IDENTIFICATION OF THE HUMAN ENZYMES INVOLVED IN THE
OXIDATIVE METABOLISM OF DASATINIB. AN EFFECTIVE APPROACH
FOR DETERMINING METABOLITE FORMATION KINETICS

Lifei Wang, Lisa J Christopher, Donghui Cui, Wenying Li, Ramaswamy Iyer, W
Griffith Humphreys, Donglu Zhang

Pharmaceutical Candidate Optimization (LW, LJC, DC, WL, RI, WGH, DZ), Bristol-
Myers Squibb Research & Development, Princeton, NJ 08543
Running title: Reaction-phenotyping for metabolism of dasatinib

Address correspondence to:
Donglu Zhang, Ph.D.
Pharmaceutical Candidate Optimization,
Bristol-Myers Squibb, PO BOX 4000,
Princeton, New Jersey 08543.
Phone: 609-252-5582.
Email: Donglu.zhang@bms.com

Abbreviations used: AUC, area under the curve; AUC(i), area under the curve in the presence of the inhibitor; CYP, cytochrome P450; DDI, drug-drug interaction; fm, fraction of dose metabolized; fmCYP3A4, fraction of dose metabolized by CYP3A4; FMO, flavin-containing monooxygenase; HLM, human liver microsomes; [I], inhibitor concentration; IS, internal standard; Ki, inhibition constant; LC/MS, liquid chromatography/mass spectrometry; NADH, nicotinamide adenine dinucleotide (reduced form); NADPH, β-nicotinamide adenine dinucleotide phosphate (reduced form); SRM, selective reaction monitoring; TFA, trifluoroacetic acid.

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ABSTRACT

Dasatinib (SPRYCEL®, BMS-354825) is a potent protein kinases inhibitor to treat chronic myeloid leukemia. In vivo studies have shown that the primary oxidative metabolites of dasatinib are M4 (N-dealkylation), M5 (N-oxidation), M6 (carboxylic acid formation), M20 and M24 (hydroxylation). To identify the enzymes responsible for the formation of these metabolites, [14C]dasatinib and non-radiolabeled dasatinib were incubated with human cDNA-expressed enzymes (CYPs and FMO3) or HLM in the presence of selective CYP inhibitors (antibodies and chemical inhibitors). The results of these experiments showed that metabolites M4, M20, and M24 were mainly generated by CYP3A4; M5 was primarily formed by FMO3; M6 was formed by a cytosolic oxidoreductase. The enzyme kinetic analysis showed that the formation of M4 and M5 in HLM followed the Michaelis-Menten kinetics and the formation data of M20 and M24 fitted well to a partial substrate inhibition kinetic model. The Km values were determined by the kinetic analysis of the substrate-dependent metabolite formation plots from a large number of incubations with the non-labeled dasatinib; the Vmax values were calculated with the pre-determined Km values and the metabolite formation rates from a limited number of incubations with [14C]dasatinib. The intrinsic formation clearance values (Vmax/Km) of 52, 14, 274, and 20 µL/mg protein/min for the formation of M4, M5, M20, and M24, respectively, suggested that the formation of M20 was more efficient than other metabolites. Collectively, multiple in vitro experiments demonstrated that dasatinib was predominately metabolized by CYP3A4.
INTRODUCTION

Dasatinib (SPRYCEL®, BMS-354825, the structure shown in Figure 1), is marketed as an oral inhibitor of BCR-ABL and SRC family kinases for the treatment of chronic myeloid leukemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL) in adults who are resistant to Gleevec® (imatinib mesylate). Studies have shown that dasatinib was extensively metabolized in animals and humans (Christopher et al., 2008a, Christopher et al., 2008b). The primary pathways of metabolism included N-dealkylation (M4), N-oxidation (M5), carboxylic acid formation (M6), and hydroxylation (M20 and M24) (Christopher et al., 2008a). Based on the human ADME study with [14C]dasatinib, the fraction clearance to form M4 (2.7%), M5 (1.4), M6 (9.0%), M20 (31.4%), M24 (4.2%), and related secondary metabolites represented approximately 81% of total dasatinib clearance (Christopher et al., 2008b).

Human liver cytochrome P450 enzymes play a key role in the clearance of many drugs and alteration of the activity of these enzymes is the major cause of drug-drug interactions. Three families of CYP enzymes (CYP1, CYP2, and CYP3) are involved in the metabolism of xenobiotics in humans, and CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4/5 are responsible for the metabolism of the majority of drugs (Nebert et al., 2002). In human livers, flavin-containing monooxygenase enzymes (FMO1, FMO3, and FMO5) are also important enzymes for metabolism of xenobiotics (Cashman et al., 1995; Parte et al., 2005; Ring et al., 1999). Human alcohol dehydrogenases (ADH) catalyze the oxidation of primary alcohols of drug (Walsh et al., 2002). Aldehyde dehydrogenases (ALDHs), NAD-dependent enzymes, also catalyze the oxidation of a wide range of
endogenous and exogenous aliphatic and aromatic aldehydes (Vasiliou et al., 2000; Vasiliou et al., 2004).

The objective of this study was to identify the human enzymes responsible for the formation of primary oxidative metabolites of dasatinib. [14C]Dasatinib was incubated with human cDNA-expressed enzymes to determine the catalytic turnover. The enzymes involved in dasatinib biotransformation were further investigated in studies with selective chemical CYP inhibitors or specific inhibitory antibodies in HLM with similar methods as described previously (Zhang et al., 2007). The kinetic parameters for metabolite formation were determined in incubations with both HLM and cDNA-expressed CYPs. The potential of drug-drug interaction of dasatinib with CYP3A4 inhibitors or inducers was estimated.
MATERIALS AND METHODS

