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, and Donglu Zhang

Pharmaceutical Candidate Optimization, Bristol-Myers Squibb Research and Development, Princeton, New Jersey

Received December 19, 2007; accepted June 9, 2008

ABSTRACT:

Dasatinib (Sprycel, BMS-354825; Bristol-Myers Squibb, Princeton, NJ) (Fig. 1) is marketed as an oral inhibitor of BCR-ABL and SRC family kinases for the treatment of chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia in adults who are resistant to Gleevec (imatinib mesylate, Novartis, Basel, Switzerland). Studies have shown that dasatinib was extensively metabolized in animals and humans (Christopher et al., 2008a,b). 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 absorption, distribution, metabolism, and excretion (ADME) study with [14C]dasatinib, the fraction clearance to form 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 $K_m$ values were determined by the kinetic analysis of the substrate-dependent metabolite formation plots from a large number of incubations with the nonlabeled dasatinib; the $V_{\text{max}}$ values were calculated with the predetermined $K_m$ values and the metabolite formation rates from a limited number of incubations with [14C]dasatinib. The intrinsic formation clearance values ($V_{\text{max}}/K_m$) 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 showed that dasatinib was predominately metabolized by CYP3A4.

Related secondary metabolites represented approximately 81% of total dasatinib clearance (Christopher et al., 2008b).

Human liver cytochrome P450 (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 (DDIs). Three families of P450 enzymes (CYP1, CYP2, and CYP3) are involved in the metabolism of xenobiotics in humans, and CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5 are responsible for the metabolism of the majority of drugs (Nebert and Russell, 2002). In human livers, flavin-containing monooxygenase enzymes (FMO1, FMO3, and FMO5) are also important enzymes for metabolism of xenobiotics (Cashman, 1995; Ring et al., 1999; Parte and Kupfer, 2005). Human alcohol dehydrogenases catalyze the oxidation of primary alcohols of drug (Walsh et al., 2002). Aldehyde dehydrogenases, NAD-dependent enzymes, also catalyze the oxidation of a wide range of endogenous and exogenous aliphatic and aromatic aldehydes (Vasiiliou and Pappa, 2000; Vasiiliou et al., 2004). The objective of this study was to identify the human enzymes responsible for the formation of primary oxidative metabolites of related secondary metabolites.

ABBREVIATIONS: dasatinib, N-(2-chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)-1-piperazinyl]-2-methyl-4-pyrimidinyl]amino]-5-thiazolocarboxamide; ADME, absorption, distribution, metabolism, and excretion; P450, cytochrome P450; DDIs, drug-drug interaction; FMO, flavin-containing monooxygenase; HLM, human liver microsome; IS, internal standard; ABT, 1-aminobenzotriazole; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; MS/MS, tandem mass spectrometry; SRM, selective reaction monitoring; AUC, area under the curve.
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 P450 inhibitors or specific inhibitory antibodies in human liver microsome (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 P450s. The potential of DDI of dasatinib with CYP3A4 inhibitors or inducers was estimated.

Materials and Methods

Materials. Dasatinib (purity 96.2%) (Fig. 1), [14C]dasatinib (specific activity 30.4 Ci/mg, radiochemical purity 99.2%, and chemical purity 98.8%), and stable isotope-labeled dasatinib prepared from (1,2,3-13C3) diethyl malonate and [13C,15N2]thiourea [internal standard (IS), purity 99.5%] were synthesized at Bristol-Myers Squibb. Standards of M4, M5, M6, M20, and M24 (Fig. 1) were also prepared at Bristol-Myers Squibb. All the P450 inhibitors [furafylline, tranylcypromine, orphenadrine, quercetin, sulfaphenazole, benzyl nirvanol, quinidine, diethyldithiocarbamate, ketoconazole, 1-aminobenzotriazole (ABT), and troleandomycin], -NADPH, and -NADH were obtained from Sigma-Aldrich Co. (St. Louis, MO). Quercetin and diethyldithiocarbamate 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 high-performance liquid chromatography (HPLC) grade were from Burdick and Jackson Inc. (Muskegon, MI). Pooled HLMs (20 subjects), human cDNA-expressed P450 enzymes (in baculovirus-insect cells, CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, and CYP4A11), human flavin-containing monoxygenase FMO3 (expressed in baculovirus insect cells), and monoclonal anti-P450 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 the chemical inhibitors were prepared in acetonitrile. Nonradiolabeled dasatinib stock solutions for substrate-dependent metabolite formation studies were prepared in methanol and dimethylsulfoxide (1:1, v/v).

