Metabolism and Disposition of Dasatinib after Oral Administration to Humans

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Abstract

SPRYCEL® (Dasatinib, BMS-354825), a multiple kinase inhibitor, is currently approved to treat chronic myelogenous leukemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL) tumors in patients that are resistant or intolerant to imatinib mesylate (Gleevec®). After a 100 mg single oral dose of [14C]dasatinib to healthy volunteers, the radioactivity was rapidly absorbed (Tmax ~0.5 h). Both dasatinib and total radioactivity (TRA) plasma concentrations declined rapidly with elimination half-life values of <4 h. Dasatinib was the major drug-related component in human plasma. At 2 h, dasatinib accounted for 25% of the TRA in plasma suggesting that metabolites contributed significantly to the total drug-related component. There were many circulating metabolites detected that included hydroxylated metabolites (M20 and M24), an N-dealkylated metabolite (M4), an N-oxide (M5), an acid metabolite (M6), glucuronide conjugates (M8a,b) and products of further metabolism of these primary metabolites. Most of the administered radioactivity was eliminated in the feces (85%). Urine recovery accounted for <4% of the dose. Dasatinib accounted for <1% and 19% of the dose in urine and feces, respectively, suggesting that dasatinib was well absorbed after oral administration and extensively metabolized prior to being eliminated from the body. The exposures of pharmacologically active metabolites M4, M5, M6, M20 and M24 in patients, along with their cell-based IC50 for Src and Bcr-Abl kinase inhibition, suggested that these metabolites were not expected to contribute significantly towards in vivo activity.
Introduction

SPRYCEL® (dasatinib, BMS-354825, \(\left[N-(2\text{-chloro-6-methylphenyl})-2-(6-(4-(2-hydroxyethyl)-piperazin-1-yl)-2\text{-methylpyrimidin-4-ylamino})\text{thiazole-5-carboxamide}\right]\)) is a multiple kinase inhibitor that potently inhibits Bcr-Abl, Src family (SRC, LCK, YES, FYN), c-KIT, EPHA2 and PDGFRβ kinases (Lombardo et al., 2004; Shah et al., 2004; Das et al., 2006). It is currently approved in US and EU to treat chronic myelogenous leukemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL) tumors in patients that are resistant or intolerant to imatinib mesylate (Gleevec®). Unlike imatinib mesylate, which binds to the closed confirmation of Bcr-Abl kinase, dasatinib was designed to bind to both the open and closed form of the enzyme (Shah et al., 2004; Tokarski et al., 2006). Because of this binding property and the ability to inhibit multiple kinases, including Src, dasatinib is effective in tumors that are resistant to imatinib mesylate (O'Hare et al., 2005; Schittenhelm et al., 2006). Clinical studies have demonstrated that dasatinib shows clinical response in patients with CML or Ph+ ALL that are resistant or intolerant to imatinib mesylate treatment (Cortes et al., 2006; Hochhaus et al., 2006; Talpaz et al., 2006; Quintas-Cardama et al., 2007).

Numerous in vitro and in vivo studies have been conducted with dasatinib in nonclinical species to understand its ADME properties and gauge the suitability of these species as toxicological models (Christopher et al., 2008; Kamath et al., 2008). The metabolic profiles from in vitro studies in liver microsomes, and hepatocytes showed good correlation with the in vivo profiles generated after a single oral dose of \(\left[^{14}\text{C}\right]\text{dasatinib}\) to rats and monkeys. The primary metabolites of dasatinib identified were products of hydroxylation on the phenyl ring (M20 and M24), \(N\)-oxidation on the
piperazine ring (M5), N-dealkylation of the hydroxyethyl moiety (M4), and oxidation of the hydroxyethyl moiety to a carboxylic acid (M6) (Christopher et al., 2008). In rat and monkey, only a small fraction of the radioactivity in bile and urine was attributed to the parent compound, suggesting that metabolism plays a major role in the elimination of dasatinib in these species (Christopher et al., 2008). In vitro studies with human liver microsomes and expressed enzyme systems showed that multiple enzymes were responsible for the oxidative metabolism of dasatinib with CYP3A4 as the primary enzyme involved.¹

The value and utility of doing ADME studies with radiolabeled compound is well understood. In drug development, these studies are performed with either C-14 or tritium labeled material to provide detailed quantitative information on circulating metabolites, the extent of metabolism and routes of excretion for drug and its metabolites (Marathe et al., 2004). This study describes the metabolism and disposition of [14C]dasatinib after a single oral dose to healthy human subjects. Furthermore, based on the metabolic profile in plasma from the human ADME study, and in vitro kinase activities of the metabolites found in the human study, the primary oxidative metabolites M4, M5, M6, M20 and M24 were monitored in plasma samples from a subsequent clinical study after administration of a single 180 mg oral dose of dasatinib. This paper describes the metabolism of dasatinib in humans along with the pharmacokinetics of its primary oxidative metabolites.
Materials and Methods

