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ORIGINAL ARTICLE

Preclinical pharmacokinetics and in vitro metabolism of dasatinib (BMS-354825): a potent oral multi-targeted kinase inhibitor against SRC and BCR-ABL

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Abstract
Purpose Dasatinib (BMS-354825), a potent oral multi-targeted kinase inhibitor against SRC and BCR-ABL, has recently been approved for the treatment of chronic myelogenous leukaemia (CML) in imatinib-acquired resistance and intolerance. In vitro and in vivo studies were conducted to characterize the pharmacokinetics and metabolism of dasatinib in mouse, rat, dog, and monkey. Possible mechanisms contributing to the incomplete oral bioavailability of dasatinib in animals were investigated.
Methods Metabolic stability of dasatinib was measured after incubation with liver microsomes (either NADPH- or UDPGA-fortified) and isolated hepatocytes obtained from mouse, rat, dog, monkey, and human. In all cases, substrate depletion over time was measured, and appropriate scaling factors were used to predict in vivo clearance. Pharmacokinetics of dasatinib were determined in mice, rats, dogs, and monkeys after administration of single intravenous or oral doses. In addition, the routes of excretion were investigated after administration of dasatinib to bile duct cannulated (BDC) rats. Absorption and first-pass metabolism were evaluated as possible reasons for the incomplete oral bioavailability using various in vitro and in vivo models like Caco-2 cells, P-glycoprotein (P-gp) knockout mice, and intra-portal dosing in rats.
Results In vivo systemic plasma clearance values of dasatinib were 62, 26, 25, and 34 ml/min/kg in mouse, rat, dog, and monkey, respectively. Scaling of in vitro hepatocyte and liver microsomal data gave reasonably good predictions of in vivo clearances across all species. Percent distribution in blood cells ranged from 43% in mouse to 57% in dog. Dasatinib showed high volumes of distribution (>3 l/kg) and high serum protein binding values (>90%) in all four species tested. Oral bioavailability of dasatinib ranged from 14% in the mouse to 34% in the dog. In rats, bioavailability after an intra-portal dose was comparable to that after intra-arterial administration. In BDC rats, less than 15% of an intravenous dose was excreted unchanged in urine, bile, and the gastrointestinal tract, suggesting that dasatinib is cleared primarily via metabolism. Dasatinib has high intrinsic permeability in Caco-2 cells, however, the efflux ratio was approximately two-fold indicating that it may be a substrate for an intestinal efflux transporter. However, in vivo studies in P-gp knockout mice versus wild-type mice showed no difference in the amount of dasatinib remaining unabsorbed in the gastrointestinal tract, suggesting that P-gp may not be responsible for the incomplete bioavailability.
Conclusions Dasatinib shows intermediate clearance in mouse, rat, dog, and monkey, and distributes extensively in those species. Oxidative metabolism appears to be the

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predominant clearance pathway. The incomplete oral bioavailability may be due to both incomplete absorption and high first-pass metabolism. However, the efflux transporter, P-glycoprotein does not appear to be limiting oral absorption.

Keywords SPRYCEL · Pharmacokinetics · Bioavailability · Metabolism · Allometric scaling

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder that is caused by the BCR-ABL oncogene [1, 2]. The current frontline therapy for CML is imatinib (Gleevec™), an orally available inhibitor of BCR-ABL [3]. However, acquired resistance to imatinib is a growing problem, mostly due to point mutations in BCR-ABL [4]. Dasatinib (BMS-354825, Fig. 1) is a novel, orally available multi-targeted kinase inhibitor against BCR-ABL that is several fold more potent than imatinib, and also potently inhibits imatinib-resistant BCR-ABL mutants [5–7]. It has been shown to be active in imatinib-resistant Philadelphia chromosome-positive leukemias [8]. Dasatinib also potently inhibits SRC kinase, a receptor tyrosine kinase that plays a critical role in the development, growth, progression, and metastasis of a number of human cancers [5, 9]. Dasatinib has recently been approved for the treatment of CML in imatinib-acquired resistance.

In this report, we describe the evaluation of dasatinib in various preclinical species. In vitro and in vivo studies were conducted to characterize the pharmacokinetics, oral bioavailability, and metabolism of dasatinib in mouse, rat, dog, and monkey. The routes of excretion of dasatinib were investigated in bile duct cannulated (BDC) rats. Absorption and first-pass metabolism were evaluated as possible reasons for the incomplete oral bioavailability observed in the different species, using various in vitro and in vivo models like Caco-2 cells, P-glycoprotein (P-gp) knockout mice, and intra-portal dosing in rats.

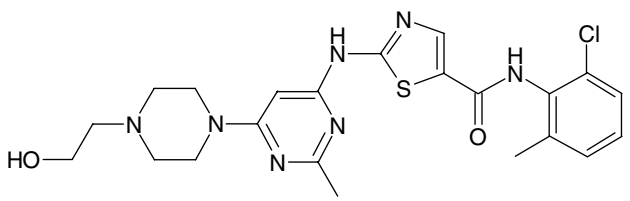

Fig. 1 Chemical structure of dasatinib

Materials and methods

Chemicals

Dasatinib was synthesized in the Chemistry Department at Bristol-Myers Squibb Pharmaceutical Research Institute. Hank's balanced salt solution (HBSS) and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals used were reagent grade or better.

Incubations with NADPH- or UDPGA-fortified liver microsomes

The in vitro oxidative metabolism of dasatinib was investigated in mouse, rat, dog, monkey, and human NADPH-fortified liver microsomes. The liver microsomes were purchased from In Vitro Technologies (Baltimore, MD, USA). The rates of oxidative metabolism were measured in duplicate under the following conditions: dasatinib, 3 μM final concentration (20 mM stock solution made in DMSO, final incubation solution contains 0.015% DMSO and 0.985% acetonitrile); final protein concentration, 1 mg/ml; NADPH, 1 mM; pH 7.4 sodium phosphate buffer, 100 mM; magnesium chloride, 6.7 mM. Incubations were conducted at 37°C for 10 min and were initiated by the addition of NADPH. Aliquots of the incubation were quenched at 0 and 10 min by addition of two volumes of acetonitrile. Samples were analyzed using an LC/MS assay and the percent metabolized was calculated based on the disappearance of the parent compound.