Incubations with cDNA-Expressed Enzymes, HLMs, and Human Liver S9. Human cDNA-expressed P450s (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, and CYP4A11) and HLM were incubated with [14C]dasatinib or nonlabeled dasatinib. The incubation mixtures (0.5 ml) contained Tris-HCl buffer (0.05 M, pH 7.5), P450 (50 pmol) or HLM (0.5 mg protein), [14C]dasatinib (2 or 20 µM), and NADPH (1.2 mM). The FMO3 incubations were similar to P450 incubations except cDNA-expressed enzymes in baculovirus-insect cells with [14C]dasatinib or nonlabeled dasatinib. The incubation mixtures (0.5 ml) contained Tris-HCl buffer (0.05 M, pH 7.5), P450 (50 pmol) or HLM (0.5 mg protein), [14C]dasatinib (2 or 20 µM), and NADPH (1.2 mM). The final incubation 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 IS (stable isotope-labeled dasatinib) was added to each sample to a final concentration of 1 µM. After centrifugation at 2000 g for 10 min, an aliquot of 50 µl of supernatant was used for liquid chromatography/mass spectrometry (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 P450 incubations except cDNA-
expressed FM03 (325 pmol) was used. Because the standard activity of FM03 was reported at pH 9.5 by the manufacturer, FM03 was also incubated in a glycine buffer (50 mM), pH 9.5, with 20 μM [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 FM03. HLM and cDNA-expressed FM03 in Tris-HCl buffer (0.05 M, pH 7.5) were preincubated 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 of 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 P450 Inhibitors.** The incubation mixtures (1 ml in duplicate) contained phosphate buffer (0.1 M, pH 7.4), HLM (0.3 mg), [14C]dasatinib or nonlabeled dasatinib (10 or 20 μM), NADPH (1 mM), and a single P450 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, benzylxirinanol (1 μM) for CYP2C19, quinidine (1 μM) for CYP2D6, diethylthiocarbamate (50 μM) for CYP2E1, ketocoxazone (1 μM) for CYP3A4, troxaloxymcin (20 μM) for CYP3A4, and ABT (1 mM) for all the P450s. Metabolism-dependent inhibitors furafylline, orphenadrine, troxaloxymcin, and ABT were preincubated 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 addition, the samples were then incubated at 37°C for 20 min with shaking.

**Dasatinib Concentration-Dependent Metabolite Formation.** To determine linear conditions of dasatinib metabolism, the following incubations were conducted: dasatinib at 10 μM incubated with 10, 30, 50, and 80 pmol/ml of CYP3A4 or 100, 200, 300, and 500 pmol/ml of HLM for 20 min, and dasatinib at 10 μM incubated with 10, 20, 30, and 50 min with CYP3A4 at 300 pmol/ml or HLM at 300 μg/ml. Incubations with 20 pmol/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 enzymic kinetics, the incubation mixtures (1 ml) contained phosphate buffer (0.1 M, pH 7.4), NADPH (1 mM), dasatinib (10 or 20 μM), and preincubated HLM mixture (50 μl, 300 μg of protein).