Chemicals: Reference standards for HPLC and mass spectrometry, namely, \(N\)-(2-chloro-6-methylphenyl)-2-(6-(4-(2-hydroxyethyl)piperazin-1-yl)-2-methylpyrimidin-4-ylamino)thiazole-5-carboxamide (dasatinib, BMS-354825), \(N\)-(2-chloro-6-methylphenyl)-2-(2-methyl-6-(piperazin-1-yl)pyrimidin-4-ylamino)thiazole-5-carboxamide (N-dealkylated amine of dasatinib, M4), 4-(6-(5-(2-chloro-6-methylphenylcarbamoyl)thiazol-2-ylamino)-2-methylpyrimidin-4-yl)-1-(2-hydroxyethyl)piperazine 1-oxide (piperazine N-oxide of dasatinib, M5) and 2-(4-(6-(5-(2-chloro-6-methylphenylcarbamoyl)thiazol-2-ylamino)-2-methylpyrimidin-4-yl)piperazin-1-yl)acetic acid (carboxylic acid metabolite of dasatinib, M6) were synthesized at Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ). Reference standards \(N\)-(2-chloro-4-hydroxy-6-methylphenyl)-2-(6-(4-(2-hydroxyethyl)piperazin-1-yl)-2-methylpyrimidin-4-ylamino)thiazole-5-carboxamide (4-phenyl-hydroxy metabolite of dasatinib, M20) and \(N\)-(2-chloro-6-(hydroxymethyl)phenyl)-2-(6-(4-(2-hydroxyethyl)piperazin-1-yl)-2-methylpyrimidin-4-ylamino)thiazole-5-carboxamide (benzylhydroxy metabolite of dasatinib, M24) were isolated from a microbial biotransformation reaction of dasatinib with \textit{Streptomyces} (Li et al., 2008). \[^{14}\text{C}]\text{Dasatinib} (17.0 \mu\text{Ci/mg}), labeled on the 2-carbon of the thiazole ring, was synthesized by the Radiochemistry Group of the Department of Chemical Synthesis, Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ) (Allentoff et al., 2008). Ecolite scintillation cocktail was obtained from MP Biomedicals (Irvine, CA) and Emulsifier-Safe and Permofluor E+ scintillation fluid were obtained from PerkinElmer Life Sciences, Inc. (Boston, MA), respectively. Reagents and solvents of analytical or HPLC grade were purchased from commercial manufacturers.
Dosing and sample collection in the human ADME study: The human ADME study with [14C]dasatinib was an open-label, non-randomized, single-dose study. A total of 8 healthy male subjects were enrolled into the study. All subjects received a single oral dose of 100 mg of [14C]dasatinib solution in 25 mM citrate buffer containing 120 µCi of total radioactivity (TRA). Blood was collected for the determination of the pharmacokinetic parameters of dasatinib, M5 and TRA at pre-dose and at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120, 144, 168, 192, 216 hours post-dose. All blood samples were collected into Vacutainer® tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Plasma was prepared from the blood samples by centrifuging for 15 min at 1000 x g and 5 °C. The plasma samples were stored at -20 °C until analysis. The entire urinary and fecal output for each subject was collected over the study period. The day 1 urine collection was in 12-hour collection increments; thereafter, urine collection was in 24-h intervals. Feces were collected in 24-h intervals. Individual urine and fecal samples were stored at -20 °C or below until analysis.

Representative pooled urine and fecal samples were prepared by combining a constant percentage of urine volume or fecal homogenate by weight across subjects. Pooled samples from 0-168 h were used for radioactivity profiling and LC/MS/MS analysis. Plasma samples were segregated by collection time and equal volumes of plasma were combined from each subject. All pooled plasma, urine and fecal samples for metabolite profiling were stored at -80 °C prior to analysis.

Analyses of radioactivity: Samples of plasma (100 µL) and urine (300-350 µL), in triplicate, were mixed with Emulsifier-Safe (PerkinElmer Life Sciences, Inc, Waltham,
MA) scintillation cocktail for the analysis of radioactivity by liquid scintillation counting (LSC). The fecal samples were transferred to tared containers and homogenized with water. The weight of the fecal homogenate was recorded for each sample. Accurately weighed duplicate portions (~0.2-0.4 g each) of the fecal homogenates were combusted to $^{14}$CO$_2$. Blood samples (0.1 mL each) were directly combusted to $^{14}$CO$_2$. Combustion of the blood and fecal samples was carried out in a model 307 or model 387 sample oxidizer (PerkinElmer Life Sciences, Inc, Waltham, MA), and the $^{14}$CO$_2$ was trapped with Carbo-Sorb E (PerkinElmer Life Sciences Inc., Waltham, MA). Perma-Fluor E+ (PerkinElmer Life Sciences Inc., Waltham, MA) scintillation cocktail was added to the $^{14}$CO$_2$-containing Carbo-Sorb E samples. $^{14}$C-labeled standards prepared in blank fecal matrix were combusted along with the study samples to determine combustion efficiency.

Radioactivity in all samples was determined in triplicate by LSC in a model LS 6000 or LS6500 liquid scintillation counter (Beckman Instruments, Inc. Fullerton, CA) equipped with automatic background subtraction and quench correction. All samples were counted for 10 min each or to a 2-sigma error of 5%. The limits of quantitation (LOQ) ranged from 132-152 ng-eq./mL for plasma (100 µL), 46.6-48.3 ng-eq./mL for urine (300-350 µL) and 56.0-62.3 ng-eq./g for feces (0.2-0.4 g).

**Analysis of dasatinib and M5 in plasma samples from the human ADME study:** The plasma concentrations of dasatinib and M5 were determined by a validated LC/MS/MS method. The internal standard (IS, BMS proprietary compound) was a close structural analog of dasatinib. The IS (200 ng/mL plasma) in 0.5% phosphoric acid was added to 0.1 mL of plasma sample before loading onto an Oasis HLB µElution 96-well solid phase extraction plate (Waters, Milford, MA). Sample components were eluted from the plate
with 50 µL of methanol. The extracts were diluted with 75 µL of water, vortexed, and a 10 µL portion of the sample was injected onto the LC/MS/MS system. The HPLC system consisted of a Shimadzu LC-10ADVP pump (Shimadzu Corporation, Columbia, MD) and a Perkin-Elmer Series 200 autosampler (Perkin-Elmer Life Sciences Inc., Waltham, MA). The column used was a Phenomenex Luna Phenyl-Hexyl (2 x 50 mm, 3 µm) (Torrance, CA). The mobile phase flow rate was 0.3 mL/min and the column was maintained at 50 °C. The components were eluted from the column with an isocratic solvent system (45% solvent A and 55% solvent B). Solvent A was a mixture of 10 mM ammonium acetate (pH 3.0)/methanol (80/20, v/v). Solvent B was a mixture of 10 mM ammonium acetate (pH 3.0)/methanol (20/80, v/v). The HPLC system was interfaced to a Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) that was operated in the positive ion ESI mode. The analysis time was 3 min with dasatinib, M5 and the IS eluting at 1.3, 1.67 and 1.0 min, respectively. For multiple reaction monitoring (MRM), the transitions monitored were m/z 488 to m/z 401 for dasatinib, m/z 504 to m/z 387 for M5 and m/z 474 to m/z 387 for the IS. The standard curve ranges in plasma were 1-1000 ng/mL for both dasatinib and M5.

