Characterization of dasatinib and its structural analogs as CYP3A4 mechanism based inactivators and the proposed bio-activation pathways

Xiaohai Li, Yuanjun He, Claudia H. Ruiz, Marcel Koenig, Michael D. Cameron

Department of Molecular Therapeutics (MC), Translational Research Institute (XL, YH, CR, MK, MC), Scripps Florida, The Scripps Research Institute, 130 Scripps Way, Jupiter, FL 33458, USA
a) Running Title: Bioactivation of dasatinib

b) Corresponding Author: Michael D. Cameron

Scripps Florida, Department of Molecular Therapeutics, 130 Scripps Way, Jupiter, FL 33458, USA.

Telephone: 561-228-2223.

E-mail: cameron@scripps.edu

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d) Non-standard abbreviations: GSH, glutathione; ACN, acetonitrile; CID, collision-induced dissociation; HLM, human liver microsomes; MRM, multiple reaction monitoring; EPI, enhanced product ion; IDA, information dependant acquisition; PI, precursor ion scan; Das, Dasatinib; DP, de-clustering potential; CE, collision energy
ABSTRACT

Dasatinib was approved in 2006 for the treatment of imatinib resistant chronic myelogenous leukemia and primarily functions through the inhibition of BCR/ABL and Src kinase. Dasatinib is extensively metabolized in humans by CYP3A4. Here we report that the bioactivation of dasatinib by CYP3A4 proceeds through a reactive intermediate that leads to CYP3A4 inactivation with $K_i = 6.3 \mu M$ and $k_{\text{inact}} = 0.034 \text{ min}^{-1}$. The major mechanism of inactivation proceeds through hydroxylation at the para position of the 2-chloro-6-methylphenyl ring followed by further oxidation forming a reactive quinone-imine, similar to the reactive intermediates formed by acetaminophen and diclofenac. Formation of a reactive imine-methide was also detected, but appears to be a minor pathway. When glutathione was added to human liver microsomal incubations, dasatinib-glutathione adducts were detected. Numerous dasatinib analogs were synthesized in an effort to understand what modifications would block the formation of reactive intermediates during dasatinib metabolism. Interestingly, blocking the site of hydroxylation with a methyl group was not effective as a reactive imine-methide was formed, nor was blocking the site with fluorine as the fluorine was removed through an oxidative defluorination mechanism and the reactive quinone-imine was still formed. Numerous analogs are presented that did effectively block the formation of glutathione adducts and prevent the inactivation of CYP3A4.
INTRODUCTION

Chronic myeloid leukemia (CML) is associated with a cytogenic abnormality referred to as the Philadelphia chromosome resulting from the translocation of the c-abl oncogene from chromosome 9 with the breakpoint cluster region (bcr) on chromosome 22 (de Klein et al., 1982; Shtivelman et al., 1985). This results in the generation of the bcr-abl fusion gene, which when translated yields a constitutively active form of abl kinase referred to as BCR-ABL leading to increased proliferation and survival of myeloid progenitor cells (Calabretta and Perrotti, 2004). Imatinib, a kinase inhibitor targeting BCR-ABL, was first introduced in phase 1 trials in mid-1998 and was a breakthrough in the treatment of CML greatly increasing patient life expectancy (Druker et al., 2001). Despite the impressive results of imatinib treatment, approximately 30% of patients receiving imatinib as first-line therapy will discontinue treatment by 5 years because of disease resistance or drug toxicity (Druker et al., 2006). The major driver of imatinib resistance is due to point mutations in the BCR-ABL gene (Deininger et al., 2005) which decrease or eliminate imatinib efficacy.

Dasatinib was approved by the FDA in June, 2006, for the treatment of imatinib-resistant acute myeloid leukemia. Because of the absence of effective alternative treatment for imatinib resistant CML, dasatinib was granted accelerated approval before the completion of phase III clinical trials. Dasatinib is structurally diverse from imatinib and is a highly potent inhibitor of BCR-ABL with biochemical potency between 0.1 nM and 3 nM for BCR-ABL and most of the common BCR-ABL mutations (Gambacorti-Passerini et al., 2005; O'Hare et al., 2005).