**Dasatinib Elimination.** To determine the elimination of dasatinib, the incubations were conducted at 37°C for 10 min in duplicate for each concentration. The final concentrations of dimethyldisulfide and methanol in all the incubations with nonradioabeled dasatinib were each kept at 0.25% (v/v). The relative formation rate (peak area ratio/min/mg protein of HLM) with respect to the corresponding specific content of 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 IS. Peak area ratios in each incubation were compared with the HLM control, and the values were then calculated based on the peak area ratio of the metabolite to the IS. Peak area ratios in each incubation were compared with 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 IS in HLM was defined as 100%. For all the incubations with expressed P450s, the metabolite formation rates (in peak area ratio/min/mg protein of HLM) with respect to the corresponding specific content of each P450 in HLM (Shimada et al., 1994; Rostami-Hodjegan and Tucker, 2007). The normalized formation rate for each P450 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 an 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 Quantum mass spectrometer. Therefore, samples from these incubations were also analyzed by an LTQ (ion trap) mass spectrometer (ThermoFinnigan) to verify the presence of M6 in the samples.

**Prediction of DDI Potential.** The extent of a potential DDI between dasatinib and ketconazole (as a prototypical strong CYP3A4 inhibitor) was predicted as follows:

\[
\text{AUC}_{i} = \frac{1}{V_{max}} \left( S_{i} [K_{i} + S] \right) + \left( 1 - S \right) \cdot f_{m, \text{CYP3A4}} \\
\text{AUC}_{c} = \frac{f_{m, \text{CYP3A4}}}{C_{\text{max}}} + \left( 1 - f_{m, \text{CYP3A4}} \right)
\]

(Ito et al., 1998)

The value for the fraction of the dose metabolized was \( f_{m} = 0.81 \), and the fraction the metabolized dose that was catalyzed by CYP3A4 was...
These values were estimated from results of a human ADME study with [14C]dasatinib (Christopher et al., 2008b) and current study. For inhibition by ketoconazole, \( \text{Cl}_{\text{int(i)}} = \frac{\text{V}_{\text{max}}}{K_I} \), where \( K_I = 0.2 \) \( \mu \)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 \( C_{\text{max}} \) value of 8.27 \( \mu \)M after administration of 200 mg of ketoconazole was used for the prediction. The mean observed area under the curve (AUC) for dasatinib was determined in a clinical drug interaction study between dasatinib (20 mg) and ketoconazole (200 mg every 12 h).
Formation of the primary oxidative metabolites in the incubations of [14C]dasatinib with human cDNA-expressed enzymes, HLMs, and S9 (samples were analyzed by HPLC separation and radioactivity detection)

<table>
<thead>
<tr>
<th>Enzymes*</th>
<th>[14C]Dasatinib Concentration</th>
<th>[14C]Dasatinib Remaining</th>
<th>Formation of Metabolites</th>
<th>Totalb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>% total</td>
<td>µM</td>
<td>% total</td>
</tr>
<tr>
<td>HLM</td>
<td>2</td>
<td>62</td>
<td>3.7</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>89</td>
<td>2.3</td>
<td>5.5</td>
</tr>
<tr>
<td>HLM + troleandomycin</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>N.D.</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>92</td>
<td>N.D.</td>
<td>5.4</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>2</td>
<td>26</td>
<td>7.8</td>
<td>25.9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>80</td>
<td>7.6</td>
<td>7.3</td>
</tr>
<tr>
<td>CYP3A4</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>N.D.</td>
<td>N.D.</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, pH 7.5</td>
<td>2</td>
<td>18</td>
<td>N.D.</td>
<td>76.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>51.5</td>
<td>N.D.</td>
<td>47.1</td>
</tr>
<tr>
<td>FMO3 + heatc</td>
<td>20</td>
<td>96</td>
<td>N.D.</td>
<td>24.4</td>
</tr>
<tr>
<td>FMO, pH 9.5</td>
<td>20</td>
<td>16</td>
<td>N.D.</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>

N.D., metabolite not detected by radioactivity detection.

*a CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP4A11 were also incubated with 2 and 20 µM [14C]dasatinib. In these incubations, >95% of the parent drug remained, and no metabolites were produced except M5, which represented ~0.4 to 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 µM incubations with CYP1A1 and CYP1B1 contained 14 and 18% of total radioactivity as metabolites that eluted before M20; the 2 and 20 µM 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.

*c 45°C for 5 min.