**Dosing and sample collection in a phase 1 clinical study:** As part of a Phase 1 dose-escalation exploratory study in patients with chronic phase chronic myelogenous leukemia (CML) who showed hematologic resistance to imatinib mesylate (Gleevec™), serial blood samples at 0.5, 1, 2, 3, 4, 5, 6, 8 and 24 h were collected after the first dose of 180 mg of dasatinib from 3 cancer patients treated once daily (QD) on a 5 day on/2 day off schedule (Q5D). Dasatinib was administered as a tablet formulation in 50 and 5 mg strengths. All blood samples were collected into Vacutainer® tubes containing
ethylenediamine tetraacetic acid (EDTA) as an anticoagulant. Plasma was prepared from the blood samples by centrifuging for 15 min at 1000 x g and 5 °C and then stored at -20 °C until the time of analysis.

**Analysis of dasatinib and metabolites M4, M5, M6, M20 and M24 in plasma samples from a phase 1 clinical study:** The plasma samples were analyzed for dasatinib and M5 with the validated LC/MS/MS method described above. Analysis of metabolites M4, M6, M20 and M24 were conducted with an LC/MS/MS method that was not fully validated. The assays for these metabolites were conducted in an exploratory mode where quality control (QC) samples were not included as would be done with a validated method. A 10 point standard curve was run in duplicate with an appropriate internal standard for each of the analyses. Metabolites M4 and M6 were analyzed together in one analytical assay while metabolites M20 and M24 were analyzed separately. The LC/MS/MS method which was used for the determination of dasatinib and M5 in human plasma was adapted for measurement of M4 and M6. The same solid phase extraction procedure and chromatography conditions used for dasatinib and M5 were used for M4 and M6. The mass spectrometer instrument parameters were optimized for the detection of M4 and M6 in 0.1 mL of human EDTA plasma. The internal standard was the same as that used in the analysis of dasatinib and M5. Chromatographic separation was achieved isocratically on a Phenomenex Luna phenyl-hexyl analytical column (2 x 50 mm, 3 μm). The mobile phase contained formic acid, water, methanol and ammonium acetate. Detection was by positive ion electrospray tandem mass spectrometry on a Sciex API 3000 system (Applied Biosystems, Foster City, CA). The analysis time was 3 min, with M4, M6 and the IS eluting at 1.3, 1.8 and 1.0 min, respectively. The ion transitions
monitored were \( m/z \) 444 to \( m/z \) 401 for M4, \( m/z \) 502 to \( m/z \) 401 for M6 and \( m/z \) 474 to \( m/z \) 387 for the IS. The standard curves, which ranged from 1 to 1000 ng/mL for M4 and from 1 to 1000 ng/mL for M6.

For extraction of metabolites M20 and M24 the same solid phase extraction procedure was used as described for dasatinib and M5. The mobile phase for the HPLC method was the same except the pH of mobile phase A was 5.0 instead of pH 3.0. Gradient chromatography was used, where mobile phase B was 35% at time=0, and increased from 35% to 65% from t=0 to t=2.0 min at a flow rate of 0.3 mL/min and a total cycle time of 4 min. The mass spectrometer instrument parameters were optimized for the detection of M20 and M24 in 0.1 mL of human EDTA plasma. A \([^{13}C_4, ^{15}N_2]\) stable labeled M4 was used as the IS. After the addition of internal standard (200 ng/mL of plasma) in 0.5% phosphoric acid to 0.1 mL of each sample and calibration standard, the samples were loaded onto an Oasis HLB µElution 96-well solid phase extraction plate. The components were eluted from the plate with 50 µL of methanol. After the addition of water to the methanol eluate and vortexing, the extracts were injected into the LC-MS-MS system. Chromatographic separation by gradient HPLC was achieved on a Luna phenyl-hexyl analytical column (2 x 50 mm, 3 µm). Detection was by positive ion electrospray tandem mass spectrometry on a Sciex API 3000 system (Applied Biosystems, Foster City, CA) operating the Analyst version 1.4 software. The analysis time was 4.4 min with M20, M24 and the IS eluting at 2.0, 2.2 and 2.4 min, respectively. The ion transitions monitored were \( m/z \) 504 to \( m/z \) 417 for M20, \( m/z \) 504 to \( m/z \) 399 for M24 and \( m/z \) 450 to \( m/z \) 407 for the IS. The standard curves, which ranged from 1 to 1000 ng/mL for M20 and M24.
Pharmacokinetic analysis methods: The pharmacokinetic parameters of dasatinib, M5 and TRA in the human ADME study were determined from plasma concentration versus time and urinary excretion data by a noncompartmental method, using Kinetica™ 4.28 in the eToolbox (InnaPhase Corporation, Philadelphia, PA). The single dose pharmacokinetic parameters assessed included: Cmax (maximum observed concentration); Tmax (time of maximum observed concentration); AUC(INF) (area under the concentration-time curve from time zero extrapolated to infinite time); AUC(0-T) (area under the concentration-time curve from time zero to the last measurable sample time); T-HALF (terminal phase half-life); %UR (percentages of the [14C]dasatinib dose excreted as dasatinib, M5 and TRA in the urine); CLR (renal clearance); %FE (percentage of TRA excreted in the feces); %TOTAL (percent of TRA excreted in feces and urine combined). The Cmax and Tmax were obtained from experimental observations. The terminal log-linear phase of the concentration-time curve was identified by least-square linear regression of at least 3 data points that yielded a maximum G-criteria, which is also referred to as adjusted R-squared, using no weighting factor. The T1/2 was calculated as Ln2/Kel, where Kel was the absolute value of the slope of the terminal log-linear phase. The AUC[0-T] was calculated using the mixed log-linear trapezoidal algorithm in Kinetica™. The AUC(INF) was estimated by summing AUC(0-T) and the extrapolated area, computed by the quotient of the last observable concentration and Kel. The amount of dasatinib, M5 and TRA excreted in urine and TRA in feces during each collection interval was calculated by multiplying the concentration of each analyte by the volume or weight of matrix collected over that interval. The total urinary recovery (UR) or fecal recovery (FE) was calculated as the
cumulative amount excreted over all the collection periods and expressed as a percentage of the administered \([^{14}\text{C}]\text{dasatinib dose (}\%\text{UR or }\%\text{FE}).\) Renal clearance (CLR) was calculated by dividing the cumulative amount excreted in the urine by the AUC(INF).