Materials. Dasatinib (BMS-354825, N-(2-chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)-1-piperazinyl]-2-methyl-4-pyrimidinyl]amino]-5-thiazolecarboxamide, purity 96.2%) (Figure 1), $[^{14}\text{C}]$dasatinib (specific activity 30.4 $\mu$Ci/mg, radiochemical purity 99.2%, and chemical purity 98.8%), and stable isotope-labeled dasatinib prepared from (1, 2, 3-$^{13}$C$_3$) diethyl malonate and $[^{13}\text{C},^{15}\text{N}_2]$thiourea (internal standard, purity 99.5%) were synthesized at Bristol-Myers Squibb (Princeton, NJ). Standards of M4, M5, M6, M20, and M24 (Figure 1) were also prepared at Bristol-Myers Squibb. All CYP inhibitors (furafylline, tranylcypromine, orphenadrine, quercetin, sulfaphenazole, benzynirvanol, quinidine, dietyldithiocarbamate, ketoconazole, 1-aminobenzotriazole (ABT), and troleandomycin), $\beta$-nicotinamide adenine dinucleotide phosphate-reduced form ($\beta$-NADPH), and $\beta$-nicotinamide adenine dinucleotide-reduced form (NADH) were obtained from Sigma-Aldrich Co. (St. Louis, MO). The quercetin and dietyldithiocarbamate are inhibitors for CYP2C8 and CYP2E1 with low isoenzyme specificities. Sodium phosphate, trifluoroacetic acid (TFA), and formic acid were obtained from EM Science (Gibbstown, NJ). Acetonitrile, methanol, and dimethylsulfoxide of HPLC grade were from Burdick & Jackson Inc. (Muskegon, MI). Pooled human liver microsomes (HLM, 20 subjects), human cDNA-expressed CYP enzymes (in baculovirus-insect cells) CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, and 4A11), human flavin-containing monooxygenase FMO3 (expressed in baculovirus-insect cells), and monoclonal anti-CYP antibodies (anti-1A2, anti-2C8, anti-2D6, and anti-3A4) were purchased from BD Biosciences (Woburn, MA).
[14C]Dasatinib stock solutions including 0.2, 0.4, 2 and 4 mM concentrations were prepared in a mixture of acetonitrile and water (1:1, v/v) and the stock solutions for all chemical inhibitors were prepared in acetonitrile. Non-radiolabeled dasatinib stock solutions for substrate-dependent metabolite formation studies were prepared in methanol and dimethylsulfoxide (1:1, v/v).

**Incubations with cDNA-expressed enzymes, human liver microsomes, and human liver S9.** Human cDNA-expressed CYPs (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5 and 4A11) and HLM were incubated with [14C]dasatinib or non-labeled dasatinib. The incubation mixtures (0.5 mL) contained Tris-HCl buffer (0.05 M, pH 7.5), CYP (50 pmole) or HLM (0.5 mg protein), [14C]dasatinib (2 or 20 µM), and NADPH (1.2 mM). The final acetonitrile concentration was 0.5% in these incubations. After 30 min incubation at 37°C with shaking (100 rpm), ice-cold acetonitrile (0.25 mL) was added to each incubation to stop the reaction and the internal standard (IS, stable isotope-labeled dasatinib) was added to each sample to a final concentration of 1 µM. After centrifugation at 2000g for 10 min, an aliquot of 50 µL of supernatant was used for LC/MS analysis, and an aliquot of 80 or 100 µL of supernatant was used for HPLC profiling. In the following experiments, similar sample treatment and analytical procedures were used.

The FMO3 incubations were similar to CYP incubations except cDNA-expressed FMO3 (325 pmole) was used. Since the standard activity of FMO3 was reported at pH 9.5 by the manufacturer, FMO3 was also incubated in a glycine buffer (50 mM), pH 9.5, with 20 µM of [14C]dasatinib. The incubations were conducted at 37°C for 30 min with shaking (100 rpm). Heat-inactivation experiments were conducted with HLM and cDNA-
expressed FMO3. HLM and cDNA-expressed FMO3 in Tris-HCl buffer (0.05 M, pH 7.5) were pre-incubated at 45°C for 5 min or on ice for 5 min before dasatinib (20 µM) and NADPH (1.2 mM) were added to the incubation mixture.

The human liver S9 incubation mixture (0.5 mL) contained Tris-HCl buffer (50 mM, pH 7.5), S9 (2 mg protein), [14C]dasatinib (20 µM), NADPH (1.2 mM) or NADH (1.2 mM). After incubation at 37°C for 55 min, ice-cold acetonitrile (0.25 mL) was used to quench the reaction.

**HLM incubations in the presence of CYP inhibitors.** The incubation mixtures (1 mL in duplicate) contained phosphate buffer (0.1 M, pH 7.4), HLM (0.3 mg), [14C]dasatinib or non-labeled dasatinib (1 or 20 µM), NADPH (1 mM), and a single CYP inhibitor. The chemical inhibitors used were furafylline (10 µM) for CYP1A2, tranylcypromine (2 µM) for CYP2A6, orphenadrine (50 µM) for CYP2B6, quercetin (20 µM) for CYP2C8, sulfaphenazole (10 µM) for CYP2C9, benzylpirvanol (1 µM) for CYP2C19, quinidine (1 µM) for CYP2D6, diethyldithiocarbamate (50 µM) for CYP2E1, ketoconazole (1 µM) for CYP3A4, troleandomycin (20 µM) for CYP3A4, and 1-aminobenzotriazole (ABT, 1 mM) for all CYPs. Metabolism-dependent inhibitors, furafylline, orphenadrine, troleandomycin, and 1-aminobenzotriazole, were pre-incubated with HLM in the presence of NADPH for 10 min before the substrate was added. After substrate addition, the samples were then incubated at 37°C for 20 min with shaking. The final acetonitrile concentration was 0.75% in control incubations and those incubations with inhibitors. Negative control incubations (without NADPH or HLM) were performed under similar conditions.
For antibody inhibition experiments, a mixture containing 60 µL of HLM, 70 µL of Tris-HCl buffer (0.05 M), and 70 µL of monoclonal anti-CYP antibody (anti-CYP1A2, 2C8, 2D6, or 3A4) was pre-incubated on ice for 20 min. The protein ratio of the antibody to HLM was 0.6:1 in these incubations. The incubation mixtures (1 mL) contained phosphate buffer (0.1 M, pH 7.4), NADPH (1 mM), dasatinib (1 or 20 µM), and pre-incubated HLM mixture (50 µL, 300 µg protein).