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. 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 with 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 because of 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 [14C]dasatinib (2 or 20 µM) with expressed enzymes, HLM, and human liver S9. Figures 3 and 4 show the metabolite profiles of [14C]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; Figs. 3 and 4). M5 was also formed in incubations with CYP1B1 and CYP1A2 (Table 2; Fig. 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 with 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 µM [14C]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 (Fig. 3) and human liver S9 incubations at 20 µM dasatinib (Fig. 4).

The results of LC/MS/MS analyses of the incubations of 20 µM [14C]dasatinib with HLM, human cDNA-expressed P450s, and FMO3 are summarized in Figs. 5A through 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, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A5, and CYP4A11 did not metabolize [14C]dasatinib to any significant extent (Figs. 5A through 8A). The results from LC/MS/MS analyses are in good agreement with the results from HPLC radiochromatographic analysis shown in Table 2.
FIG. 3. Typical HPLC-radiochromatographic profiles of 30-min incubations of [¹⁴C]-
dasatinib (20 μM) with cDNA-expressed
CYP1A1, CYP1A2, CYP1B1, CYP3A4,
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 de-
scribed under Materials and Methods.
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 Fig. 3, respectively. Troleandomycin did not inhibit the formation of M5 but did inhibit overall metabolism of dasatinib in HLM by $\approx 70\%$ 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 ABT inhibited the formation of M4, M20, and M24 to a near background level but did not inhibit the formation of M5 (Figs. 5B–8B). Ketoconazole inhibited the formation of M20 and M24 in HLM incubations at 1 $\mu$M dasatinib (Figs. 7B and 8B). However, ketoconazole was less effective to inhibit the formation of M4, M20, and M24 in HLM incubations at 20 $\mu$M dasatinib (Figs. 5B, 7B, and 8B). The inhibitors for other P450 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 $\approx 50\%$ at 1 or 20 $\mu$M dasatinib (Figs. 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 before incubation with [14C]dasatinib. These treatment conditions would be expected to inactivate the heat-sensitive FMO3 en-
zyme but do not appreciably affect P450 enzyme activities (Tugnait et al., 1997). After heat treatment, the formation activity of M5 was inactivated by 73% in HLM (Fig. 3) and 95% in the expressed FMO3 (Table 2), respectively, whereas the P450-catalyzed formation of M4, M20, and M24 remained intact (Fig. 3).

Metabolite M6, the carboxylic acid metabolite of dasatinib, was a minor metabolite in HLM incubation in the presence of NADPH (Fig. 3) or human liver S9 incubation in the presence of NADH or NADPH (Fig. 4). M6 was not detected in the incubations of dasatinib with human cDNA-expressed P450s.

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: 

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

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: 

\[ \frac{V}{V_{\text{max}}} = \frac{S}{K_m + S} \]

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 Fig. 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 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 the values falling within 1.8 to 10.5 \( \mu M \). The \( V_{\text{max}} \) values for M4, M5, M20, and M24 (Table 4) were calculated from the formation rates from the incubations of HLM or CYP3A4 at three different concentrations (2,
10, and 20 µM of [14C]dasatinib and predetermined \( K_m \) values. The catalytic efficiencies \( (V_{\text{max}}/K_m) \) were approximately 52, 274, and 20 \( l/mg \text{ protein/min} \) (equivalent to 0.68, 3.6, and 0.26 \( l/pmol/min \), respectively, when an average CYP3A4 content of 76 pmol/mg HLM proteins were used) for the formation of M4, M20, and M24 in HLM and 0.82, 4.8, and 0.4 \( l/pmol \text{ P450/min} \) 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-pmol CYP3A4/mg HLM protein is used (Rostami-Hodjegan and Tucker, 2007). The calculated Clint \( (V_{\text{max}}/K_m) \) 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, whereas M4 and M24 each accounted for <4.2\% of the dose (Christopher et al., 2008b).

**Prediction of the DDI Potential.** The predicted \( \text{AUC}(i)/\text{AUC}(c) \) would be 3.5 in a clinical DDI study by the CYP3A4 inhibitor ketoconazole (200 mg, b.i.d.); the clinical observation was 4.5.