Pharmacokinetic parameters of dasatinib, M4, M5, M6, M20 and M24 in patients administered 180 mg oral dose were derived from plasma concentration-time data by a noncompartmental method, using Kinetica\textsuperscript{TM} 4.2 in the eToolbox (InnaPhase Corporation, Philadelphia, PA). The single dose PK parameters assessed included: Cmax (maximum observed plasma concentration); Tmax (time of maximum observed plasma concentration); AUC(0-T) (area under the plasma concentration-time curve from zero to the last time of the last quantifiable concentration). The Cmax and Tmax were obtained from experimental observations. The AUC(0-T) was calculated using the mixed log-linear trapezoidal algorithm in Kinetica\textsuperscript{TM}. AUC(0-T) was calculated for the metabolites as the circulating concentrations were low and could not be measured in all of the 24 hour samples.

**Preparation of Samples for Radioactivity Profiling:**

**Plasma:** Pooled plasma samples obtained from the human ADME study were extracted with methanol/acetonitrile (1:1, v/v) at a ratio of 2 volumes solvent to 1 volume of plasma. The mixtures were vortex mixed, sonicated for 5 min, and centrifuged at 3500 rpm for 10 min. The supernatants were transferred into new tubes. The remaining pellets were extracted two more times with 3 mL of methanol/acetonitrile (1:1, v/v). The supernatants were combined and evaporated to dryness in a Savant Speed-Vac (Savant Instruments, Inc., Holbrook, NY). The residue was reconstituted in 0.35 to 0.45 mL of 30% acetonitrile in water, vortex mixed and centrifuged at 13,000 rpm for 5 min.
(American Scientific Products Biofuge A centrifuge, Heraeus Sepatech GmbH, Germany). The supernatant was subjected to HPLC analysis. Aliquots of the reconstituted samples (30 µl) were also counted on the liquid scintillation counter (LSC) to determine the radioactivity extraction recovery.

**Urine:** A pooled 0-168 h human urine (5 mL) sample was extracted with 2 volumes of acetonitrile/acetone (1:1, v/v). The mixtures were vortex mixed, sonicated and centrifuged at 3500 rpm for 10 min. The supernatants were evaporated to dryness. The residue was suspended in 0.3-0.5 mL of 30% acetonitrile in water and centrifuged at 13,000 rpm for 5 min. Aliquots of the supernatants were subjected to HPLC analysis or analyzed by LSC for radioactivity recovery.

**Feces:** Human 0-168 h pooled fecal homogenate (5.0 g) was extracted by addition of 3 volumes of acetonitrile/methanol (1:1, v/v) and mixed on a vortex mixer. The mixture was sonicated for 5 min and then centrifuged at 3000 rpm for 10 min. The supernatants were removed and saved. The extraction was repeated two additional times and the supernatants were combined and evaporated to dryness on a Savant Speed-Vac (Savant Instruments Inc., Holbrook, NY). The residue was suspended in 0.5 mL of 35% acetonitrile in water, vortex mixed for 5 min and centrifuged at 13,000 rpm for 10 min. An aliquot of the supernatant was injected onto the HPLC for metabolite profiling and a portion was analyzed by LSC for radioactivity extraction recovery.

**HPLC for biotransformation analysis:** Metabolite profiling was performed on an Agilent 1100™ system equipped with binary pumps, autoinjector, and a column heater (Agilent Technologies, Santa Clara, CA). A Phenomenex Synergy Polar-RP® column
(4.6 x 250 mm, 5 micron, Phenomenex, Torrance, CA) was used for metabolite separation. All HPLC analyses were performed at 1 mL/min flow rate at 35°C. The HPLC mobile phase consisted of (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The linear gradient was as follows: 0 min, 15% B; 3 min, 15% B; 53 min, 32% B; 53.5 min, 32% B; 60 min, 90% B; 63, 90% B. The column was re-equilibrated at 15% B for 6 min before the next injection.

For quantification of radioactivity, the HPLC effluent was collected in 15-sec intervals into 96-well Packard Lumaplates® (PerkinElmer Life Sciences Inc., Waltham, MA) with a Gilson Model FC 204 fraction collector (Gilson, Middleton, WI). Each fraction of column eluent was evaporated to dryness on a Savant Speed-Vac (Savant Instruments Inc., Holbrook, NY) and counted for radioactivity with a Packard Top Count® microplate scintillation analyzer (PerkinElmer Life Sciences Inc., Waltham, MA). Radiochromatographic metabolite profiles were prepared by plotting the net CPM values obtained from the Top Count versus time after injection with Microsoft Excel (Microsoft Corporation, Seattle, WA). The relative abundance of the metabolites were determined based on the percentage of total radioactivity in each peak relative to the entire radiochromatogram (Zhu et al., 2005). For metabolites that were not baseline resolved by HPLC, the percent of radioactivity of these metabolites were obtained by splitting the partially resolved peaks on the radiochromatogram.