Dasatinib has five primary phase I metabolites. Three of these are catalyzed by CYP3A4: hydroxylation at the para position of the chloromethylphenyl ring (major metabolite),...
hydroxylation of the C5-methyl of the chloromethylphenyl ring and N-dealkylation of the hydroxyethyl moiety. FMO can catalyze the formation of an N-oxide on the piperazine ring and an unidentified cytosolic oxidoreductase converts the hydroxyethyl group to an acid. Additional secondary metabolites were detected as were phase II metabolites due to sulfation or glucuronidation (Christopher et al., 2008a; Christopher et al., 2008b; Kamath et al., 2008; Wang et al., 2008).

The product information sheet for dasatinib states that hepatotoxicity is a possible side effect of treatment and dasatinib is a possible mechanism-based inactivator of CYP3A4; however, no details are given and there is nothing in the literature to allow quantitation of the risk. Details of a case of dasatinib-induced acute hepatitis published in July 2008 (Bonvin et al., 2008) were similar to reports of immune-mediated idiosyncratic hepatotoxicity which have been reported for compounds with similar functional groups such as diclofenac (Schapira et al., 1986). An additional report of dasatinib-induced lupus was reported in August 2008 and was concluded to be immune-mediated (Rea et al., 2008). Evaluation of the structure of dasatinib and the known CYP3A4 catalyzed oxidations on the ortho-methyl and the para position of the chloromethylphenyl ring, would make the formation of reactive quinone-imine and imine-methide intermediates plausible. Quinone-imine and imine-methides are reactive electrophiles and may adduct to cysteine and lysine residues of proteins and may act as haptens and initiate immunological responses (Pirmohamed et al., 2002).
MATERIALS AND METHODS

Chemicals used. Midazolam, carbamazepine, ketoconazole, and glutathione were purchased from Sigma Chemical Co. (St. Louis, MO). Dasatinib and its structural analogs were synthesized at Scripps Florida. All solvents used for LC/MS were of chromatographic grade. HLM (pooled) were purchased from Xenotech (Lenexa, KS). All solutions were prepared from Mili-Q treated water with a specific resistance $\geq$ 17.8 MΩ.

Synthesis of dasatinib analogs. Dasatinib was synthesized using the published method of Lombardo et al (Lombardo et al., 2004) in five linear steps beginning with the condensation of the lithium anion of 2-chlorothiazole with 2-chloro-6-methylphenyl isocyanate. A modified method was used for the synthesis of dasatinib analogs. The analogs bearing the (hydroxyethyl)piperazine group were synthesized in two linear steps beginning with the coupling of 2-bromothiazole-5-carboxylic acid and the corresponding anilines followed by a Buchwald-Hartwig amination reaction with 2-(4-(6-amino-2-methylpyrimidin-4-yl)piperazin-1-yl)ethanol. The analogs 984, 1920 and 1235 were synthesized using similar methods.