Dasatinib was also incubated with mouse, rat, dog, monkey, and human liver microsomes along with cofactors for glucuronidation. The experiment was conducted in duplicate under the following conditions: dasatinib, 10 μM final concentration (2 mM stock solution made in acetonitrile); final protein concentration, 1 mg/ml; UDPGA, 30 mM; pH 7.5 tris buffer, 100 mM, magnesium chloride, 10 mM, alamethicine, 2.5 mg/ml. Incubations were conducted at 37°C for 60 min and were initiated by the addition of UDPGA. Aliquots of incubation were quenched at different time points by addition of one volume of acetonitrile. Samples were analyzed using HPLC with UV detection and the percent glucuronidated was calculated based on the disappearance of the parent compound.

Incubations with hepatocytes

The metabolic stability of dasatinib was evaluated in suspensions of hepatocytes isolated from mouse, rat, dog, monkey, and human. Mouse and rat hepatocytes were prepared in-house as cell suspensions based on a literature protocol [10]. The freshly isolated rat and mouse

hepatocytes were subjected to Percoll purification by centrifuging a mixture of cell suspension (12.5 ml) and Percoll solution (12.5 ml) at 50×*g* for 5 min at 4°C. After a further wash in suspension buffer (Krebs-Henseleit buffer, pH 7.4), the cells were resuspended in the incubation buffer (Krebs-Henseleit buffer fortified with glucose). Fresh dog and monkey hepatocytes were purchased from CellzDirect corporation (formerly, CEDRA, Austin, TX, USA). Cryopreserved human hepatocytes were obtained from In Vitro Technologies. The gel-entrapped dog, monkey and human hepatocytes were processed in a stepwise manner, according to the instructions provided by the vendors, and washed in suspension buffer (Krebs-Hensleit buffer, pH 7.4), following which the cells were resuspended in the incubation buffer (Krebs-Hensleit buffer fortified with glucose). The incubations with mouse, rat, dog and monkey hepatocytes were performed with cells from one donor, whereas the incubations with the cryopreserved human hepatocytes were performed with a pool created from hepatocytes of three different donors. Cell viabilities, determined by trypan blue exclusion, of the fresh hepatocytes were >75% and of the cryopreserved hepatocytes were >60%. Dasatinib (3 μM) was incubated at 37°C for 1 h, and aliquots of samples (0.2 ml) were taken at 0, 20, 40, and 60 min. The reactions were terminated by adding an equal volume of acetonitrile. The incubations were performed in duplicate at a cell density of 0.67×10^6 cells/ml in Krebs-Hensleit buffer fortified with glucose, in an incubator at 37°C, 95% humidity, in an environment of 5% CO_2. The samples were analyzed by LC/MS and the percent metabolized was calculated based on the disappearance of the parent compound.

Metabolism of dasatinib by specific CYP enzymes and FMO3 (reaction phenotyping)

Reaction phenotyping studies were performed for dasatinib using human CYPs and FMO3 obtained from insect cells that expressed a single CYP enzyme (Supersomes™ obtained from Gentest Co., Woburn, MA, USA). The incubations were conducted using the following conditions: 50 pmol/ml CYP enzyme or 0.5 mg/ml of FMO3, 1 or 10 μM dasatinib (10 mM stock solution made in acetonitrile), 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM of NADPH and 3.3 mM of magnesium chloride. After a 5 min pre-incubation at 37°C the reaction was initiated by the addition of NADPH and incubations were conducted for a further 30 min. Aliquots of the incubation were quenched at 0 and 30 min by addition of two volumes of acetonitrile. Samples were analyzed using a LC/MS/MS assay and the percent metabolized was calculated based on the disappearance of the parent compound.

Serum protein binding

The extent of protein binding of dasatinib was determined in fresh mouse, rat, dog, monkey and human sera using equilibrium dialysis. All experiments were done in fresh, pooled serum ($n = 10$ for mice, $n = 3$ for rat, dog, monkey and human) obtained from Bioreclamation Inc. (Hicksville, NY, USA). Dasatinib (1 mM) in acetonitrile was added into serum at a ratio of 1:100 to give a final concentration of 10 μM. Serum samples were dialyzed against 134 mM phosphate buffer (pH 7.4). The Micro-Equilibrium Dialyzer™ (500 μl chamber volume, Amika Corp., Holliston, MA, USA) containing spiked serum was incubated in a shaking water bath maintained at 37°C for 4 h. A 10,000 Da molecular weight cutoff dialysis membrane (Amika Corp., Holliston, MA, USA) was used. All experiments were carried out in triplicate. Aliquots of buffer and serum were taken at 4 h, and analyzed using an LC/MS/MS method. The stability of dasatinib in sera was also determined over the 4 h incubation period. From each dialysis cell, the free and bound drug percentages were calculated as follows:

% free = 100 × (concentration in buffer)/(concentration in serum)

% bound = 100 – % free

Blood cell partitioning

The extent of blood cell partitioning of dasatinib was determined in mouse, rat, dog, monkey and human blood. All experiments were done in fresh, pooled blood obtained from Bioreclamation Inc. Dasatinib (1 mM) in acetonitrile was added into blood at a ratio of 1:100 to give a final concentration of 10 μM. Samples were incubated at 37°C in a shaking water bath for 2 h. Aliquots of blood were removed at 0.5 and 2 h and the remaining blood was centrifuged to obtain plasma. Blood samples were treated with a 0.5 volume of water and centrifuged to obtain supernatant. All experiments were carried out in triplicate. Samples were analyzed using an LC/MS/MS method. A blood to plasma partitioning ratio (C_B/C_P) for dasatinib was calculated from the concentrations in blood (C_B) and plasma (C_P). Blood cell distribution was calculated from the values of C_B, C_P, and hematocrit (HCT) as follows:

$$\%\,\text{blood cell distribution} = 100 \times [C_B - C_P(1 - \text{HCT})]/C_B$$

Permeability studies using Caco-2 cells

Caco-2 cells (American Type Culture Collection, Rockville, MD, USA) were seeded onto 12 well polycarbonate filter membranes at a density of 60,000 cells/cm^2. The permeability studies were conducted with the monolayers cultured for approximately 21 days in culture. The transport

medium buffer was modified Hank's balanced salt solution containing 10 mM HEPES. The pH of both the apical and basolateral compartments was 7.4. The bi-directional permeability studies were initiated by adding an appropriate volume of buffer containing dasatinib (final conc 50 μM; 20 mM stock solution made in DMSO) to either the apical (apical to basolateral transport) or basolateral (basolateral to apical transport) side of the monolayer ($n = 3$). Samples were taken from both the apical and basolateral compartments at the end of a 2 h incubation period and the concentrations of test compound were analyzed for dasatinib using an HPLC method with UV detection. Permeability coefficient (Pc) was calculated according to the following equation: $Pc = dA/(dt \times S \times Co)$, where dA/dt is the flux of dasatinib across the monolayer (nmole/s), S is the surface area of the cell monolayer, and Co is the initial concentration of dasatinib in the donor compartment. The Pc values are expressed in nm/s.