**LC/MS and LC/MS/MS analysis:** The LC/MS system used for analysis of plasma, urine and fecal samples consisted of an Agilent 1100™ HPLC system interfaced to a Finnigan LCQ-DecaXP mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). Samples were analyzed using positive electrospray ionization. The capillary temperature
used for analysis was 320°C, and the nitrogen gas flow rate, spray current, and voltages were adjusted to give maximum sensitivity. The HPLC conditions were the same as used for biotransformation analysis.

**Cell Assays:** Dasatinib and metabolites M4, M5, M6, M20 and M24 were tested for antiproliferative affects in mSRC-A4 and K562 CML cell lines. The development and use of mSRC-A4 cells, which are mammary carcinoma cells from a transgenic mouse harboring the activated SRC oncogene, in antiproliferative assays will be the subject of a separate publication. The use of K562 cell lines for measurement of antiproliferative activities has been reported (O'Hare et al., 2005). Cell growth assays were conducted in 6-well culture plates. A solution of dasatinib or its metabolites in DMSO was added at various concentrations to the culture plates. The concentration of DMSO in the cell growth assay never exceeded 0.1%. The cells were exposed to the compounds for 72 h and the cell viability was determined by the direct counting of cell number with a Coulter Channelyzer (Beckman Coulter, Miami, FL).
Results

Pharmacokinetics of Total Radioactivity, Dasatinib and M5 in Healthy Subjects: The mean total radioactivity (TRA), dasatinib, and M5 plasma concentration versus time profiles following oral administration of 100 mg of [14C]dasatinib (120 µCi) are presented in Figure 1. The last quantifiable time point for TRA was different from the last quantifiable time point for dasatinib and M5 because the TRA assay was less sensitive compared to the LC/MS/MS assays. Upon oral administration, radioactivity was rapidly absorbed with a median Tmax of 0.5 h. The concentrations of TRA were substantially higher than unchanged dasatinib in the plasma. After the completion of the absorption phase, concentrations of dasatinib declined rapidly with a mean terminal phase half life of 3.6 h. By 24 h post dose, the mean concentrations of dasatinib were ~1% of the mean Cmax values. The pharmacokinetic parameters, determined by a non-compartmental method, are summarized in Table 1. Metabolite M5 was rapidly formed after administration of dasatinib as Cmax was attained at a median Tmax of 1.5 h. The plasma concentration of M5 declined with a mean terminal half-life of 3.1 h. At 2 h, dasatinib accounted for 25% of the TRA in plasma suggesting that metabolites contributed significantly to the total drug-related species.

Recovery of radioactivity in healthy subjects dosed with [14C]dasatinib: The recovery of radioactivity in urine and feces from 8 healthy human volunteers administered a single 100 mg (120 µCi) oral dose of [14C]dasatinib was 3.58 ± 1.17% and 85.32 ± 17.28%, respectively (data not shown). Only one of the eight subjects at 9 days post-dose, showed low recovery (45.89%). The reason for the low recovery for this one patient is not known.
The majority of TRA that was recovered in urine and feces was recovered within 24 and 72 h after dosing, respectively.

**Biotransformation Profile in Plasma:** The recovery of radioactivity after acetonitrile/methanol extraction of the pooled plasma sample was >90%. The 2 h plasma sample was the only sample with sufficient radioactivity for profiling and analysis of metabolites. The biotransformation profile of the human 2 h plasma sample is shown in Figure 2 and the distribution of metabolites is summarized in Table 2. In addition to dasatinib, multiple circulating metabolites were detected by radioactivity in human plasma. Dasatinib was the major circulating species and represented approximately 26% of total radioactivity in the 2 h plasma sample. Metabolite M20, a phenyl hydroxylated metabolite and its sulfate conjugate (M21) were also detected in significant amounts in human plasma.

**Biotransformation Profile in Urine:** Excretion of radioactivity in urine accounted for 4% of the radioactive dose. The biotransformation profile of pooled human urine (0-168 h) is shown in Figure 2 and the distribution of urinary metabolites is summarized in Table 2. The recovery of radioactivity after an extraction and a concentration step was 92%. M5, a piperazine N-oxide, was the predominant metabolite accounting for 1.4% of the radioactive dose (Table 2). Dasatinib accounted for <1% of the dose. Multiple metabolites, in addition to dasatinib and M5, including products from both oxidative and conjugated pathways, were also identified in urine, but none of these metabolites accounted for >1% of the radioactive dose.

**Biotransformation Profile in Feces:** The biotransformation profile of pooled fecal extract (0-168 h) is shown in Figure 2 and the distribution of fecal metabolites is summarized in
Table 2. After extraction, recovery of radioactivity was 75% in the pooled human fecal sample. Unchanged parent drug was a prominent component in the fecal profile accounting for 19% of the dose. Metabolites M20 and M6 were also detected in significant amounts. Other fecal metabolites included M23a,b, M24 and M4. No conjugated or N-oxide metabolites were observed in feces.

**LC/MS/MS analysis of metabolites:** Metabolites in plasma, urine and fecal homogenates were detected by LC/MS/MS analysis. The identity of the primary metabolites M4, M5, M6, M20 and M24 were based on a match of their retention times and mass spectrometric fragmentation patterns to synthetic standards. The rest of the metabolites were identified based on their MS/MS fragmentation (data not shown). Detailed identification of the metabolites of dasatinib is described in a separate paper (Christopher et al., 2008).