**Dasatinib concentration-dependent metabolite formation.** In order to determine linear conditions of dasatinib metabolism, the following incubations were conducted: dasatinib at 10 µM incubated with 10, 30, 50, and 80 pmole/mL of CYP3A4 or 100, 200, 300, and 500 µg/mL of HLM for 20 min; dasatinib at 10 µM incubated with 10, 20, 30, and 50 min with CYP3A4 at 30 pmole/mL or HLM at 300 µg/mL. Incubations with 20 pmole/mL of CYP3A4 or with 150 µg/mL of HLM for 15 min were within linear conditions and used in the subsequent kinetic studies. For enzyme kinetic studies, the incubation mixtures (1 mL) contained phosphate buffer (50 mM, pH 7.5), NADPH (1 mM), HLM (150 µg) or CYP3A4 (20 pmole), and dasatinib. Nine substrate concentrations, 0, 1, 2, 5, 10, 20, 50, 100, and 200 µM were evaluated in triplicate. The incubation was conducted at 37°C for 15 min. The final concentrations of dimethylsulfoxide and methanol in all incubations with non-radiolabeled dasatinib were each kept at 0.25% (v/v). The relative formation rate (peak area ratio/min/pmole CYP or mg protein of HLM) of each metabolite was calculated and plotted against substrate concentration. The formation rates of M4, M5, M20, and M24 from incubations with a broad substrate concentration range were evaluated by fitting the data to the Michaelis-
Menten equation or to a partial substrate inhibition model (Cornish-Bowden, 1995): 

\[ V = \frac{V_{\max} \cdot S}{K_m + S \cdot (1 + S/K_i)} \]

using a nonlinear regression analysis program (KaleidaGraph, version 3.6, Synergy Software Reading, PA). \( K_m \) is the Michaelis-Menten constant, \( K_i \) is the substrate inhibition constant, and \( S \) is the substrate concentration. To determine the \( V_{\max} \) value, three concentrations (2, 10, and 20 \( \mu \)M) of \(^{14}\)C\text{-}dasatinib were separately incubated with HLM (150 \( \mu \)g/ml) or expressed CYP3A4 (20 pmole/ml) for 15 min. After quenching with ice-cold acetonitrile, the samples were centrifuged at 2000g for 10 min, and an aliquot (50 \( \mu \)L) of supernatant from each sample was used for HPLC radioactivity profiling and the formation rate (\( V \)) of each metabolite in these incubations was calculated based on the radioactivity. The \( V_{\max} \) value was then calculated based on the Michaelis-Menten equation or the partial substrate inhibition model, 

\[ V = \frac{V_{\max} \cdot S}{K_m + S \cdot (1 + S/K_i)} \],

using the \( V \) value and predetermined \( K_m \) value.

**Metabolite profiling.** Metabolites in incubation samples were analyzed using a Shimadzu LC-10AT system equipped with a photodiode array ultraviolet (UV) detector (Shimadzu Scientific Instruments, Kyoto, Japan). Samples were injected onto a Phenomenex® Synergi 4 \( \mu \) polar-RP 80Å column (4.6 x 250 mm). The mobile phase consisted of two solvents: A) 0.1% formic acid in water and B) 0.1% formic acid in acetonitrile. The gradient was as follows: Solvent B started at 5%, then linearly increased to 20% at 5 min, to 30% at 50 min, to 35% at 55 min, to 90% at 65 min, held at 90% for 2 min, and then decreased to 5% at 69 min. The HPLC effluent (1 mL/min) was collected into Deepwell LumaPlate™-96 plates (PerkinElmer Life and Analytical Sciences, Shelton, CT) at 0.25 min intervals for 75 min with a Gilson Model 202 fraction collector (Gilson Medical Electronics, Middleton, WI). The plates were dried with a Savant Speed-
Vac System (Global Medical Instrumentation, Inc., Ramsey, MN) and counted for 10 min per well with a TopCount analyzer (PerkinElmer Life and Analytical Sciences, Shelton, CT). Biotransformation profiles were prepared by plotting the resulting net CPM values vs HPLC time and radiochromatograms were reconstructed from the Topcount data using Microsoft® Excel software.

**Metabolite identification and quantification.** For determining the metabolite formation in CYP and HLM incubations, LC/MS/MS analyses were performed on a Finnigan TSQ Quantum mass spectrometer (ThermoFinnigan, San Jose, CA) with an ESI probe and a Waters 2695 HPLC system equipped with a Waters™ 996 photodiode array detector (Waters Corporation, Milford MA). Samples were analyzed in the positive ionization mode and the TSQ capillary temperature was set at 300°C. The flow rate of nitrogen gas, spray current, and voltages were adjusted to give maximum sensitivity for the parent drug. The HPLC chromatography was performed on a Waters YMC ODS-AQ™ S-3 µm 120 Å column (3.0 x 150 mm) maintained at 35°C. A gradient consisting of two solvent systems, A and B, was used for HPLC separation. Solvent A consisted of 0.1% TFA in water and Solvent B consisted of 0.1% TFA in acetonitrile. The mobile phase flow rate was 0.4 mL/min. The gradient employed as follows: Solvent B started at 5%, then linearly increased to 15% at 5 min, to 32% at 52 min, to 95% at 55 min, held at 95% for 5 min, and then decreased to 5% at 61 min. Under these HPLC conditions, the metabolites M4, M5, M20, M24, and parent compound eluted at 31.0, 36.2, 19.5, 20.5, and 31.6 min, respectively. Dasatinib, IS, and metabolites were monitored using selective reaction monitoring (SRM). The specific transitions monitored were dasatinib (m/z 488→401), internal standard (IS, m/z 494→407), M4 (m/z 444→303), M5 (m/z 504→460), M6 (m/z...
502→361), M20 (m/z 504→417), and M24 (m/z 504→399). Since the amounts of available metabolite standards were not adequate to develop quantification methods for each metabolite, the relative amount of each metabolite formed in each incubation was calculated based on the peak area ratio of the metabolite to the internal standard. Peak area ratios in each incubation were compared to the HLM control and the values were then used to measure the relative amount of metabolite formation between different incubations. In these analyses, the peak area ratio of a metabolite to the internal standard in HLM was defined as 100%. For all incubation with expressed CYPs, the metabolite formation rates (in peak area ratio/min/pmole of CYP) were normalized (in peak areas ratio/min/mg protein of HLM) with respect to the corresponding specific content of each CYP in HLM (Shimada et al., 1994, Rostami-Hodjegan and Tucker, 2007). The normalized formation rate for each CYP was expressed as a percentage of the formation rate obtained with HLM. In inhibition studies, the relative amount of each metabolite formed in the control HLM incubation (without inhibitors) was defined as 100%. The relative amount of each metabolite formed in HLM incubation with a inhibitor was expressed as a percentage of the formation rate of the control HLM incubation.