**Discussion**

Several analytical methods have been developed for P450 reaction phenotyping and for determining the enzyme kinetic parameters using recombinant P450s 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 is very time-consuming (Zhao et al., 2008). LC/MS/MS quantification...
methods have also been used extensively in P450 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 comprising both LC/MS/MS and radioactivity profiling analyses. In the LC/MS/MS method we used, stable isotope-labeled dasatinib was added as an IS for each incubation. All the relevant metabolites were analyzed by LC/MS/MS SRM after chromatographic separation. The peak area ratio (metabolite to IS) 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 P450 or mg protein of HLM) for each metabolite was calculated; the data were plotted against the substrate concentration; and the \( K_m \) values were obtained by fitting the plot to the equation of partial substrate inhibition:

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

[\(14C\)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

---

**FIG. 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 IS. The details of the incubation and sample analyses are described under Materials and Methods.

**TABLE 3**

The determined \( K_m \) values for the formation of primary oxidative metabolites in the incubations of nonlabeled dasatinib with HLM and human cDNA-expressed CYP3A4

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Metabolite</th>
<th>( K_m ) a,b (( \mu M ))</th>
<th>( K_i ) (( \mu M ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM</td>
<td>M4</td>
<td>173.2 ± 0.1</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>M5</td>
<td>79.3 ± 13.9</td>
<td>N.A.</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>N.A.</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>

N.A., not applicable.

a Kinetic values were estimated from extrapolation of \( V = V_{\text{max}} \cdot \frac{S}{(K_m + S)} \cdot (1 + S/K_i) \) analysis for M20 and M24 or \( V = V_{\text{max}} \cdot \frac{S}{S + K_m + S} \) analysis for M4 and M5.

b The maximal peak area ratio of a metabolite to the internal standard (a relative \( V_{\text{max}} \) 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.

---
of dasatinib was carried out with initial screening of metabolic turn-over by cDNA-expressed enzymes followed by evaluation of the effects of selective antibodies and chemical inhibitors on metabolism of dasatinib in HLM. On initial screening with cDNA-expressed enzymes, multiple P450s (CYP1A1, CYP1B1, CYP1A2, and CYP3A4/3A5) and FMO3 were found to be involved in the oxidation of dasatinib. CYP1A1, CYP1B1, and CYP3A4 were shown to catalyze the formation of M4, whereas CYP3A4 and CYP3A5 were the major enzymes catalyzing dasatinib hydroxylation (M20 and M24). CYP1A1 and CYP1B1 are mainly expressed in the extrathoracic tissues with low levels detected in HLMs (Shimada et al., 1996; Drahushuk et al., 1998; Chang et al., 2003). Therefore, these P450 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 μM for the formation of M4 and 224 μM in HLM. The low $K_m$ values of 2 to 8 and 10 to 10 μM 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, whereas 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/pmol, 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 P450 was normalized to relative formation per picomole of a specific P450 enzyme in HLM based on their abundance/concentration in the HLMs (Shimada et al., 1994; Rodrigues, 1999; Rostami-Hodjegan and Tucker, 2007). The results indicate that CYP3A4 is the major enzyme responsible for formation of M4, M20, and M24 (Figs. 5, 7, and 8).

ABT potently inhibited formation of M4, M20, and M24 by 100% in HLM incubations but not M5 at 20 μM concentrations of dasatinib (Figs. 5B–8B). Troleandomycin completely blocked the formation of M4 in HLM incubation (Fig. 3). Antibodies of CYP3A4 also appreciably decreased the formation of M4, M20, and M24 in HLM incubations (Figs. 5B, 7B, and 8B). Ketoconazole at 1 μM inhibited M20 and M24 formation at 1 μM dasatinib but had a limited effect on formation of M4, M20, and M24 in HLM at 20 μM dasatinib (Figs. 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 (20/1.8 μM = 11) was comparable with that of ketoconazole concentration to its binding affinity ($K_i$) to CYP3A4 (1/0.05–0.2 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 (Figs. 3 and 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 toward mild heat treatment has been shown 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 ABT 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 P450 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 P450 enzymes and cytosolic oxidoreductase (Table 2; Figs. 3 and 4). There was significantly less M6 formed in the HLM incubation than in human liver S9. Human alcohol dehydrogenases have been shown to catalyze the oxidation of primary alcohols of many drugs to carboxylic acids (Asmoe et al., 1998; Walsh et al., 2002). Aldehyde dehydrogenases, NAD-dependent enzymes, also catalyze the oxidation of a wide range of endogenous and exogenous aliphatic and aromatic aldehydes (Vasiliou and Pappa, 2000; Vasiliou et al., 2004). It is not clear which enzyme is responsible for the cytosolic formation of M6.