**Activities of dasatinib and its primary metabolites against SRC and BCR-ABL kinases:** The inhibitory activities of dasatinib and its primary metabolites M4, M5, M6, M20 and M24 were determined in two in vitro cell models: m-SRC-A4 mammary carcinoma and K562 CML cell lines, whose proliferation is dependent on Src and BCR-Abl kinases, respectively. Dasatinib was highly active in both the cell lines, producing inhibitory IC\textsubscript{50} values of 0.7 and 2.7 nM in the K562 and mSRC-A4 cell lines, respectively (Table 3). The metabolites exhibited a range of potency against both cell lines (Table 3), with M4 being the most potent (approximately equivalent to dasatinib), M5 and M20 of intermediate potency (10-20 fold less potent than dasatinib), and M6 and M24 the least potent (>50 fold less potent than dasatinib).

**Pharmacokinetics of dasatinib, M4, M5, M6, M20 and M24 in patients:** The plasma concentration vs. time profiles for dasatinib and metabolites M4, M5, M6, M20 and M24
after a single oral dose of 180 mg to 3 patients are shown in Figure 3 and the pharmacokinetic parameters are summarized in Table 4. There was a dose-dependent increase in exposures to dasatinib and M5 when compared to the 100 mg dose in the human ADME study. All five metabolites had mean Cmax and mean AUC(0-T) values less than dasatinib. M20 was the most prominent of the metabolites with an AUC value 45% of that of dasatinib. Median Tmax values for dasatinib and the 5 metabolites ranged from 1 to 3 hours.
Discussion

In healthy human subjects administered a single oral solution dose of $^{[14C]}$dasatinib, the drug was rapidly absorbed as indicated by the early Tmax of 0.5 h for both TRA and dasatinib (Table 1). Circulating metabolites contributed a significant portion of the AUC of the TRA. This was further confirmed by the metabolic profiles generated with the 2-h plasma sample where, in addition to dasatinib, 19 metabolites were identified (Table 2). The decline of TRA and dasatinib concentration with half-life values of <4 h suggested that the clearance of the drug-related material from plasma was rapid. A similar rapid plasma clearance of dasatinib was observed in Phase 1 and 2 studies.

Metabolite M5 was a prominent rodent metabolite (Christopher et al., 2008) and was thus monitored in early clinical studies. It was the only metabolite monitored in the human ADME study with a specific LC/MS/MS method and showed a similar plasma concentration versus time profile to that of dasatinib, suggesting that M5 exhibits formation rate-limited kinetics. Similar pharmacokinetics for dasatinib and M5 were also observed in healthy subjects who received a single 100 mg tablet formulation of dasatinib. The decision to monitor M5 was also based on the potential for the metabolite to inhibit Src and Bcr-Abl kinases. However, the data generated here indicate that M5 is a minor circulating metabolite in humans and, therefore, is unlikely to contribute significantly to in vivo efficacy.

Most of the radioactive dose was recovered in feces (85%) with a small amount (<4%) recovered in urine for a mean total recovery of 88.9%. One subject showed a recovery value (<46%) significantly lower than the other subjects. If this subject was
excluded, the overall recovery improved to 95%. Since the bulk of the TRA was in the 
feces it was thought that the low recovery in this subject could be due to incomplete 
homogenization of the fecal samples. However, re-homogenization and re-analysis of the 
fecal homogenate from the low recovery subject gave similar results to the initial values. 
Therefore, the reason for the low recovery is not known. Interestingly, the same subject 
had radioactivity counts in plasma below LLOQ at all time points even though 
LC/MS/MS analysis of plasma samples showed exposure to both dasatinib and M5 that 
were similar to other subjects (data not shown).

Intact dasatinib accounted for approximately 19% of the recovered dose. These 
data suggest that dasatinib is well absorbed after oral administration. The presence of 
dasatinib in human feces could be due to a combination of one or more of the following, 
namely: 1) incomplete absorption of the oral dose, 2) reduction of the N-oxide metabolite 
M5 to dasatinib by the GI bacteria, and 3) hydrolysis of the conjugated metabolites of 
dasatinib (M8a,b) in the GI. In rat, M5 was a prominent metabolite in bile but was not 
observed in the feces. Instead, dasatinib was observed in rat feces, indicating that M5 was 
reduced during transit through the GI by the gut bacteria (Christopher et al., 2008). 
Reduction of N-oxide metabolites by the GI gut bacteria is known and has been reported 
for a number of compounds (Powis et al., 1979; Jaworski et al., 1991; Mitchell et al., 
1997).

The results of the current study indicate that dasatinib is extensively metabolized 
in vivo. The proposed metabolic pathway for dasatinib in humans is shown in Figure 4. 
Dasatinib was the major drug-related component in human plasma. The many circulating 
metabolites of dasatinib in humans included products of hydroxylation, N-dealkylation,
N-oxidation, alcohol oxidation, sulfate and glucuronide conjugation and products of further metabolism of the primary metabolites. All of the human circulating metabolites identified here were also present in monkey plasma and many in rat plasma (Christopher et al., 2008). One interesting interspecies difference in metabolite profiles was the lack of a direct sulfate conjugate in monkey and human while rat produced significant amounts of this metabolite. This interspecies difference in sulfation of primary alcohols has been observed before (Nakano et al., 2004).

The metabolites identified in plasma were also present in human microsomal and hepatocyte incubations (Christopher et al., 2008). Similar to the human plasma profile metabolites, M20 and M24 were the most abundant oxidative metabolites in liver microsome and hepatocyte incubations. Metabolite M21, a prominent metabolite in human plasma, was also a major metabolite in human hepatocyte incubations. The other minor metabolites of dasatinib identified in human hepatocytes and microsomal incubations were also identified as minor metabolites in human plasma. These data suggest a good correlation between in vitro and in vivo metabolism profiles for dasatinib. A similar correlation between in vitro and in vivo profiles was also observed in rat and monkey. These data support the utility of microsomal and hepatocyte system to predict in vivo metabolism.