M6 was observed in the HPLC-radiochromatograms of samples from [14C]dasatinib incubations with HLM and human liver S9, however, there was no corresponding peak in the SRM channel for M6 in the LC/MS/MS analysis on the TSQ Quantum mass spectrometer. Samples from these incubations were therefore also analyzed by an LTQ (ion trap) mass spectrometer (ThermoFinnigan, San Jose, CA) to verify the presence of M6 in the samples.
Prediction of drug-drug interaction potential. The extent of a potential drug-drug interaction between dasatinib and ketoconazole (as a prototypical strong CYP3A4 inhibitor) was predicted as follows:

\[
\frac{AUC_{(i)}}{AUC_{(c)}} = \frac{1}{fm \cdot fm_{(CYP3A4)} \cdot \frac{Cl_{int(c)}}{Cl_{int(i)}} + (1 - fm \cdot fm_{(CYP3A4)})}
\]

(Ito et al., 1998)

The value for the fraction of the dose metabolized was \( fm = 0.81 \) and the fraction the metabolized dose that was catalyzed by CYP3A4 was \( fm_{(CYP3A4)} = 0.80 \). These values were estimated from results of a human ADME study with \(^{14}\text{C}\)dasatinib (Iyer et al., 2008) and current study. For inhibition by ketoconazole, \( Cl_{int(c)} = V_{max}/K_m \); \( CL_{int(i)} = [1/(1 + I/K_i)] \cdot V_{max}/K_m \), where \( K_i = 0.2 \ \mu\text{M} \), an average \( K_i \) value obtained from a survey of the literature with midazolam and testosterone as substrates (Galetin et al., 2005). The plasma \( C_{max} \) value of 8.27 \( \mu\text{M} \) after administration of 200 mg ketoconazole was used for the prediction. The mean observed \( AUC_{(i)}/AUC_{(c)} \) for dasatinib was determined in a clinical drug interaction study between dasatinib (20 mg) and ketoconazole (200 mg every 12 h).
RESULTS

**In vitro metabolite profile.** The LC/MS properties of the five primary oxidative metabolites of dasatinib are summarized in Table 1. LC/MS/MS analysis was performed on a TSQ Quantum mass spectrometer for dasatinib incubations conducted with HLM and cDNA-expressed enzymes. Selective reaction monitoring (SRM) was used to monitor each metabolite in the LC/MS/MS analyses. Figure 2 shows the ion chromatograms of dasatinib, its metabolites, and IS in the incubation samples with HLM. Similar ion chromatograms of dasatinib, its metabolites, and IS were observed in the incubation samples with the expressed CYP3A4 except that M5 peak was absent. Under these conditions, dasatinib, IS, M4, M5, M6, M20, and M24 were well separated with minimal or no cross-interference. The metabolites formed in HLM and CYP3A4 incubations had similar retention times, fragmentation patterns, and SRM transitions as the metabolite standards. The relative amount of each metabolite in different samples (incubations) was determined by comparing the peak area ratios of metabolite to IS. The relative amounts of different metabolites within the same sample were not compared because ionization properties could be very different between components. M6 was not detected on the TSQ Quantum mass spectrometer presumably due to insufficient sensitivity under these analytical conditions. When the samples were reanalyzed on a LTQ mass spectrometer, the presence of M6 was confirmed in both HLM and human liver S9 incubations.

**[14C]Dasatinib metabolism by human cDNA-expressed enzymes and human liver fractions.** Table 2 shows the distribution of radioactive metabolites in incubations of
[$^{14}$C]dasatinib (2 or 20 $\mu$M) with expressed enzymes, HLM, and human liver S9. Figures 3-4 show the metabolite profiles of [$^{14}$C]dasatinib in representative cDNA-expressed enzyme incubations (CYP1A1, CYP1A2, CYP1B1, CYP3A4, FMO3), HLM, and human liver S9, respectively. Under these HPLC profiling conditions, metabolites M20 and M24 were not completely separated and they are grouped together in Table 2. The results generated from these experiments indicate that M4 was formed in the incubations with CYP1A1, CYP1B1, CYP3A4, CYP3A5, HLM, and human liver S9. M5 was efficiently generated in the incubations with FMO3, HLM, and human liver S9 (Table 2, Figures 3 and 4). M5 was also formed in incubations with CYP1B1 and CYP1A2 (Table 2 and Figure 3). M6 was formed in HLM in the presence of NADPH and human liver S9 in the presence of NADH or NADPH. M20 and M24 were mainly formed in incubations of dasatinib with CYP3A4, HLM and human liver S9. CYP3A5 showed a lower level of activity for the metabolism of dasatinib relative to CYP3A4 (Table 2). At 2 $\mu$M [$^{14}$C]dasatinib, CYP3A4 extensively metabolized dasatinib to additional metabolites that eluted earlier than the primary metabolites on the HPLC profile (data not shown); presumably, these early-eluting metabolites were formed from further metabolism of the primary metabolites. Several similar minor peaks (secondary metabolites) were also observed in CYP3A4 incubations (Figure 3) and human liver S9 incubations at 20 $\mu$M dasatinib (Figure 4).

The results of LC/MS/MS analyses of the incubations of 20 $\mu$M [$^{14}$C]dasatinib with HLM, human cDNA-expressed CYPs, and FMO3 are summarized in Figures 5A-8A. The LC/MS/MS analyses confirmed that the metabolites M4, M20, and M24 are
predominately generated by CYP3A4; and M5 was mainly produced by FMO3. CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A5, and 4A11 did not metabolize [14C]dasatinib to any significant extent (Figures 5A-8A). The results from LC/MS/MS analysis are in good agreement with the results from HPLC radiochromatographic analysis shown in Table 2.

**Inhibition studies.** The effects of chemical inhibitors or antibodies on the formation of the oxidative metabolites of dasatinib were evaluated in HLM incubations by radioactivity profiling. The inhibition results generated from CYP3A4 inhibitors, ketoconazole and troleandomycin, are shown in Table 2 and Figure 3, respectively. Troleandomycin did not inhibit the formation of M5 but did inhibit overall metabolism of dasatinib in HLM by >70% and inhibited formation of M20 and M24 (Table 2). Ketoconazole inhibited the formation of M4, M5, M20, and M24 in the CYP3A4 incubation (Table 2). The LC/MS/MS analysis results showed that 1-aminobenzotriazole inhibited the formation of M4, M20 and M24 to a near background level, but did not inhibit the formation of M5 (Figures 5B-8B). Ketoconazole inhibited the formation of M20 and M24 in HLM incubations at 1 µM dasatinib (Figures 7B-8B). However, ketoconazole was less effective to inhibit the formation of M4, M20, and M24 in HLM incubations at 20 µM dasatinib (Figures 5B, 7B, and 8B). The inhibitors for other CYP enzymes did not significantly inhibit the formation of any of these primary oxidative metabolites. The CYP3A4 antibody inhibited the formation of M4, M20, and M24 by >50% at 1 or 20 µM dasatinib (Figures 5B, 7B, and 8B).
To provide additional evidence for the contribution of FMO3 to M5 formation, HLM and expressed FMO3 were heat-treated at 45°C for 5 min prior to incubation with [14C]dasatinib. These treatment conditions would be expected to inactivate the heat-sensitive FMO3 enzyme, but do not appreciably affect CYP enzyme activities (Tugnait et al., 1997). After heat-treatment, the formation activity of M5 was inactivated by 73% in HLM (Figure 3) and 95% in the expressed FMO3 (Table 2), respectively, while the CYP-catalyzed formation of M4, M20 and M24 remained intact (Figure 3).