The ability of dasatinib to inhibit P-gp was evaluated by using the inhibition of the transport of digoxin, a P-gp substrate, in Caco-2 cell monolayer. Both the apical to basolateral (A-to-B) transport as well as the basolateral to apical transport (B-to-A) of [^{3}H]-digoxin was measured in the absence and presence of dasatinib. The transport medium was Hank's balanced salt solution, pH 7.4, on both the apical and basolateral side. The concentration of digoxin used was 5 μM, and dasatinib was tested at two concentrations (1 and 10 μM). Verapamil, a P-gp inhibitor, was used as a positive control in each experiment at a concentration of 10 μM. The studies were initiated by adding an appropriate volume of buffer containing digoxin to either the apical (apical to basolateral transport) or basolateral (basolateral to apical transport) side of the monolayer. Dasatinib was added to both sides of the monolayer. The monolayers were then incubated for 2 h at 37°C. Samples were taken from either the apical (basolateral to apical transport) or basolateral (apical to basolateral transport) compartment at the end of the 2-h period and analyzed for total radioactivity. The A-to-B and B-to-A permeability coefficient (Pc) of digoxin was calculated in the presence and absence of dasatinib. Results are reported as percent inhibition of digoxin transport by dasatinib.

In vivo animal studies

All procedures used for the animal studies were approved by the Bristol-Myers Squibb Institutional Animal Care and Use Committee.

Pharmacokinetics and oral bioavailability in mice

The pharmacokinetics of dasatinib were investigated in female nude mice (Harlan Sprague Dawley Co., Indianapolis, IN, USA). A total of 45 mice were divided into three groups to receive one of the following treatments, a single dose of 10 mg/kg intravenously (IV) and 5 mg/kg and 15 mg/kg orally by gavage. The mice were fed ad lib throughout the study. The vehicle used was propylene glycol:water (1:1) for both routes of administration. Serum concentrations of dasatinib in mice ($n = 3$ mice at each time point) were measured at 5, 15 min, 1, 3 and 6 h after IV dosing and at 15 min, 1, 2, 4 and 8 h after oral dosing. Composite serum concentration-time profiles were constructed for pharmacokinetic analysis.

A study was conducted in wild type and P-gp knockout mice (Taconic, Germantown, NY, USA) to determine the amount of dasatinib remaining in the gastrointestinal tract (GIT). Dasatinib was administered to both groups orally at a dose of 10 mg/kg in a 50 mM sodiun acetate buffer, pH 4.6. Serum samples were collected at 0.25, 0.5, 1, 3, 6, and 8 h post dose and gastrointestinal tract was collected at 8 h ($n = 3$ replicates per time point). GIT samples were homogenized with 3 volumes of water and 3 volumes of acetonitrile per 1 volume of GIT. Samples were analyzed for dasatinib by LC/MS/MS.

Pharmacokinetics and oral bioavailability in rats

The pharmacokinetics of dasatinib were investigated in male Sprague-Dawley rats following a single dose of 10 mg/kg either intra-arterially (IA) as a 10 min infusion or orally by gavage. The intra-arterial route was chosen for drug administration since blood samples for pharmacokinetic analysis were obtained from the cannula placed in the jugular vein. There were three rats per group. The dosing vehicle used was propylene glycol:water (1:1). A second study was also conducted in hepatic portal vein cannulated rats ($n = 3$), where the rats were dosed intraportally (IPT) at a dose of 10 mg/kg as a 30 min infusion. The vehicle used was 50 mM sodium acetate buffer, pH 4.6. In both studies the rats were fasted overnight and fed 4 h post dose. Blood samples were collected at 15, 30, 45 min, 1, 2, 4, 6, 8 and 10 h after intraarterial, oral and intraportal dosing. An additional 10 min sample was collected after intraarterial and intraportal dosing. Approximately 0.3 ml of blood was collected from the jugular vein in tubes containing EDTA and plasma was obtained by centrifugation. Urine was collected over the 10 h study period.

The routes of excretion and metabolism of dasatinib were investigated in bile duct cannulated (BDC) rats following a single dose of 10 mg/kg either IV as a 10 min infusion or orally by gavage. There were two rats per group. The vehicle used was 50 mM sodium acetate buffer, pH 4.6. The rats were fasted overnight and for the duration of the study. Urine and bile were collected over a 9 h period. The gastrointestinal tract (GIT) and feces were

collected at the end of the 9 h study period. GIT and feces were homogenized with 3 volumes of water and 3 volumes of acetonitrile per 1 volume of GIT/feces. Blood samples were collected at 15, 30 min, 1, 2, 4, 6 and 9 h after IV and oral dosing in tubes containing EDTA and plasma was prepared by centrifugation. All samples were analyzed for dasatinib by LC/MS/MS.

Pharmacokinetics and oral bioavailability in dogs

The pharmacokinetics of dasatinib were investigated in male beagle dogs ($n = 3$) following a single intravenous dose of 1.2 mg/kg as a 10 min infusion and as an oral dose of 3 mg/kg by gavage in a cross-over study design. The vehicle used for both the IV and oral route was 50 mM sodium acetate buffer, pH 4.6. The dogs were fasted overnight and fed 4 h post dose. Blood samples were collected at 10, 15, 30, 45 min, 1, 2, 4, 6, 8, 10 and 24 h after IV and oral dosing. Approximately 1 ml of blood was collected in tubes containing EDTA and plasma was obtained by centrifugation. Urine was collected over a 24 h period. Samples were analyzed for dasatinib by LC/MS/MS.

Pharmacokinetics and oral bioavailability in monkeys

The pharmacokinetics of dasatinib were investigated in male cynomolgus monkeys ($n = 3$) following a single intravenous dose of 2 mg/kg as a 10 min infusion or an oral solution dose of 5 mg/kg by gavage in a cross-over study design. The vehicle used for both the IV and oral route was 50 mM sodium acetate buffer, pH 4.6. The monkeys were fasted overnight and fed 4 h post dose. Blood samples were collected at 10, 15, 30, 45 min, 1, 2, 4, 6, 8, 10 and 24 h after IV and oral dosing. Approximately 1 ml of blood was collected in tubes containing EDTA and plasma was obtained by centrifugation. Urine was collected over a 24-h period. Samples were analyzed for dasatinib by LC/MS/MS.