The results from HLM incubations and 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 ($f_{met}$) by CYP3A4. These results predict that dasatinib would be susceptible to DDIs when it is coadministered with drugs that are CYP3A4 inhibitors or inducers (e.g., ketoconazole and rifampin). A quantitative prediction of in vivo DDIs caused by metabolic inhibition can be made based on the inhibitor concentration in plasma ($[I]$), the in vitro inhibition constant ($K_i$), and the $f_{met}$ of the substrate (Ito et al., 1998). We predicted the $AUC_{i}/AUC_{(c)}$ ratio of dasatinib coadministered with ketoconazole based on

### TABLE 4

<table>
<thead>
<tr>
<th>Enzymes and Metabolites</th>
<th>2 μM</th>
<th>10 μM</th>
<th>20 μM</th>
<th>Mean $V_{max}$</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V$</td>
<td>$V_{max}$</td>
<td>$V$</td>
<td>$V_{max}$</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td>HLM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>15.7</td>
<td>1175</td>
<td>129</td>
<td>14550</td>
<td>165</td>
</tr>
<tr>
<td>M5</td>
<td>30.3</td>
<td>1231</td>
<td>132</td>
<td>1178</td>
<td>194</td>
</tr>
<tr>
<td>M20</td>
<td>212</td>
<td>407</td>
<td>287</td>
<td>553</td>
<td>288</td>
</tr>
<tr>
<td>M24</td>
<td>10.2</td>
<td>53</td>
<td>30.3</td>
<td>158</td>
<td>52.2</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>0.27</td>
<td>16</td>
<td>1.96</td>
<td>111</td>
<td>2.65</td>
</tr>
<tr>
<td>M4</td>
<td>1.68</td>
<td>11</td>
<td>9.25</td>
<td>37</td>
<td>9.69</td>
</tr>
<tr>
<td>M20</td>
<td>0.30</td>
<td>1.9</td>
<td>0.59</td>
<td>3.7</td>
<td>1.05</td>
</tr>
<tr>
<td>M24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The unit for $V$ and $V_{max}$ was pmol/mg protein/min for HLM and pmol/pmol CYP3A4 for M4, M20, and M24.

$^b$ The $K_m$ values used for calculations were from Table 3.

$^c$ The unit of $V_{max}/K_m$ for M5 was μl/min/mg proteins for M5.
plasma concentrations (II) of ketoconazole along with \( K_v \) values obtained from published literature (Galetin et al., 2005). The predicted \( \text{AUC}_{0\rightarrow\infty}/\text{AUC}_{0\rightarrow\infty} \) 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 coadministered 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 to 90% decrease in dasatinib exposure was observed when coadministered with rifampin. These results confirmed the major role of CYP3A4 in the metabolic clearance of dasatinib.

In summary, the combined application of LC/MS/MS and radioactivity analysis for metabolite detection and kinetic analysis of data used in this study provided an effective approach for determining metabolite formation kinetic parameters (\( K_m \) and \( V_{max} \) values). Incubations with human liver fractions generated all the important metabolic clearance pathways of dasatinib. The studies with cDNA-expressed enzymes, P450 inhibitors (chemical and antibody), and kinetic analysis showed that dasatinib was predominately metabolized by CYP3A4.

References


Address correspondence to: Dr. Dongliu Zhang, Pharmaceutical Candidate Optimization, Bristol-Myers Squibb, P.O. Box 4000, Princeton, NJ 08543. E-mail: dongliu.zhang@bms.com

Downloaded from dmd.aspetjournals.org at ASPET Journals on July 7, 2017