Metabolite M6, the carboxylic acid metabolite of dasatinib was a minor metabolite in HLM incubation in the presence of NADPH (Figure 3), or human liver S9 incubation in the presence of NADH or NADPH (Figure 4). M6 was not detected in the incubations of dasatinib with human cDNA-expressed CYPs.

**Substrate concentration-dependent metabolite formation.** Inhibition was observed for the formation of M20 and M24 at higher substrate concentration in HLM incubations. Therefore, the equation of partial substrate inhibition: \( V = \frac{V_{max} \cdot S}{K_m + S \cdot (1 + S/K_i)} \) was fitted well to the experimental data for the formation of M20 and M24 by cDNA-expressed CYP3A4 or HLM. The formation of M4 and M5, on the other hand, fits very well to the Michaelis-Menten kinetic equation: \( V = \frac{V_{max} \cdot S}{K_m + S} \). The data fitting was statistically significant from a t test analysis with a \( p \) value of <0.05. Substrate concentration dependent formation of M4, M5, M20, and M24 in the incubations of dasatinib with HLM is illustrated in Figure 9 and similar profiles were observed for the formation of M4, M20, and M24 in cDNA-expressed CYP3A4. Table 3 summarizes the experimentally determined \( K_m \) and \( K_i \) values for the primary oxidative metabolites of dasatinib.
dasatinib in HLM and expressed CYP3A4. The $K_m$ value for the formation of M4 was approximately 224 and 111 $\mu$M in HLM and CYP3A4, respectively. The $K_m$ value for the formation of M5 was 79.3 $\mu$M in HLM. The $K_m$ values were relatively low for the formation of M20 and M24 in both HLM and CYP3A4 with all values falling within 1.8-10.5 $\mu$M. The $V_{\text{max}}$ values for M4, M5, M20, and M24 (Table 4) were calculated from the their formation rates from the incubations of HLM or CYP3A4 at three different concentrations (2, 10, and 20 $\mu$M) of $[^{14}\text{C}]$dasatinib and pre-determined $K_m$ values. The catalytic efficiencies ($V_{\text{max}}/K_m$) were approximately 52, 274, and 20 $\mu$L/mg protein/min (equivalent to 0.68, 3.6, and 0.26 $\mu$L/pmole/min, respectively, when an average CYP3A4 content of 76 pmole/mg HLM proteins were used) for the formation of M4, M20, and M24 in HLM, and 0.82, 4.8, and 0.4 $\mu$L/pmole CYP/min for formation of M4, M20, and M24 by CYP3A4, respectively. The catalytic efficiency values for HLM and expressed CYP3A4 are in good agreement when a value of 76-pmole CYP3A4/mg HLM protein is used (Rostami-Hodjegan and Tucker, 2007). The calculated $C_{\text{int}}$ ($V_{\text{max}}/K_m$) values suggest that CYP3A4 has a much higher catalytic efficiency for the formation of M20 relative to M4 or M24. This was confirmed in vivo, where M20 accounted for 31% of the dasatinib dose in humans while M4 and M24 each accounted for <4.2% of the dose.

**Prediction of the drug-drug interaction potential.** The predicted $AUC_{(i)}/AUC_{(c)}$ would be 3.5 in a clinical drug-drug interaction study by the CYP3A4 inhibitor ketoconazole (200 mg, BID); the clinical observation was 4.5.
DISCUSSION

Several analytical methods have been developed for CYP reaction phenotyping and for determining the enzyme kinetic parameters using recombinant CYPs or HLM. Radioactive drugs are often preferred because of sensitive detection and no need of metabolic reference standards for quantification of multiple metabolites. However, a complete reaction phenotyping study requires a large number of in vitro incubations and radioactive sample profiling and the total process are very time consuming (Zhao et al., 2008). LC/MS/MS quantification methods have also been used extensively in CYP phenotyping work, but synthetic metabolite standards are required for quantification. Very often metabolic reference standards are not available at the time of reaction phenotyping. Here, we report a combined approach comprised of both LC/MS/MS and radioactivity profiling analyses. In the LC/MS/MS method we used, stable isotope labeled dasatinib was added as an internal standard for each incubation. All relevant metabolites were analyzed by LC/MS/MS selective reaction monitoring (SRM) after chromatographic separation. The peak area ratio (metabolite to internal standard) was obtained for each metabolite. The peak area ratios for a given metabolite can be compared between samples, or in a particular set of samples such as HLM incubation within the same series of experiments. Although standard curves were not available in the quantification method we used, different volumes of the metabolite mixtures in incubations at low and high substrate concentrations were injected to ensure linearity in the LC/MS/MS response. The relative formation rate (V) (peak area ratio/min/pmol of CYP or mg protein of HLM) for each metabolite was calculated and the data was plotted against the substrate concentration and the $K_m$ values were obtained by fitting the plot to
the equation of partial substrate inhibition, $V = \frac{V_{\text{max}} \cdot S}{(K_m + S \cdot (1 + S/K_i))}$ or the Michaelis-Menten equation $V = \frac{V_{\text{max}} \cdot S}{K_m + S}$. 

[14C]Dasatinib was also incubated at three different concentrations and the radioactive profiling was completed to determine the formation rate ($V$) of each metabolite by radioactivity measurement. The $V_{\text{max}}$ values were then obtained by calculation using the above equations. Determining the $K_m$ and $V_{\text{max}}$ values for metabolism of dasatinib represents the first application example for the relative LC/MS/MS quantification method and for the combination approach to determine $K_m$ and to calculate $V_{\text{max}}$ values.

Identification of the enzymes involved in the oxidative metabolism of dasatinib was carried out with initial screening of metabolic turnover by cDNA-expressed enzymes followed by evaluation of the effects of selective antibodies and chemical inhibitors on metabolism of dasatinib in HLM. Upon initial screening with cDNA-expressed enzymes, multiple CYPs (CYP1A1, 1B1, 1A2, and 3A4/3A5) and FMO3 were found to be involved in the oxidation of dasatinib. CYP1A1, 1B1, and 3A4 were shown to catalyze the formation of M4 while CYP3A4 and 3A5 were the major enzymes catalyzing dasatinib hydroxylation (M20 and M24). CYP1A1 and CYP1B1 are mainly expressed in the extrahepatic tissues with low levels detected in human liver microsomes (Chang et al., 2003; Drahushuk et al., 1998; Shimada et al., 1996). Therefore, these CYP enzymes are not expected to play a significant role in the hepatic clearance of dasatinib in humans.