Sample analysis

Samples from all the pharmacokinetic studies and samples from the protein binding and blood cell partitioning studies were analyzed by the following LC/MS/MS method. Plasma or serum samples were treated with two volumes of acetonitrile containing 200 ng/ml of the internal standard (IS, BMS proprietary compound). After centrifugation to remove precipitated proteins, a 10-µl portion of the clear supernatant was analyzed by LC/MS/MS. Bile, urine, and gastrointestinal tract (GIT) samples were first diluted 1:10 in the corresponding blank plasma and followed by the plasma sample extraction procedure. The HPLC system consisted of two Shimadzu LC10AD pumps, a HTS PAL autosampler, and a Hewlett Packard Series 1,100 column compartment. The column used was a YMC C18-AQ, 2 mm × 50 mm, 3 µm particle size, maintained at 60°C and a flow rate of 0.3 ml/min. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Initial mobile phase composition was 85% solvent A and 15% solvent B. After sample injection, the mobile phase was changed using a linear gradient to 5% solvent A and 95% solvent B over 1 min and held at that composition for an additional 1 min. The mobile phase was then returned to initial conditions and the column re-equilibrated for 1.0 min. The total analysis time was 3 min. The HPLC was interfaced to either a Micromass Quattro Micro triple quadrupole mass spectrometer equipped with an electrospray interface, or a Sciex API 3000 triple quadrupole mass spectrometer with a turbo ionspray interface. On Micromass Quattro Micro UHP nitrogen was used as the nebulizing and desolvation gas at flow rates of 100 l/h for nebulization and 1,000 l/h for desolvation. The desolvation temperature was 300°C and the source temperature was 150°C. Data acquisition employed selected reaction monitoring (SRM). Positively charged ion representing the [M + H] + for dasatinib and the IS were selected in MS^1 and collisionally dissociated with argon at a pressure of 2×10^{-3} Torr to form specific product ions which were subsequently monitored by MS^2. All dwell times were 100 ms. The SRM transitions monitored were m/z 488 → 401 for dasatinib, m/z 459 → 338 for the IS. Cone voltage was optimized at 45 V for dasatinib and 30 V for the IS, while the collision energy was 30 eV for dasatinib and 20 eV for the IS. The retention times for dasatinib and the IS, were approximately 1.0 and 1.2 min, respectively. The Sciex API 3000 triple quadrupole mass spectrometer used the turbo ionspray interface with an ionspray voltage of 4,500 V. Gas 1 and gas 2 were set at 8 and 800, respectively. The turbo ionspray temperature was set at 400°C. The declustering potential (DP) was 41 V for dasatinib and 61 V for the IS. The focusing potential (FP) was 170 V for dasatinib and 100 V for the IS. The collision energy was 41 eV and 29 eV for dasatinib and the IS, respectively. The nitrogen collision gas setting was 6. The standard curves ranged from 1 to 20,000 nM. The standards were analyzed in duplicate. Quality control (QC) samples at concentrations of 80, 800, and 8,000 nM were analyzed in duplicate with the analytical set. Predicted concentrations of more than 80% of the QCs were within 20% of nominal concentrations, indicating acceptable assay performance.

Liver microsomal samples for determination of metabolic stability were analyzed using a high throughput LC/MS assay. The HPLC system consisted of Shimadzu LC10AD pumps (Shimadzu, Columbia, MD, USA), and a SIL-10AD autosampler. Hepatocyte samples were analyzed by a LC/MS assay using a Quattro Ultima triple quadrupole mass spectrometer interfaced to a Waters 2790 gradient

HPLC. Samples from the Caco-2 permeability and glucuronidation studies were analyzed by HPLC-UV. The HPLC system consisted of the 2690 Waters separation module and a Waters 996 photodiode array detector.

Data analysis

Plasma concentration data was analyzed with standard non-compartmental methods [11] with the KINETICA™ software program. Composite plasma concentration–time profiles were constructed for pharmacokinetic analysis in the mouse. The C_{max} and T_{max} values were recorded directly from experimental observations. The AUC_{tot} values were calculated using a combination of linear and log trapezoidal summations. The total body clearance (CL), mean residence time (MRT), and the steady state volume of distribution (V_{ss}) were also calculated after IV or IA administration. The in vivo plasma clearance was converted to the blood clearance using the C_B/C_P values obtained experimentally for each species. The absolute oral bioavailability (%F) was estimated by taking the ratio of dose-normalized AUC values after oral doses to those after IV or IA doses.

Human plasma CL and V_{ss} values of dasatinib were also predicted using allometry [12, 13]. Allometric scaling was done by fitting the relationship between the values of plasma CL and V_{ss} found in animals (mouse, rat, dog, monkey) and the body weight (BW) of the animal. The equations used were: $CL = a \times (BW)^b$ and $V_{ss} = a \times (BW)^b$ where a is the allometric coefficient, and b is the allometric exponent. The plasma CL was then converted to blood clearance using the C_B/C_P ratio determined for human.

The in vitro intrinsic clearance of dasatinib in microsomes was calculated as follows [14]: CL_{int} (ml/min/mg protein) = rate/C, where rate is the rate of metabolism in microsomes (nmol/min/mg protein), and C is the initial concentration of dasatinib in the incubation. The in vitro intrinsic clearance of dasatinib in hepatocytes (CL_{int}) was calculated in a similar manner where CL_{int} (ml/min/million cells) = rate/C, where rate is the rate of metabolism in hepatocytes (pmole/min/million cells), and C is the concentration of dasatinib in the incubation.

The in vivo intrinsic hepatic clearance of dasatinib ($CL_{int,in\ vivo}$) estimated from in vitro hepatocyte incubations was calculated as follows [14]:

$$CL_{int,\,in\,vivo}(ml/\,min\,/kg) = CL_{int} \times \frac{120\,(million\,cells)}{g\,liver} \times \frac{\chi\,g\,liver}{kg\,body\,weight}$$

where χ is 88, 40, 32, 32 and 21 g liver/kg body weight for the mouse, rat, dog, monkey and human, respectively [15].

For calculation of intrinsic hepatic clearance of dasatinib from in vitro microsomal incubations, 45 mg microsomal protein/g liver was substituted in the above equation instead of 120-million cells/g liver. Protein binding in the incubation mixture or plasma was not factored into these calculations. The hepatic clearance CL_h was calculated from the following equation using the well-stirred model:

$$CL_h(ml/\,min\,/kg) = \frac{Qh \times CL_{int,\,in\,vivo}}{Qh + CL_{int,\,in\,vivo}}$$

where Qh is the liver blood flow of 90, 55, 31, 44 and 21 ml/min/kg for the mouse, rat, dog, monkey and human, respectively [15].

