMS-204352**

<table>
<thead>
<tr>
<th>Species</th>
<th>In Liver Microsomal Incubations</th>
<th>In Plasma following i.v. Doses</th>
<th>In Liver Homogenate following i.v. Doses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/mg protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>1215.8 ± 271.8</td>
<td>16.5 ± 7.2 (at 24h)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Dog</td>
<td>302.0 ± 25.2</td>
<td>55.3 ± 10.5 (at 24h)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Rat</td>
<td>703.6 ± 110.3</td>
<td>447.3 ± 33.5 (at 4h)</td>
<td>633.4 ± 45.2 (at 4h)</td>
</tr>
</tbody>
</table>

N.D., not determined.

* Incubations were for 30 min at 37°C in 1 mg/ml microsomal proteins at 25 μM [14C]BMS-204352. Protein covalent binding was normalized to pmol/mg · h.
* Dogs and rats were i.v. dosed with 6 and 2 mg/kg, respectively, and human subjects were i.v. dosed with 10 mg of [14C]BMS-204352 (5 μCi/mg).
glutathione or cysteine. Covalent binding through nucleophilic addition reactions of amino acids, such as cysteine or lysine residues, represents irreversible-type covalent binding. Protein covalent binding may lead to such consequences as the production of autoantibodies, inflammation, and cancer. Protein covalent binding has been observed with drugs that are either generally considered safe (e.g., captopril, aspirin, valproic acid), or drugs that have been associated with hepatic damage (e.g., acacetaminophen overdose) and drugs associated with idiosyncratic or allergic reactions (e.g., diflunisal and zomepirac) (Uetrecht, 1992; Pulmford and Halmes, 1997). The BMS-204352 lysine adduct identified in our studies represents the first example of a lysine residue forming a covalent bond with a methide-reactive intermediate via a nucleophilic addition reaction (Zhang et al., 2003).

Ortho-Quinone from catechols (Dehal and Kupfer, 1999) and iminoquinone (Lai et al., 2000) are adverse events were observed in a large population of patients. Drug-induced hepatotoxicity and idiosyncratic reactions are complex issues that can pose challenges in drug development (Uetrecht, 2003; Velayudham and Farrell, 2003; Evans et al., 2004). They are a major impediment, especially for developing drugs that are either generally considered safe (e.g., captopril, aspirin, valproic acid), or drugs that have been associated with hepatic damage (e.g., acacetaminophen overdose) and drugs associated with idiosyncratic or allergic reactions (e.g., diflunisal and zomepirac) (Uetrecht, 1992; Pulmford and Halmes, 1997). The BMS-204352 lysine adduct identified in our studies represents the first example of a lysine residue forming a covalent bond with a methide-reactive intermediate via a nucleophilic addition reaction (Zhang et al., 2003). Ortho-Quinone from catechols (Dehal and Kupfer, 1999) and iminoquinone (Lai et al., 2000) are adverse events were observed in a large population of patients. Drug-induced hepatotoxicity and idiosyncratic reactions are complex issues that can pose challenges in drug development (Uetrecht, 2003; Velayudham and Farrell, 2003; Evans et al., 2004). They are a major impediment, especially for developing drugs that are either generally considered safe (e.g., captopril, aspirin, valproic acid), or drugs that have been associated with hepatic damage (e.g., acacetaminophen overdose) and drugs associated with idiosyncratic or allergic reactions (e.g., diflunisal and zomepirac) (Uetrecht, 1992; Pulmford and Halmes, 1997). The BMS-204352 lysine adduct identified in our studies represents the first example of a lysine residue forming a covalent bond with a methide-reactive intermediate via a nucleophilic addition reaction (Zhang et al., 2003).

References


Given the intended low clinical dose of <10 mg administration over the course of a day, and the nature of the indication of the drug, which was developed for stroke, protein covalent binding does not appear to adversely impact the development of BMS-204352.

Acknowledgments. We thank I. E. Fulmor for assistance with clinical trial monitoring, Mumul Hemrajani and Marc Fancher for collection of animal samples and fecal extraction, Drs. Burde Kamath and Robert J. Noveck at Clinical Research Center (New Orleans, LA) in the conduct of the clinical study, and Dr. Zheng Yang for helpful discussions.

Drug-induced hepatotoxicity and idiosyncratic reactions are complex issues that can pose challenges in drug development (Uetrecht, 2003; Velayudham and Farrell, 2003; Evans et al., 2004). They are a major impediment, especially for developing high dose and chronic administration drugs. Some cases of hepatotoxicity and idiosyncratic reactions might have been caused by bioactivation/formation of reactive intermediates and protein covalent bindings. However, recent literature suggests that protein covalent binding alone may not be sufficient to cause hepatotoxicity and idiosyncratic reactions (Uetrecht, 2003). Relatively high levels of protein covalent binding of MaxiPost in vitro did not translate into meaningful toxicological consequences in animals and humans. Covalent binding to large pools of albumin may, in fact, have provided a buffering effect to attenuate the bioactivation of BMS-204352. In this context, excellent preclinical toxicity profiles have been observed with BMS-204352, and no serious adverse events were observed in a large population of patients.

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