The cDNA-expressed CYP3A4 had a $K_m$ value of 111 \(\mu\text{M}\) for the formation of M4 and 224 \(\mu\text{M}\) in HLM. The low $K_m$ values of 2-8 and 6-10 \(\mu\text{M}\) for the formation of two major metabolites M20 and M24 in cDNA-expressed CYP3A4 and HLM suggest that dasatinib has a high binding affinity to CYP3A4. It is not known why the high $K_m$ reactions for M4
and M5 formation followed the simple Michaelis-Menten kinetic model while the low $K_m$ reactions for M20 and M24 formation followed a partial substrate inhibition kinetic model. The $Cl_{int}$ values ($V_{max}/K_m$) of CYP3A4 for the formation of M4, M20, and M24 were 0.68, 3.6, and 0.26 µL/min/pmole, respectively, suggesting that the formation of M20 in human liver was more efficient than that of M4 or M24. To better estimate the contribution of each enzyme to the overall metabolism of dasatinib, the enzyme activity of each CYP was normalized to relative formation per pmole of a specific CYP enzyme in HLM based on their abundance/concentration in the human liver microsomes (Rostami-Hodjegan and Tucker, 2007; Rodrigues, 1999; Shimada et al., 1994). The results indicate that CYP3A4 is the major enzyme responsible for formation of M4, M20, and M24 (Figures 5, 7, and 8).

1-Aminobenzotriazole (ABT) potently inhibited formation of M4, M20, and M24 by 100% in HLM incubations, but not M5 at 20 µM concentrations of dasatinib (Figures 5B-8B). Troleandomycin completely blocked the formation of M4 in HLM incubation (Figure 3). Antibodies of CYP3A4 also appreciably decreased the formation of M4, M20, and M24 in HLM incubations (Figures 5B, 7B, and 8B). Ketoconazole at 1 µM inhibited M20 and M24 formation at 1 µM of dasatinib, but had a limited effect on formation of M4, M20, and M24 in HLM at 20 µM of dasatinib (Figures 5B, 7B, and 8B). Although the reason for this discrepancy is not clear, a reasonable explanation is that the high substrate concentration along with a relative high binding affinity (low $K_m$ value) for CYP3A4, allowed dasatinib to compete effectively with ketoconazole. The ratio of dasatinib concentration to its binding affinity ($K_m$) to CYP3A4 (20 µM/1.8 µM = 11) was
comparable to that of ketoconazole concentration to its binding affinity ($K_i$) to CYP3A4 (1 µM/0.05-0.2 µM = 5-20). Overall, the data support the conclusion that the formation of M4, M20, and M24 was catalyzed by CYP3A4.

The formation of the $N$-oxide of dasatinib was evaluated in HLM and cDNA-expressed enzymes. Results generated from the incubations with expressed enzymes indicated that FMO3 was the most catalytically efficient enzyme for the formation of M5 and that CYP1A2, CYP1B1, CYP2C9, and CYP3A4 were also capable of catalyzing M5 formation (Figures 3, 6A). Heat treatment of HLM incubations at 45°C for 5 min significantly decreased the activity for M5 formation. The liability of the FMO enzyme towards mild heat treatment has been demonstrated previously (Kitchell et. al, 1978) and can be used as evidence to support the contribution of FMO to microsomal biotransformation. Additional results that confirmed the primary involvement of FMO3 included the lack of effect of 1-aminobenzotriazole on M5 formation in HLM incubations. The relatively high contents of FMO3 in HLM (Overby et al., 1997), the low formation rates of M5 by other cDNA-expressed CYP enzymes, and the results from inhibitory studies, all provide confirmatory evidence that FMO3 plays a primary role in the formation of M5.

Multiple enzymes seemed to be capable of forming M6 with evidence for involvement of CYP enzymes and cytosolic oxidoreductase (Table 2, Figures 3 and 4). There was significantly less M6 formed in the HLM incubation than in human liver S9. Human alcohol dehydrogenases (ADHs) have been demonstrated to catalyze the oxidation of primary alcohols of many drugs to carboxylic acids (Aasmoe et al., 1998; Walsh et al.,
2002). Aldehyde dehydrogenases (ALDHs), NAD-dependent enzymes, also catalyze the oxidation of a wide range of endogenous and exogenous aliphatic and aromatic aldehydes (Vasiliou et al., 2000; Vasiliou et al., 2004). It is not clear which enzyme is responsible for the cytosolic formation of M6.

The results from HLM incubations as well as the hepatocyte incubations and in vivo studies all support the conclusion that dasatinib is mainly metabolized by CYP3A4 in humans. The results from the human ADME study (Christopher et al., 2008b) along with the reaction phenotyping results presented in this study allow an estimation of 0.80 as the fraction of dose metabolized ($f_m$) by CYP3A4. These results predict that dasatinib would be susceptible to drug-drug interactions (DDIs) when it is co-administered with drugs that are CYP3A4 inhibitors or inducers (e.g., ketoconazole and rifampin). A quantitative prediction of in vivo DDIs due to metabolic inhibition can be made based on the inhibitor concentration in plasma ([I]), the in vitro inhibition constant ($K_i$), and the $f_m$ of the substrate (Ito et al., 1998). We predicted the $\frac{AUC_{(i)}}{AUC_{(c)}}$ ratio of dasatinib co-administered with ketoconazole based on plasma concentrations ([I]) of ketoconazole along with $K_i$ values obtained from published literature (Galetin et al., 2005). The predicted $\frac{AUC_{(i)}}{AUC_{(c)}}$ ratio (~3.5 fold) correlated well with the actual findings from the clinical studies, in which the exposure (AUC) of dasatinib increased about 4.5-fold when co-administered with ketoconazole. The significant fraction metabolized via CYP3A4 metabolism would also predict a substantial effect of enzyme inducers on dasatinib pharmacokinetics. Indeed, an approximate 80-90% decrease in dasatinib exposure was observed when co-administered with rifampin. These results confirmed the major role of CYP3A4 in the metabolic clearance of dasatinib.
In summary, the combination applications of LC/MS/MS and radioactivity analysis for metabolite detection and kinetic analysis of data used in this study provide an effective approach for determining metabolite formation kinetic parameters ($K_m$ and $V_{max}$ values). Incubations with human liver fractions generated all of the important metabolic clearance pathways of dasatinib. The studies with cDNA-expressed enzymes, CYP inhibitors (chemical and antibody), and kinetic analysis showed that dasatinib was predominately metabolized by CYP3A4.
REFERENCES


Footnote: ¹ Current affiliation: Department of Drug Metabolism, Merck and Co., Inc., West Point, Pennsylvania.
Legends for figures:

Figure 1. Chemical structures of dasatinib, $^{13}$C$_4,^{15}$N$_2$-dasatinib and metabolites M4, M5, M6, M20, and M24.