Results

Incubations with NADPH- or UDPGA-fortified liver microsomes

In vitro studies in NADPH-fortified liver microsomes showed that dasatinib undergoes oxidative metabolism in mouse, rat, dog, monkey and human and the in vitro rates of metabolism when tested at a concentration of 3 μM, were found to be 0.06, 0.13, 0.23, 0.13, and 0.22 nmol/min/mg protein, respectively. These rates predict moderate clearance of dasatinib in mouse, rat, and monkey, and high clearance in dog and human (Table 1). The rates of glucuronidation of dasatinib were determined in UDPGA-fortified liver microsomes from mouse, rat, dog, monkey, and human at a concentration of 10 μM and were found to be very low at 0.024, 0.034, 0.029, 0.026, and 0.017 nmol/min/mg protein, respectively (data not shown).

Incubations with hepatocytes

In vitro rates of metabolism of dasatinib in hepatocytes when tested at a concentration of 3 μM, were found to be 30, 49, 24, 20, and 26 pmol/min/10^6 cells in mouse, rat, dog, monkey, and human, respectively. These rates predict moderate clearance of dasatinib in all five species tested (Table 1).

Metabolism of dasatinib by specific CYPs and FMO3 (reaction phenotyping)

Preliminary reaction phenotype data, obtained with a panel of recombinant human CYPs, and FMO3 indicate that dasatinib is primarily metabolized by CYP3A4. In this instance, the turnover was related to the known abundance

Table 1 Rates of oxidative metabolism of dasatinib in liver microsomes and hepatocytes and predicted in vivo hepatic clearances

Species	Liver microsomes		Hepatocytes		In vivo blood CL (observed) (ml/min/kg)
	Rate (nmol/min/mg prot)	Predicted CL (ml/min/kg)	Rate (pmol/min/10^6 cells)	Predicted CL (ml/min/kg)	
Mouse	0.06	42	30	49	46
Rat	0.13	32	49	32	21
Dog	0.23	24	24	15	16
Monkey	0.13	26	20	16	23
Human	0.22	16	26	11	–

of each CYP form in human tissue. All recombinant human CYP isoforms tested in our panel appeared to be capable of metabolizing dasatinib (Table 2). Some of these enzymes are expressed extrahepatically and may contribute to the total metabolic clearance. However, CYP3A4 is the most abundant CYP form in human gut and liver and thus plays a major role in the oxidative metabolism of dasatinib metabolism. Human FMO3 was also capable of metabolizing dasatinib, however, its contribution to the total turnover of dasatinib could not be estimated since the enzyme concentration in the incubation was not known.

Serum protein binding

The serum protein binding of dasatinib was determined by equilibrium dialysis at 10 μM and was found to be 92, 97, 96, 97, and 94% in mouse, rat, dog, monkey and human, respectively. The stability of dasatinib was determined in serum at 10 μM for 4 h at 37°C. Dasatinib was stable under these conditions in mouse, rat, dog, monkey and human serum.

Table 2 Rates of oxidative metabolism of dasatinib by cDNA expressed human CYPs and FMO3 (reaction phenotyping)

CYP enzyme	Turnover in 30 min (%)		Rate of metabolism (pmol/min/pmol CYP)	
	1 μM	10 μM	1 μM	10 μM
1A1	63.3	14.8	0.422	0.989
1A2	2.1	9.1	0.014	0.605
2C9	20.5	23.2	0.136	1.550
2C19	12.2	8.4	0.082	0.559
2D6	7.9	16.3	0.053	1.090
2E1	33.8	3.6	0.225	0.238
3A4	69.5	81.2	0.463	5.416
FMO3	14.2	29.6	–*	–*
1B1	60.0	28.5	0.400	1.901
2A6	12.9	17.2	0.086	1.147
2B6	18.6	6.1	0.124	0.405
2C8	17.5	9.4	0.117	0.623
3A5	6.9	9.1	0.046	0.609
4A11	19.0	5.4	0.126	0.362

*Not estimated

Blood cell partitioning

The extent of blood cell partitioning of dasatinib was determined in mouse, rat, dog, monkey and human blood at a nominal concentration of 10 μM at 37°C. The ratio of concentration in blood to plasma (C_{blood}/C_{plasma}) was 1.2, 1.1, 1.3, 1.5, and 1.8 in mouse, rat, dog, monkey and human, respectively, after a 30-min incubation. The hematocrit values were 0.31, 0.49, 0.43, 0.34, and 0.21, and the percent distribution of dasatinib in blood cells was 43, 55, 57, 54 and 55%, in mouse, rat, dog, monkey and human, respectively. Dasatinib appears to be equally distributed between plasma and blood cells in all species tested.

Permeability studies using Caco-2 cells

Caco-2 cells are derived from a human colon carcinoma and grow to become a confluent monolayer on a semipermeable membrane. They become polarized and form tight intercellular junctions, and thereby strongly resemble the intestinal epithelium. The rate of passage of compounds through the barrier is used to determine a permeability coefficient (Pc), which can be related to the in vivo absorption of the compound. The Pc of dasatinib in the apical to basolateral direction was 102 nm/s at an initial concentration of 50 μM at pH 7.4. This value is comparable to compounds that exhibit good absorption in humans. Under similar conditions, the average basolateral to apical Pc value of dasatinib was 222 nm/s. The higher B-to-A permeability resulted in a BA/AB efflux ratio of 2.2, suggesting that dasatinib may be a substrate of an intestinal efflux transporter such as P-gp.

To evaluate whether dasatinib inhibits the transport of P-gp substrates, the permeability of digoxin (a P-gp substrate) was measured across Caco-2 cell monolayers in the presence and absence of dasatinib at two different concentrations of 1 and 10 μM. Dasatinib showed weak inhibition of

digoxin transport (10% at 1 μM and 11% at 10 μM). This level of inhibition is consistent with typical negative controls in this assay. The inhibition value for verapamil (a known P-gp inhibitor) was 59% at 10 μM in this experiment. These results suggest that dasatinib is unlikely to be a P-gp inhibitor and may not alter the absorption and disposition characteristics of compounds that are P-gp substrates.