Both imatinib and dasatinib bind to the ATP-binding site of Bcr-Abl kinase. An x-ray crystal structure of dasatinib bound to the Bcr-Abl kinase and docking studies to ATP-binding site of Src kinase showed that the piperazine ring and the terminal ethyl alcohol group were not in the active site of either enzyme (Lombardo et al., 2004; Shah et al., 2004; Das et al., 2006). Thus it was not surprising that metabolic modifications on the
piperazine ring yielded compounds that retained Src and Bcr-Abl kinase activity. This was confirmed with metabolites M4, M5 and M6 which all showed activity towards Src and Bcr-Abl kinases in the nanomolar range (Table 3). Metabolite M4 was approximately equipotent to dasatinib, whereas the other metabolites M5 and M6 were >10 fold less active than dasatinib (Table 3). M20 and M24 were modified on a portion of the dasatinib molecule expected to be in the active site, however, these metabolites still maintained reasonable potency. In general, among the metabolites and dasatinib, the rank order of potency was similar for Src and BCR-Abl, indicating that the Src/Ab1 dual kinase inhibitory activity of parent was also maintained by the metabolites.

Since only the 2-h plasma sample in the human ADME study contained sufficient radioactivity for profiling, no exposure versus time data was generated for metabolites M4, M6, M20 and M24 from the 14C-profiling experiments. M5 was analyzed by a validated LC/MS/MS assay, as mentioned above, and found to be a minor circulating metabolite. Exposure values for metabolites M4, M6, M20 and M24, were obtained with specific LC/MS/MS assays from 3 patients dosed with a single oral dose of 180 mg dasatinib. This data was used to make an informed decision with regard to monitoring metabolites in future clinical studies. The results showed that M20 and M24 were prominent circulating metabolites, whereas M4, M5 and M6 were relatively minor circulating metabolites (Figure 3). These data along with the relative potency values suggest that these metabolites are unlikely to contribute significantly to the pharmacological activity in humans. Moreover, since this study provided sufficient data regarding exposure to the prominent human metabolites, a decision was made not to monitor them in future clinical studies.
The current study clearly demonstrates that the major route of clearance for
dasatinib is via oxidative biotransformation. Along with the in vitro data\textsuperscript{1}, these data
suggest that CYP3A4 will play a major role in dasatinib clearance. Furthermore, key
drug-drug interaction studies have clearly demonstrated that perturbation of the CYP3A4
pathway strongly impacts the pharmacokinetics of dasatinib. Co-administration of
dasatinib with ketoconazole increased the exposures of dasatinib by 4-5 fold whereas,
rifampin, reduced the exposure of dasatinib by \~80\%.\textsuperscript{1}

In summary, the human ADME study of dasatinib showed that the drug was well
absorbed and extensively metabolized in vivo, with all the circulating metabolites
detected in human plasma also present in plasma of at least one of the species used in
toxicological evaluations. LC/MS/MS monitoring of key primary metabolites, which had
in vitro kinase activity, showed that these metabolites were not expected to contribute
significantly towards in vivo pharmacological activity.

\textbf{Acknowledgments:} We would like to thank the Clinical Pharmacology Unit (CPU) of
Bristol-Myers Squibb for the conduct of the human ADME study.


Hochhaus A, Kim DW, Rousselot P, Drolhia-Llacer PE, Milone J, Francis S, Bleickardt E and Kantarjian H (2006) Dasatinib (SPRYCEL(R)) 50mg or 70mg BID Versus 100mg or 140mg QD in Patients with Chronic Myeloid Leukemia in Chronic Phase (CML-CP) Resistant or Intolerant to Imatinib: Results of the CA180-034 Study. *ASH Annual Meeting Abstracts* **108**:166.


Schittenhelm MM, Shiraga S, Schroeder A, Corbin AS, Griffith D, Lee FY, Bokemeyer C, Deininger MW, Druker BJ and Heinrich MC (2006) Dasatinib (BMS-354825), a dual SRC/ABL kinase inhibitor, inhibits the kinase activity of wild-type,


Footnote

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1Part of this work has been presented previously at the 2006 ISSX meeting, 2005 AAPS meeting and 2007 AACR meeting.

2Current address: Genentech, 1 DNA Way, South San Francisco, CA 94080-4990.
Figure Legends

Figure 1  Mean plasma concentration-time profiles for total radioactivity (TRA), dasatinib and metabolite M5 following a single oral administration of \([^{14}C]\)dasatinib (100 mg, 120 \(\mu\)Ci) to healthy subjects (n=8).

Figure 2  Biotransformation profiles of pooled plasma (A), urine (B) and feces (C) after a single oral dose of \([^{14}C]\)dasatinib to healthy human volunteers. The profiles are background-subtracted, reconstructed radiochromatograms of 15-sec fractions collected from an HPLC run.

Figure 3  Mean plasma concentration-time profiles of dasatinib and its metabolites (M4, M5, M6, M20 and M24) on day 1 after a single oral dose of 180 mg to 3 patients.

Figure 4  Proposed biotransformation pathway based on metabolites identified in plasma, urine and fecal samples from humans administered a single oral dose of \([^{14}C]\)dasatinib (100 mg, 120 \(\mu\)Ci).
Table 1  Pharmacokinetic parameters of total radioactivity (TRA), dasatinib and metabolite M5 after a single oral solution dose of 100 mg (120 µCi) of [14C]dasatinib to healthy subjects