Human liver microsomal incubations – CYP3A4 inactivation. The CYP3A4 activity in HLM incubations was assessed using a selective marker reaction, oxidation of midazolam to 1’-hydroxymidazolam. Time-dependent inhibition was assessed using a two step procedure. Primary incubations (250 µl, containing 2 mM NADPH, 0.5 mg/ml HLM and various concentrations of dasatinib or its analogs in 0.1 M potassium phosphate buffer, pH 7.4) were incubated with human liver microsomes. At various time points, e.g. 0, 4, 8, 15, 22 and 30 min, 10 µl of the primary reaction was removed and added into a secondary reaction containing midazolam, NADPH, and buffer. The final reaction volume and concentrations in the secondary
reactions were 200 µl with 20 µM midazolam, 1 mM NADPH, 0.05 mg/ml HLM, and 0.1 M potassium phosphate buffer, pH 7.4). Both reactions were conducted in 96 well plates on a shaking incubator maintained at 37 °C. The secondary incubation was stopped after 5 minutes by adding acetonitrile in a 1:1 ratio (v/v) containing carbamazapine 0.2 µM as an internal standard. At the end of the assay, the incubations were centrifuged and passed through a Millipore Multiscreen Solvinter 0.45 micron low binding PTFE hydrophilic filter plate and analyzed by LC-MS/MS for the formation of 1´-hydroxymidazolam using an API4000 mass spectrometer (Applied Biosystems, Foster City, CA) interfaced with an Agilent 1200 HPLC (Agilent Technologies, Palo Alto, CA). Chromatographic separation was achieved by using a Phenomenex Synergi Fusion RP C18 column (2.0 x 50 mm, 4 µm). Mobile phases consisted of 0.1% aqueous formic acid (solvent-A) and acetonitrile with 0.1% formic acid (solvent-B) run at a constant flow rate of 0.375 ml/min. A 2.5 minute HPLC method was used with % B equal to 2% at t=0 min, 80% at t=1.35-1.6 min, and 2% at t=1.61-2.5 min (all gradients were linear). 1´-hydroxymidazolam was detected using the transition from m/z 342.2 to m/z 203.1, , DP = 90, CE = 38. All inhibition studies were conducted in triplicate.

HLM incubations for GSH adduct identification. Incubations to test for the formation of GSH adducts contained 40 µM dasatinib or analog (from DMSO stock resulting in 0.2% DMSO v:v), and 1 mg/ml HLM in 0.1 M potassium phosphate buffer, pH 7.4, in the presence or absence of NADPH (1 mM) and reduced glutathione (5 mM). The total incubation volume for each sample was 0.5 mL. After 3 minutes preincubation at 37 °C, the incubations were initiated by adding NADPH. Incubations were quenched after 1 h with 1 mL ice cold ACN and vortexcled for 1 minute. These mixtures were centrifuged at 14,000 rpm for 10 minutes and the supernatants
were transferred to clean 1.5 mL microcentrifuge tubes and dried at 25 °C in a temperature
controlled Speedvac for up to 4 h. Once dried, extracts were reconstituted in 100µL of a 30%
ACN solution and transferred to HPLC autosampler vials for analysis. Experiments to show the
time dependent production of dasatinib-glutathione adducts and to evaluate the effect of the
CYP3A4 inhibitor ketoconazole were done using similar procedures except dasatinib was
lowered to 20 µM. Ketoconazole (1 µM), an inhibitor of P450 3A4, was from a methanol stock
and resulted in 0.1% v:v methanol in the final reaction.

**LC-MS/MS setting for detection of glutathione adducts.** LC-MS/MS analyses were
performed on an API4000 Q-Trap mass spectrometer (Applied Biosystems, Foster City, CA)
interfaced with an Agilent 1200 HPLC (Agilent Technologies, Palo Alto, CA). Chromatographic
separation was achieved by using a ZORBAX RX-C18 column (2.1 x 150 mm, 5 µm, Agilent
Technologies, Palo Alto, CA). Mobile phases consisted of 0.1% aqueous formic acid (solvent-A)
and acetonitrile with 0.1% formic acid (solvent-B) run at a constant flow rate of 0.4 ml/min. A
35 minute HPLC method was used with %B equal to 5% at t=0-3 min, 10% at t=3.5 min, 50% at
t=23 min, 80% at t=28-29 min, and 5% at t=29.5-35 min (all gradients were linear).