Figure 2. Ion chromatograms of dasatinib, internal standard, and metabolites for HLM incubation at 20 µM dasatinib in the presence of NADPH at 37°C for 30 min. The details of the incubation and sample analyses are described in the Materials and Methods section.

Figure 3. Typical HPLC-radiochromatographic profiles of 30-min incubations of $[^{14}$C]dasatinib (20 µM) with cDNA-expressed CYP1A1, 1A2, 1B1, 3A4, FMO3, HLM, HLM plus troleandomycin, or HLM with heat treatment at 45°C in the presence of NADPH. The details of the incubation and sample analyses are described in the Materials and Methods section.

Figure 4. Typical HPLC-radiochromatographic profiles of a 30-min incubation of $[^{14}$C]dasatinib (20 µM) with human liver S9, human liver S9 plus NADPH, or human liver S9 plus NADH. The details of the incubation and sample analyses are described in the Materials and Methods section.

Figure 5. A: Relative formation of M4 in incubations of dasatinib (20 µM) with HLM, cDNA-expressed CYPs, or FMO3. B: Relative formation of M4 in incubations of dasatinib (20 µM) with HLM plus selective CYP inhibitors.
The details of the incubation and sample analyses are described in the Materials and Methods section.

Figure 6. A: Relative formation of M5 in the incubations of dasatinib (20 µM) with HLM, cDNA-expressed CYPs, or FMO3. B: Relative formation of M5 in incubations of dasatinib (20 µM) with HLM plus selective CYP inhibitors. The details of the incubation and sample analyses are described in the Materials and Methods section.

Figure 7. A: Relative formation of M20 in incubations of dasatinib (20 µM) with HLM, cDNA-expressed CYPs, or FMO3. B: Relative formation of M20 in incubations of dasatinib (1 or 20 µM) with HLM plus selective CYP inhibitors. The details of the incubation and sample analyses are described in the Materials and Methods section.

Figure 8. A: Relative formation of M24 in incubations of dasatinib (20 µM) with HLM, cDNA-expressed CYPs, or FMO3. B: Relative formation of M24 in the incubations of dasatinib (1 or 20 µM) with HLM plus selective CYP inhibitors. The details of the incubation and sample analyses are described in the Materials and Methods section.
Figure 9. Dasatinib concentration-dependent relative metabolite formation in HLM incubations in the presence of NADPH. The relative formation of each metabolite was calculated by measuring the peak area ratio of the metabolite to the internal standard. The details of the incubation and sample analyses are described in the Materials and Methods section.
Table 1. Primary in vitro oxidative metabolites of \[^{14}\text{C}]\text{dasatinib}\ in humans

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>[M+H](^+) *</th>
<th>MW Change from parent</th>
<th>Biotransformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4</td>
<td>444</td>
<td>-44</td>
<td>N-Dealkylation</td>
</tr>
<tr>
<td>M5</td>
<td>504</td>
<td>+16</td>
<td>N-Oxidation</td>
</tr>
<tr>
<td>M6</td>
<td>502</td>
<td>+14</td>
<td>Oxidation of ethanol group to carboxylic acid</td>
</tr>
<tr>
<td>M20</td>
<td>504</td>
<td>+16</td>
<td>Hydroxylation at 4-position of phenyl ring</td>
</tr>
<tr>
<td>M24</td>
<td>504</td>
<td>+16</td>
<td>Hydroxylation on methyl group of chloromethylphenyl ring</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>488</td>
<td>0</td>
<td>Parent</td>
</tr>
</tbody>
</table>

* The analysis was by positive ESI LC/MS.
Table 2. Formation of the primary oxidative metabolites in the incubations of \([^{14}C]\)dasatinib with human cDNA-expressed enzymes, human liver microsomes and S9 (samples were analyzed by HPLC separation and radioactivity detection)

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>[(^{14}C)]Dasatinib Concentration ((\mu)M)</th>
<th>[(^{14}C)]Dasatinib Remaining (% total)</th>
<th>Formation of Metabolites (% total)</th>
<th>Totalb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>M4</td>
<td>M5</td>
</tr>
<tr>
<td>HLM</td>
<td>2</td>
<td>25</td>
<td>3.7</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>62</td>
<td>2.3</td>
<td>5.5</td>
</tr>
<tr>
<td>HLM + Troleandomycin</td>
<td>20</td>
<td>89</td>
<td>ND</td>
<td>6.8</td>
</tr>
<tr>
<td>HLM + Heatc</td>
<td>20</td>
<td>70.3</td>
<td>3.2</td>
<td>1.8</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>2</td>
<td>23</td>
<td>10.5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>80</td>
<td>9.5</td>
<td>2.5</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>2</td>
<td>96</td>
<td>ND</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>92</td>
<td>ND</td>
<td>5.4</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>2</td>
<td>26</td>
<td>7.6</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>80</td>
<td>7.6</td>
<td>7.3</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>2</td>
<td>87</td>
<td>2.3</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>92</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>2</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>19</td>
<td>3.6</td>
<td>1.8</td>
</tr>
<tr>
<td>CYP3A4 + Ketoconazole</td>
<td>2</td>
<td>79</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>91</td>
<td>0.8</td>
<td>1.1</td>
</tr>
<tr>
<td>FMO3 pH7.5</td>
<td>2</td>
<td>18</td>
<td>ND</td>
<td>76.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>51.5</td>
<td>ND</td>
<td>47.1</td>
</tr>
<tr>
<td>FMO3 + Heatc</td>
<td>20</td>
<td>96</td>
<td>ND</td>
<td>2.4</td>
</tr>
<tr>
<td>FMO pH 9.5</td>
<td>20</td>
<td>16</td>
<td>ND</td>
<td>82</td>
</tr>
<tr>
<td>S9 + NADPH</td>
<td>20</td>
<td>46</td>
<td>2.6</td>
<td>5.1</td>
</tr>
<tr>
<td>S9 + NADH</td>
<td>20</td>
<td>57</td>
<td>2.2</td>
<td>11.9</td>
</tr>
</tbody>
</table>

\(^{a}\) CYP2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 4A11 were also incubated with 2 and 20 \(\mu\)M of \([^{14}C]\)dasatinib. In these incubations, >95% of the parent drug remained and no metabolites were produced except M5, which represented ~0.4-1.3% of the radioactivity in each sample.