Pharmacokinetics and oral bioavailability in mice

Pharmacokinetic parameters of dasatinib in mice obtained after single intravenous and oral doses are summarized in Table 3, and the serum concentration–time profiles are presented in Fig. 2. The systemic plasma clearance of dasatinib in mice was 62 ml/min/kg, which is equivalent to a blood clearance of 46 ml/min/kg. The blood clearance of dasatinib is ~50% of the hepatic blood flow of 90 ml/min/kg in mice [15]. The V_{ss} was high (4.2 l/kg), greater than the blood volume of 0.085 l/kg [15], indicating significant extravascular distribution. The estimated half-life was 0.9 h and the MRT was 1.1 h. The oral bioavailability of dasatinib in mice was 14 and 17% at doses of 5 and 15 mg/kg, respectively. The T_{max} after both oral doses was 2 h. The values for oral bioavailability are lower than those previously reported [16]. Although the reason for this discrepancy in not obvious, the previous study used tumor-bearing mice while mice used in this study were non-tumor-bearing.

To investigate whether P-gp plays a role in the incomplete oral absorption of dasatinib, a study was conducted in wild type and P-gp knockout mice (Table 4). Dasatinib was administered to both groups orally at a dose of 10 mg/kg, and GIT was collected at 8 h after dosing. The values of C_{max} and AUC were similar in the two groups of mice. More importantly, the percentage of dose remaining in the GIT was similar between the two groups suggesting that P-gp does not play a role in the oral absorption of dasatinib.

Table 3 Pharmacokinetic parameters of dasatinib in female nude mice

Parameter	Intravenous (n = 3)	Oral (n = 3)	
Dose (mg/kg)	10	5	15
C_{max} (μM)	–	0.104	0.32
T_{max} (h)	–	2	2
AUC_{tot} (μM × h)	5.6	0.46	1.2
CL (ml/min/kg)	61.7	–	–
V_{ss} (l/kg)	4.2	–	–
$t_{1/2}$ (h)	0.9	2.5	2.0
MRT (h)	1.1	4.2	3.7
Bioavailability (%)	–	17	14

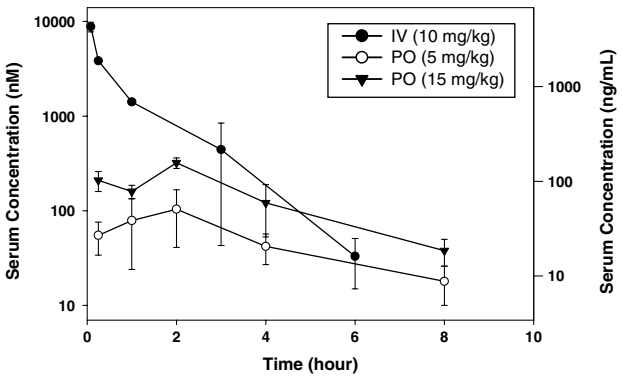

Fig. 2 Serum concentration versus time profiles of dasatinib following IV (10 mg/kg) and oral (5 and 15 mg/kg) administration in mice (mean ± SD)

Pharmacokinetics and oral bioavailability in rats

Pharmacokinetic parameters of dasatinib in rats after single IA, oral, and intraportal doses are summarized in Table 5 and the plasma concentration time profile is presented in Fig. 3. The systemic pharmacokinetic parameters have been reported previously [5] are included here for completion and comparison to other nonclinical species. The systemic plasma clearance of dasatinib was 26 ± 7.8 ml/min/kg, which is equivalent to a blood clearance of 21 ml/min/kg. The blood clearance of dasatinib corresponds to ~38% of the hepatic blood flow of 55 ml/min/kg [15]. The V_{ss} was high (6.3 ± 2.2 l/kg), greater than blood volume of 0.054 l/kg [15], indicating significant extravascular distribution. The estimated elimination half-life was 3.3 ± 0.9 h and the MRT was 4.1 ± 1.2 h. The oral bioavailability of dasatinib was 27%. The Tmax after an oral dose was 2.3 ± 3.3 h in the rat. The bioavailability after an intraportal dose of 10 mg/kg was comparable to that after intra-arterial administration. In intact rats, the percent of dose excreted unchanged in the urine over a 10 h period was 1.6 ± 0.5% after an IA dose of 10 mg/kg and 0.2 ± 0.04% after an oral dose of 10 mg/kg. In bile duct cannulated rats, the percent of dose excreted unchanged in the urine and bile over a 9 h period was 0.8 and 9.6%, respectively, after an IV dose of 10 mg/kg, and 0.4 and 4.7%, respectively, after an oral dose of 10 mg/kg. The percent of dose in the GIT at 9 h post dose was 3.5% after the IV dose and 33.4% after the oral dose.

Pharmacokinetics and oral bioavailability in dogs

Pharmacokinetic parameters of dasatinib in the dog are summarized in Table 6 and the plasma concentration time profiles are presented in Fig. 4. The systemic plasma clearance of dasatinib was 25 ± 6.3 ml/min/kg, which is equivalent to a blood clearance of 16 ml/min/kg. The blood

Table 4 Pharmacokinetic parameters of dastinib in wild type and P-gp knockout mice

Parameter	Wild-type (n = 3)	P-gp Knockout (n = 3)
Oral dose (mg/kg)	10	10
C_{max} (μM)	0.70	0.40
T_{max} (h)	0.25	0.25
AUC (0–8 h) (μM × h)	2.1	1.1
Percentage of dose in GIT at 8 h	15.0 ± 4.5	14.2 ± 6.9

Table 5 Pharmacokinetic parameters of dasatinib in rats

Parameter	Intraarterial (n = 3)	Oral (n = 3)	Intraportal (n = 3)
Dose (mg/kg)	10	10	10
C_{max} (μM)	–	0.49 ± 0.18	7.5 ± 1.2
T_{max} (h)	–	2.3 ± 3.3	0.5 ± 0.0
AUC_{tot} (μM × h)	13.9 ± 4.6	3.8 ± 2.1	15.7 ± 5.5
CL (ml/min/kg)	26.4 ± 7.8	–	–
V_{ss} (l/kg)	6.3 ± 2.2	–	–
$t_{1/2}$ (h)	3.3 ± 0.9	3.1 ± 0.3	6.7 ± 3.0
MRT (h)	4.1 ± 1.2	6.7 ± 0.6	6.8 ± 2.8
Bioavailability (%)	–	27	~100
Percentage of dose excreted in urine as parent (0–10 h)	1.6 ± 0.5	0.2 ± 0.04	0.5 ± 0.2