MS/MS analyses incorporated polarity switching, using conditions similar to those
reported in recent publications by Bo Wen (Wen and Fitch, 2008; Wen et al., 2008a; Wen et al.,
2008b). The polarity switching method utilizes improved specificity and lower background of
the negative ion precursor scan to detect the presence of GSH-adducts and then obtains a more
informative positive ion fragmentation pattern in a single injection. Briefly, a negative ion
precursor ion scan (precursor = m/z 272, DP = -75, CE = -30) was used with a range from m/z
350 to m/z 900 followed by a positive ion enhanced resolution scan and an information-
dependent acquisition (IDA) triggered positive ion EPI spectra with m/z 80-900 (DP=75 and CE=40). A settling time of 700 ms was required to accommodate the switch in polarity and the total cycle time was 4.6 seconds.
RESULTS

Initial incubations of dasatinib with human liver microsomes verified that oxidation of the chloromethylphenyl ring yielded two major hydroxylated metabolites and that the addition of ketoconazole inhibited their formation (Wang et al., 2008). Preliminary incubations using 10 µM dasatinib also determined that dasatinib caused the NADPH- and time-dependent inactivation of CYP3A4 (data not shown). Based on these results, more detailed experiments were employed to determine the kinetic constants for dasatinib mediated loss of CYP3A4 activity.

Dasatinib-mediated time-dependent inactivation of CYP3A4. A series of incubations with a range of dasatinib concentrations were allowed to incubate with HLM and NADPH. At various time points, the remaining CYP3A4 activity was determined by the marker reaction of midazolam conversion to 1’-hydroxymidazolam. Appropriate controls, lacking dasatinib, were included to assure that the loss in activity was not due to thermal inactivation. The data is represented graphically in Figure 1. The observed first-order rate constants (k_{obs}) for the inactivation of CYP3A4 by individual concentrations of dasatinib were obtained from the slope of the individual lines. This was fit to a Kitz-Wilson plot and is shown in the insert of Figure 1. The calculated K_{I} was 6.3 µM and the k_{inact} was 0.034 min^{-1}. The addition of 2 mM reduced glutathione to the reaction did not protect the enzyme and the calculated K_{I} and k_{inact} were virtually identical suggesting that bioactivated dasatinib inactivates CYP3A4 prior to releasing from the active site into the bulk solution. Incubations of dasatinib with recombinant CYP3A4 also showed time-dependent inhibition (K_{I} = 21 µM and k_{inact} = 0.095 min^{-1}).

Removal or modification of the piperazine or methylpyrimidine rings of dasatinib did not eliminate the time-dependent inactivation properties of the molecule. Kinetic constants for three
Dasatinib analogs are shown in Table 1. Additional analogs were made with modifications on the chloromethylphenyl ring of dasatinib. Time-dependent CYP3A4 inactivation was detected for six of the seven analogs tested. The one compound that did not show CYP3A4 inactivation had the methyl removed and the para position of the chloromethylphenyl ring blocked with chlorine, Table 2.

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Dasatinib-glutathione adduct formation. Glutathione has a free sulfhydryl group which is nucleophilic and can spontaneously react with electrophiles. The addition of glutathione to microsomal incubations containing dasatinib and NADPH resulted in the detection of dasatinib-glutathione adducts. Six distinct adducts were observed (Figure 2): two dasatinib-glutathione adducts (D-GSH a and b), two hydroxy-dasatinib-GSH adducts (D-OH-GSH a and b), and two dihydroxy-dasatinib-GSH adducts (D-2OH-GSH a and b). The D-OH-GSH (a) adduct appeared to be the major adduct. If we assume that all of the glutathione adducts have similar ionization efficiency, then the relative concentrations of D-2OH-GSH:D-OH-GSH:D-GSH were 25%:67%:8%. The addition of 1 µM ketoconazole to the incubations inhibited the formation of all adducts (Figure 2B), implying that the formation of all of the glutathione adducts were predominately catalyzed by CYP3A4. This was further verified with the detection of D-2OH-GSH, D-OH-GSH, and D-GSH in incubations using recombinant CYP3A4 (data not shown). Repeating the incubation with both rat and mouse microsomes gave the same profile of glutathione adducts with only minor alterations in the relative concentrations (data not shown). No adducts were observed in any of the microsomal incubations if either glutathione or NADPH were eliminated from the reaction.
The product ion spectra of dasatinib has characteristic fragment ions at m/z 319 and 347 corresponding to loss of the chloromethylphenyl ring through fragmentation on both sides of the carboxyl group of the amide bond, Figure 3. Because dasatinib contains chlorine, the $^{35}\text{Cl}$ and $^{37}\text{Cl}$ isotopes were useful in fragment pattern evaluation of dasatinib and the glutathione adducts (data not shown). All of the detected dasatinib-glutathione adducts had characteristic m/z 319 and 347 fragments indicating that glutathione was not bound to that portion of the molecule and that all adducts were located on the chloromethylphenyl ring.