\(^{b}\) Some of the totals are less than 100% because metabolites other than the five primary oxidative metabolites were formed: e.g. the 2 \(\mu\)M incubations with CYP1A1 and 1B1 contained 14 and 18% of total radioactivity as metabolites that eluted before M20; the 2 \(\mu\)M and 20 \(\mu\)M incubations with CYP3A4
contained early eluting metabolites representing 91 and 19% of the total radioactivity; the incubations with HLM and S9 also contained additional metabolites. Presumably, these early eluting metabolites are secondary metabolites produced from further metabolism of the primary metabolites.

ND = metabolite not detected by radioactivity detection.
Table 3. The determined $K_m$ values for the formation of primary oxidative metabolites in the incubations of non-labeled dasatinib with HLM and human cDNA-expressed CYP3A4

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Metabolite</th>
<th>$K_m$ (µM) $^{a,b}$ (Mean ± SE)</th>
<th>Ki</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM</td>
<td>M4</td>
<td>173.2 ± 0.1</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>M5</td>
<td>79.3 ± 13.9</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>M20</td>
<td>1.8 ± 0.3</td>
<td>426</td>
</tr>
<tr>
<td></td>
<td>M24</td>
<td>8.2 ± 2.2</td>
<td>416</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>M4</td>
<td>111.1 ± 0.1</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>M20</td>
<td>6.0 ± 1.0</td>
<td>3209</td>
</tr>
<tr>
<td></td>
<td>M24</td>
<td>10.5 ± 2.6</td>
<td>1118</td>
</tr>
</tbody>
</table>

NA = not applicable.

$^a$Kinetic values were estimated from extrapolation of $v=V_{max}\times S/(K_m+S*(1+S/K_i))$ analysis for M20 and M24 or $v=V_{max}\times S/(K_m+S)$ analysis for M4 and M5.

$^b$The maximal peak area ratio of a metabolite to the internal standard (a relative $V_{max}$ value) was 0.046 ± 0.004, 0.021 ± 0.002, 0.41 ± 0.02, and 0.086 ± 0.010, respectively, for M4, M5, M20, and M24 in HLM and 0.046 ± 0.004, 1.94 ± 0.12, and 0.24 ± 0.03, respectively, for M4, M20, and M24 in expressed CYP3A4.
Table 4. The calculated $V_{\text{max}}$ values for the formation of primary oxidative metabolites in the incubation of $[^{14}\text{C}]$dasatinib with HLM and human cDNA-expressed CYP3A4

<table>
<thead>
<tr>
<th>Enzymes and metabolites</th>
<th>Determination of $V_{\text{max}}^{a,b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$2 \mu\text{M}$</td>
</tr>
<tr>
<td></td>
<td>$V$</td>
</tr>
<tr>
<td>HLM</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>15.7</td>
</tr>
<tr>
<td>M5</td>
<td>30.3</td>
</tr>
<tr>
<td>M20</td>
<td>212</td>
</tr>
<tr>
<td>M24</td>
<td>10.2</td>
</tr>
<tr>
<td>CYP3A4</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>0.27</td>
</tr>
<tr>
<td>M20</td>
<td>1.68</td>
</tr>
<tr>
<td>M24</td>
<td>0.30</td>
</tr>
</tbody>
</table>

$^a$The unit for $V$ and $V_{\text{max}}$ was pmole/mg protein /min for HLM and pmole/pmole CYP/min for CYP3A4. CYP3A4 content used was 76 pmole/mg in human liver microsomes (Rostami-Hodjegan and Tucker, 2007). The unit for $V_{\text{max}}/K_{\text{m}}$ was $\mu\text{L}/\text{min}$/pmole CYP3A4 for M4, M20, and M24.

$^b$The $K_{\text{m}}$ values used for calculations were from Table 3.

$^c$The unit of $V_{\text{max}}/K_{\text{m}}$ for M5 was $\mu\text{L}/\text{min}$/mg proteins for M5.
Figure 1.

Dasatinib, *denotes $^{14}$C label

$^{13}$C$_4$,$^{15}$N$_2$-Dasatinib

M4

M5

M6

M20

M24
Figure 2.
Figure 3

- **HLM**
  - M20
  - M24
  - M4
  - M6
  - M5

- **1A1**
  - M4
  - M5

- **1A2**
  - M5

- **1B1**
  - M4
  - M5

- **3A4**
  - M20
  - M24
  - Secondary metabolites

- **FMO3**
  - M5

- **HLM, pre-treated @ 45°C (5 min)**
  - M20
  - M24
  - M4
  - M6

- **HLM and troleandomycin (20 µM)**
  - M5
Figure 4.
Figure 5.

A. Relative formation of Metabolite M4 at 20 µM.

B. Relative formation of Metabolite M4 at 20 µM.
Figure 6.

A

Relative formation of Metabolite M6

20 µM

B

Relative formation of Metabolite M6

20 µM

HLM control
CYP1A1
CYP1A2
CYP1B1
CYP2A6
CYP2B6
CYP2C8
CYP2C9
CYP2C19
CYP2D6
CYP2E1
CYP3A4
CYP3A5
CYP4A11
FMO3 pH7.5
FMO3 pH9.5

Tranylcypromine (CYP2A6)
Orphenadrine (CYP2B6)
Sulfaphenazole (CYP2C9)
Benzylnirvanol (CYP2C19)
Quinidine (CYP2D6)
Diethyldithiocarbamate (CYP2E1)
Ketoconazole (CYP3A4)
ABT (all CYPs)
Figure 7.

A

HLM control  CYP1A1  CYP1A2  CYP1B1  CYP2A6  CYP2B6  CYP2C8  CYP2C9  CYP2C19  CYP2D6  CYP2E1  CYP3A4  CYP3A5  CYP4A11  FMO3 pH7.5  FMO pH9.5

0  20  40  60  80  100  120  140

Relative formation of Metabolite M20

20 µM

B

HLM control  Furafylline (CYP1A2)  Omeprazole (CYP2C19)  Sulfaphenazole (CYP2C9)  Tranylcypromine (CYP2A6)  Benzylimidazole (CYP2B6)  Diethylldithiocarbamate (CYP2E1)  Ketoconazole (CYP3A4)  Anti-CYP2C8  Anti-CYP2D6  Anti-CYP2E1  Anti-CYP3A4  Anti-CYP4A11

1µM  20 µM

0  50  100  150  200
Figure 8.
Figure 9

Dasatinib concentration (μM)

Relative formation of metabolites in human liver microsomes

M4

M5

M20

M24

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