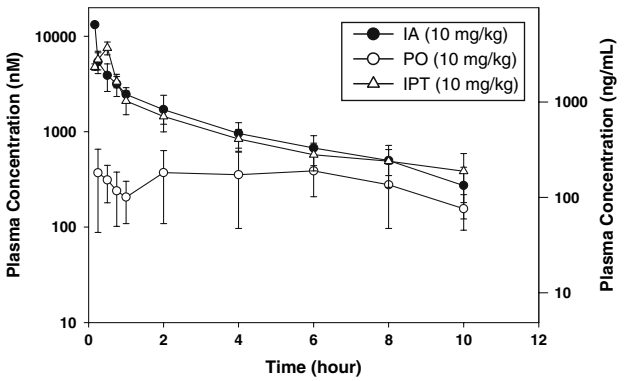

Fig. 3 Plasma concentration versus time profiles of dasatinib following intraarterial (IA, 10 mg/kg), oral (PO, 10 mg/kg), or intraportal (IPT, 10 mg/kg) administration in rats (mean ± SD)

clearance of dasatinib corresponds to ~50% of the hepatic blood flow of 31 ml/min/kg. The V_{ss} was high (4.7 ± 0.8 l/kg), greater than blood volume of 0.09 l/kg, indicating significant extravascular distribution. The estimated elimination half-life was 4.2 ± 2 h and the MRT was 3.2 ± 0.8 h. The oral bioavailability of dasatinib was 34 ± 13%. The T_{max} after an oral dose was 0.75 ± 0.25 h in the dog. The percent of dose excreted unchanged in the urine over a 24 h period was <1% after both the IV and oral doses.

Pharmacokinetics and oral bioavailability in monkeys

Pharmacokinetic parameters of dasatinib in the monkey are summarized in Table 6 and the plasma concentration time profiles are presented in Fig. 5. The systemic plasma clearance of dasatinib was 34 ± 4.1 ml/min/kg, which is equivalent to a blood clearance of 23 ml/min/kg. The blood clearance corresponds to ~50% of the hepatic blood flow of 44 ml/min/kg. The V_{ss} was high (3.5 ± 0.1 l/kg), greater than blood volume of 0.073 l/kg, indicating significant extravascular distribution. The estimated elimination half-life was 2.1 ± 0.1 h and the MRT was 1.7 ± 0.2 h. The oral bioavailability of dasatinib was 15 ± 2.1%. The T_{max} after an oral dose was 0.6 ± 0.1 h in the monkey. The percent of dose excreted unchanged in the urine over a 24 h period was <1% after both the IV and oral doses.

Prediction of clearance and V_{ss} values in humans

Allometric scaling by body weight was used to predict the systemic plasma clearance and V_{ss} of dasatinib in humans (Fig. 6). The linear regression of clearance or V_{ss} versus body weight, using data from mouse, rat, and dog, showed very good correlations with a coefficient of correlation (R^2) of 0.99 for both parameters. The equation obtained after regression was $CL = 33.5 \times (BW)^{0.90}$ and $V_{ss} = 4.55 \times (BW)^{0.98}$, giving a predicted systemic plasma clearance of 21.5 ml/min/kg and V_{ss} of 4.2 l/kg. This predicted plasma clearance is equivalent to a blood clearance of 11.9 ml/min/kg, which corresponds to ~57% of hepatic blood flow. The blood clearance value obtained by allometry is comparable to the hepatic clearance estimated from human hepatocytes (11 ml/min/kg) and slightly lower than that estimated from human liver microsomes (16 ml/min/kg).

Discussion

In vivo systemic plasma clearance values of dasatinib were 62, 26, 25, and 34 ml/min/kg in mouse, rat, dog, and monkey, respectively. Dasatinib showed high volumes of distribution (>3 l/kg) and high serum protein binding values (>90%) in all four species tested. The oral bioavailability of dasatinib was incomplete and ranged from 14% in the mouse to 34% in the dog. This incomplete oral bioavailability could be due to poor absorption and/or extensive first pass metabolism. In order to understand the mechanism behind the low bioavailability, dasatinib was

Table 6 Pharmacokinetic parameters of dasatinib in dogs and monkeys

Parameter	Dog (n = 3)		Monkey (n = 3)	
	IV	Oral	IV	Oral
Dose (mg/kg)	1.2	3	2	5
C_{max} (μM)	–	0.30 ± 0.09	–	0.34 ± 0.06
T_{max} (h)	–	0.75 ± 0.25	–	0.6 ± 0.1
AUC_{tot} (μM × h)	1.67 ± 0.41	1.4 ± 0.36	2.01 ± 0.23	0.75 ± 0.05
CL (ml/min/kg)	25 ± 6.3	–	34 ± 4.1	–
V_{ss} (l/kg)	4.7 ± 0.8	–	3.5 ± 0.1	–
$t_{1/2}$ (h)	4.2 ± 2.0	5.0 ± 1.8	2.1 ± 0.1	2.2 ± 0.4
MRT (h)	3.2 ± 0.8	5.8 ± 1.1	1.7 ± 0.2	3.0 ± 0.2
Bioavailability (%)	–	34 ± 13	–	15.2 ± 2.1
Percentage of dose excreted in urine as parent (0–24 h)	0.7 ± 0.3	0.8	0.7 ± 0.3	0.1 ± 0.1

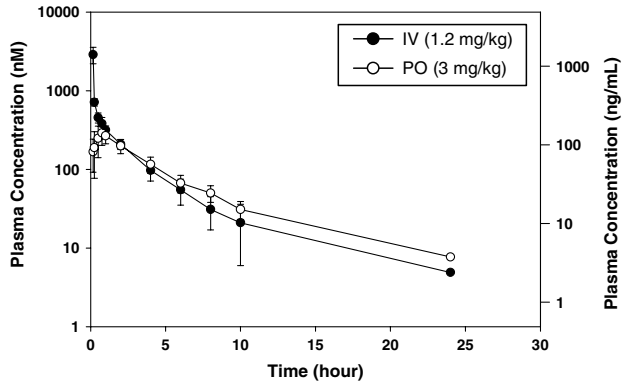

Fig. 4 Plasma concentration versus time profiles of dasatinib following IV (1.2 mg/kg) and oral (3 mg/kg) administration in dogs (mean ± SD)