The MS/MS spectra and proposed fragments of D-OH-GSH and D-2OH-GSH are shown in Figure 3. Only the spectra for the most intense D-OH-GSH (a) and D-2OH-GSH (a) adducts are shown because the mass and fragmentation are identical for the corresponding lower abundance hydroxylated adducts. We have drawn the structures with glutathione adducted at the 3 position as we believe this to be the major site of adduction for a para quinone-imine due to the electron withdrawing effect of the chlorine and the electron donating properties of the methyl group. The MS/MS spectra is shown for both of the D-GSH adducts, Figure 4, because the two had different fragmentation patterns. D-GSH (a) was drawn as having glutathione bound at the C5 position and has a fragment at m/z 520 corresponding to dasatinib plus sulfur caused by fragmentation on the glutathione side of the thioether bond. Binding sulfur to the aromatic ring should result in a stronger bond than adduction through the methyl. D-GSH (b) has a fragment at 486 corresponding to fragmentation on the dasatinib side of the thioether bond and was assigned as glutathione bound directly to the benzylic methyl group. The proposed structures are based on expected sites of adduction for an ortho imine-methide intermediate.
Bioactivation of dasatinib analogs. Two mass spectroscopy methods were utilized to detect glutathione adducts for microsomal incubations containing dasatinib analogs. Polarity switching, as described in the methods section, utilizes a diagnostic glutathione fragment and is suitable for detecting glutathione bound to any part of the molecule. A second enhanced product ion scan utilizing a characteristic m/z 347 ion was particularly sensitive due to excellent signal intensity, reduced background, and shorter cycle time for the acquisition without the need to switch from negative to positive ion mode during each scan. This second method is limited to detecting adducts where glutathione is bound to the chloromethylphenyl ring. While we did not use the common methodology of scanning for loss of the pyroglutamate group (neutral loss of 129), all detected adducts contained the characteristic loss, Table 3.

Twelve dasatinib structural analogs on the chloromethylphenyl ring were evaluated for their potential to form adducts with glutathione, Table 3. The role of the methyl group was investigated by replacing the methyl group with fluorine (1698). As previously noted in Table 2, 1698 retains the ability to inactivate CYP3A4. As would be expected, the fluorine did not prevent hydroxylation at the para position and 1698-OH-GSH and 1698-2OH-GSH adducts were detectable, whereas 1698-GSH adducts were not. The +GSH (non-hydroxylated) adducts were hypothesized to be generated through oxidation of the methyl group to form an imine-methide which would then undergo nucleophilic attack on the methyl group and on the adjacent C5 carbon (Figure 4). When tested, all five analogs that retained the methyl group had detectable +GSH adducts. On the contrary, +GSH adducts were not observed for any of the six analogs that lacked the methyl group.
Several analogs were prepared that blocked the para position of the methylchlorophenyl ring. When a methyl group was added to the para position (1660), no hydroxylated glutathione adducts were detected. However, the additional methyl group provided an additional site for the formation of a reactive imine-methide and incubations of 1660 with glutathione resulted in the formation of a single +GSH adduct. The k_{inact} of 1660 was the highest of all the analogs tested.