<table>
<thead>
<tr>
<th></th>
<th>Cmax (ng/mL)</th>
<th>AUC(INF) (ng·h/mL)</th>
<th>AUC(0-T) (ng·h/mL)</th>
<th>Tmax (h) Median (Min, Max)</th>
<th>T-HALF (h) Mean (SD)</th>
<th>CLR (mL/h) Mean (SD)</th>
<th>UR (%) Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRA (n=7)</td>
<td>224.6</td>
<td>-b</td>
<td>400.4</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>3.58</td>
</tr>
<tr>
<td></td>
<td>(23)</td>
<td>(41)</td>
<td>(41)</td>
<td>(0.25, 1.5)</td>
<td>-</td>
<td>-</td>
<td>(1.17)</td>
</tr>
<tr>
<td>Dasatinib (n=8)</td>
<td>104.5</td>
<td>313.9</td>
<td>298.8</td>
<td>0.5</td>
<td>3.6</td>
<td>404.8</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>(29)</td>
<td>(42)</td>
<td>(44)</td>
<td>(0.25, 1.5)</td>
<td>(1.0)</td>
<td>(168.7)</td>
<td>(0.05)</td>
</tr>
<tr>
<td>M5 (n=8)</td>
<td>2.9</td>
<td>15.3</td>
<td>8.9</td>
<td>1.5</td>
<td>3.2</td>
<td>-</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(55)</td>
<td>(53)</td>
<td>(74)</td>
<td>(0.75, 3)</td>
<td>(1.2)</td>
<td>-</td>
<td>(0.49)</td>
</tr>
</tbody>
</table>

aN = 7 because 1 subject had no quantifiable levels of total radioactivity in plasma. The limits of quantitation (LOQ) in plasma for radioactivity ranged from 132-152 ng- eq./mL.

bThe AUC(INF) and T-HALF for radioactivity was not calculated since radioactivity was measurable only up to 4 h.

cN = 6 because 1 subject had no quantifiable levels of total radioactivity in plasma and the extrapolated AUC could not be estimated in a second subject.
Table 2  Relative percent distribution of radioactive metabolites in pooled plasma, urine and fecal samples after oral administration of [14C]dasatinib to humans

| Metabolite ID | % Distribution | | | | | |
|---------------|----------------|----------------|----------------|----------------|----------------|
|               | Plasma (2 h)   | Urine (0-168 h) | Feces (0-168 h) | | |
|               | % Radioactivity | % Radioactivity | % Dose | % Radioactivity | % Dose |
| M3a, ba       | 3.3            | 6.8            | 0.2    | nd             | nd     |
| M4            | MS             | 1.3            | 0.05   | 3.1            | 2.6    |
| M5            | 4.5            | 39.8           | 1.4    | nd             | nd     |
| M6            | 3.6            | 1.3            | 0.05   | 10.4           | 8.9    |
| M7            | 3.3            | 2.1            | 0.08   | nd             | nd     |
| M8a           | 3.4            | 5.5            | 0.2    | nd             | nd     |
| M8b, M23a, b  | 1.4            | nd             | nd     | nd             | nd     |
| M9            | nd             | nd             | 1.8    | nd             | nd     |
| M20           | 12.5           | 4.1            | 0.2    | 36.6           | 31.2   |
| M21           | 9.5            | 7.8            | 0.3    | nd             | nd     |
| M23a,b        | nd             | nd             | nd     | 14.7           | 12.5   |
| M24           | 3.1            | 6.0            | 0.2    | 4.7            | 4.0    |
| M30           | 6.9            | nd             | nd     | nd             | nd     |
| M31           | 3.6            | nd             | nd     | nd             | nd     |
| M34           | 1.1            | 2.3            | 0.08   | nd             | nd     |
| M35a          | 3.6            | 4.2            | 0.2    | nd             | nd     |
| M36           | nd             | 4.4            | 0.2    | nd             | nd     |
| M37a, b       | 4.1            | 2.5            | 0.1    | nd             | nd     |
| Dasatinib     | 25.5           | 3.6            | 0.1    | 22.4           | 19.1   |
| Total         | 89.4           | 91.7           | 3.4    | 93.7           | 79.8   |

MS = mass spectrometry; nd = not detected

aMetabolites (M3a, b), (M23a, b) and (M37a, b) were pairs of positional isomers that were not well resolved on HPLC.

bMetabolites M8b and M23a, b were not well resolved on HPLC. The % of distribution is the total percentage of all three metabolites.
Table 3  Inhibitory activities of dasatinib and 5 of its metabolites on the in vitro growth of mSRC-A4 mammary carcinoma and K562 CML cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₅₀, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dasatinib</td>
</tr>
<tr>
<td>mSRC-A4ᵃᵇ</td>
<td>2.7</td>
</tr>
<tr>
<td>K562ᵇ</td>
<td>0.7</td>
</tr>
</tbody>
</table>

ᵃDerived from a mammary carcinoma from a transgenic mouse harboring the activated SRC oncogene
ᵇProliferation of mSRC-A4 and K562 cells is dependent on Src and Bcr-Abl kinases, respectively.
Table 4  Pharmacokinetic parameters for dasatinib and metabolites M4, M5, M6, M20 and M24 in patients (n=3) after a single oral dose of 180 mg.

<table>
<thead>
<tr>
<th></th>
<th>Cmax (ng/mL)</th>
<th>AUC(0-T) (ng·h/mL)</th>
<th>Tmax (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Geometric Mean (SD)</td>
<td>Geometric Mean (SD)</td>
<td>Median (Min, Max)</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>247.7 (52.0)</td>
<td>1151 (213)</td>
<td>1.5 (0.5, 2.0)</td>
</tr>
<tr>
<td>M4</td>
<td>10.4 (6.8)</td>
<td>53 (53)</td>
<td>2.1 (2.0, 4.9)</td>
</tr>
<tr>
<td>M5</td>
<td>11.2 (3.5)</td>
<td>56 (22)</td>
<td>2 (2.0, 2.0)</td>
</tr>
<tr>
<td>M6</td>
<td>21.9 (7.2)</td>
<td>115 (45)</td>
<td>3.0 (1.5, 5.0)</td>
</tr>
<tr>
<td>M20</td>
<td>200.2 (101.6)</td>
<td>513 (188)</td>
<td>1.5 (1.0, 1.5)</td>
</tr>
<tr>
<td>M24</td>
<td>71.4 (26.7)</td>
<td>288 (102)</td>
<td>1 (1.0, 2.0)</td>
</tr>
</tbody>
</table>
Figure 1

![Graph showing plasma concentration over time for TRA, Dasatinib, and M5](image-url)
Figure 2
Figure 3