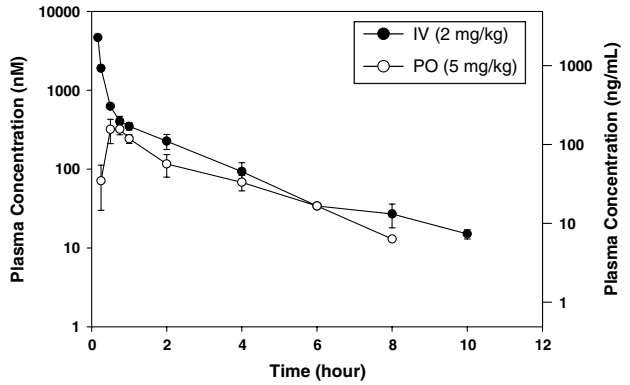

Fig. 5 Plasma concentration versus time profiles of dasatinib following IV (2 mg/kg) and oral (5 mg/kg) administration in monkeys (mean ± SD)

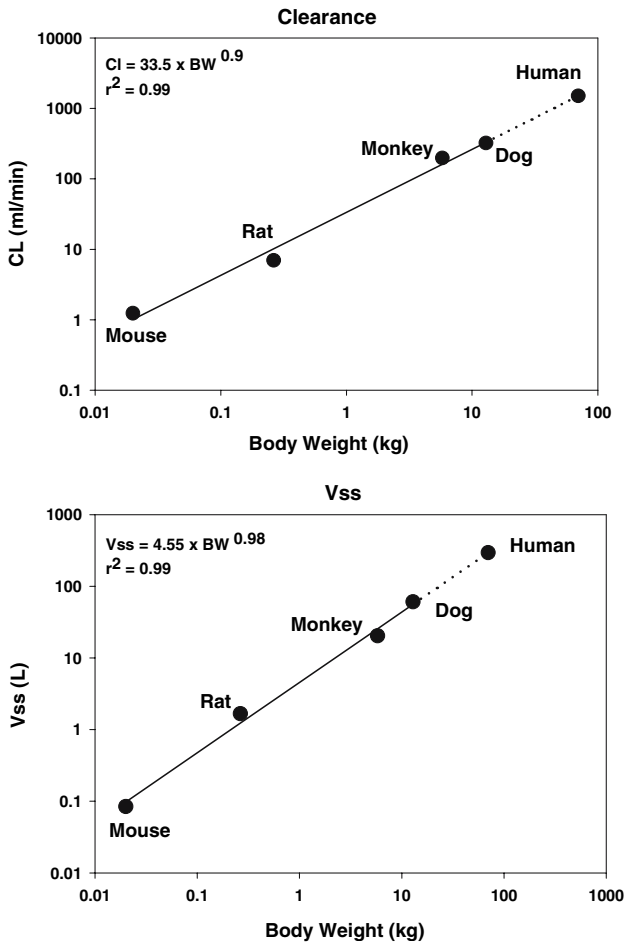

Fig. 6 Allometric scaling of clearance and V_{ss} values

dosed intraportally in rats. The bioavailability after an intraportal dose was comparable to that after intra-arterial administration. This increase in exposure after an intraportal dose suggested that absorption rather than presystemic liver first pass metabolism was responsible for the low oral bioavailability seen in rats. This was contrary to the data obtained in Caco-2 cells showing that dasatinib has high intestinal permeability, and the data in rat liver microsomes and hepatocytes showing intermediate metabolic

rates suggesting the possibility of first pass metabolism. High plasma concentrations seen after intraportal dosing may have led to some saturation of first pass metabolism.

Further studies in BDC rats showed that less than 15% of an intravenous dose was excreted unchanged in urine, bile, and the gastrointestinal tract, suggesting that dasatinib is primarily cleared via metabolism. This was supported by the low urinary excretion of unchanged dasatinib in intact rats, dogs, and monkeys. After an oral dose of 10 mg/kg in BDC rats, 33% of the dose was found in the GIT as unchanged dasatinib. The oral bioavailability depends on absorption, metabolism in the walls of the GIT, and finally metabolism by the liver before the compound reaches the systemic circulation. Thus, the oral bioavailability can be represented as $F = f_A \times f_G \times f_H$ where f_A = fraction of oral dose absorbed, f_G = fraction of oral dose that is not metabolized in the GIT, and f_H = fraction of oral dose escaping liver extraction during first pass. Assuming that all of the 33% of the dose found in the GIT in BDC rats was due to unabsorbed compound, 67% of the dose should have been absorbed i.e. $f_A = 0.67$. However, the oral bioavailability in rats was only 27% i.e., $F = 0.27$. Assuming no metabolism in the GIT ($f_G = 1$), the f_H is calculated to be 0.4, i.e., 40% of the dose escapes liver first pass metabolism. Hence, of the percent of dose that was absorbed, 60% was metabolized in the liver before reaching the systemic circulation. These studies suggest that the incomplete oral bioavailability of dasatinib is due to a combination of incomplete absorption and high first pass metabolism.

P-gp is an ATP-dependent efflux transporter that is expressed on the intestinal epithelial cells and has been implicated in restricting the oral absorption of various compounds thereby decreasing their bioavailability [17]. Dasatinib had high intrinsic permeability in Caco-2 cells however, the efflux ratio was approximately two-fold indicating that it may be a substrate for an intestinal efflux transporter. To investigate whether P-gp plays a role in the incomplete oral absorption of dasatinib, a study was conducted in wild type and P-gp knockout mice. P-gp knockout mice and wild-type mice showed no differences in the amount of dasatinib remaining unabsorbed in the gastrointestinal tract, suggesting that P-gp may not be responsible for the incomplete bioavailability. In addition, dasatinib does not inhibit P-gp and may not alter the absorption and disposition characteristics of compounds that are P-gp substrates.

Scaling of in vitro hepatocyte and liver microsomal data gave reasonably good predictions of in vivo clearances across all species. The systemic clearance in humans was predicted to be intermediate to high, ranging from 11 to 16 ml/min/kg, based on in vitro human liver microsomal and hepatocyte stability data, and allometric scaling using clearance parameters obtained from four species. Both in vitro hepatocyte studies and allometry predicted similar clearance values which were also reasonably close to that predicted by in vitro microsomal studies. These results demonstrate that for compounds like dasatinib, where metabolism is the predominant clearance pathway, a good prediction of in vivo clearance can be obtained using liver microsomes and hepatocytes employing a simple substrate depletion approach. Multiple human CYP enzymes appear to have the potential to metabolize dasatinib, and the major route of hepatic elimination is predicted to be CYP3A4.

In summary, dasatinib shows intermediate clearance in mouse, rat, dog, and monkey, and distributes extensively in those species. Oxidative metabolism appears to be the predominant clearance pathway. The incomplete oral bioavailability may be due to both lower absorption and high first-pass metabolism. However, the efflux transporter, P-gp does not appear to be limiting oral absorption.

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