Two analogs were synthesized with fluorine in the para position. We expected this modification to prevent the formation of the hydroxylated glutathione adducts and only the +GSH adduct arising from oxidation of the methyl group to an imine-methide would be detected. 5826 differed from dasatinib only in the presence of fluorine at the para position. 1701 had the additional modification of replacing the chlorine with a methyl group. The expected +GSH adducts was detected in both analogs. Surprisingly, the major adducts detected for both compounds were hydroxylated glutathione adducts and the fluorine group appeared to be removed. The hydroxylated glutathione adducts had identical masses, fragmentation patterns, and chromatographic retention times to dasatinib and 1695 (data not shown). Accurate mass MS/MS confirmed that the trapped adducts did not contain fluorine. Purity tests showed that 5826 and 1701 were pure and no dasatinib or 1695 was detectable.

The presence of chlorine in the para position (6704) gave similar results. The major glutathione adduct appeared to be due to hydroxylation and loss of chlorine. The distinctive chlorine isotope pattern seen with all of the chlorine containing molecules was absent. No adducts were detected with four compounds. Each of these had the methyl group removed and contained two or more halogens.
DISCUSSION

It is not our intention to imply that dasatinib is a particularly dangerous drug. Dasatinib fills an important niche in CML treatment and several ongoing clinical trials may prove dasatinib effective in the treatment of other cancers as well. However, in light of the recent report of dasatinib-induced acute hepatitis (Bonvin et al., 2008) and dasatinib-induced lupus (Rea et al., 2008), further studies on the metabolic fate of dasatinib is warranted to better understand the relationship between dasatinib metabolism and adverse effects. Additionally, because so many drugs in the clinic today have common structural moieties or are direct analogs of existing compounds, it is important to discern what can be modified to increase drug safety.

The finding that dasatinib was a mechanism-based inhibitor of CYP3A4 and that it is bioactivated to a reactive electrophile by CYP3A4 has significant patient implications. These results imply that dasatinib may potentially have pharmacokinetic drug-drug interactions when it is co-administered with drugs that are CYP3A4 substrates. Additionally, in a small percentage of individuals, immune mediated idiosyncratic hepatotoxicity may be observed by dasatinib adducting to cellular proteins in a manner similar to diclofenac (Schapira et al., 1986), carbamazepine (Moore et al., 1985; Wu et al., 2006), tienilic acid (Homberg et al., 1984), or dihydralazine (Beaune et al., 1996; Masubuchi and Horie, 2007).

Pharmacokinetic drug-drug interactions are usually a result of one drug altering the metabolism or elimination of another. The prescribing information of Sprycel/dasatinib notes a clinical drug-drug interaction study when dasatinib was codosed with simvastatin. Single dose administration of 100 mg dasatinib with simvastatin in 54 healthy subjects resulted in mean simvastatin $C_{max}$ and AUC increases of 37% and 20%, respectively. Full details of the
experimental conditions are not published, nor are additional studies where dasatinib is predosed, so it is not possible to approximate what percentage of the observed simvastatin exposure is due to irreversible mechanism-based inhibition of CYP3A4 or if the observed interaction would increase with repeated dosing. It is also not clear how the simvastatin hydroxy acid metabolite was accounted for, as its formation is not catalyzed by CYP3A4.

Pharmacokinetic drug-drug interactions with dasatinib are no doubt minimized by the high potency of dasatinib. Biochemical data for dasatinib inhibition of BCR-ABL and known BCR-ABL mutants found IC₅₀s between 0.1 and 3 nM and cell based values between 0.6 and 11 nM (O'Hare et al., 2005). Plasma dasatinib levels after a single 100 mg oral dose had a Cₘₐₓ of 215 nM (105 ng/ml) (Christopher et al., 2008b). While the plasma Cₘₐₓ is significantly below the Kᵢ determined in this study, the irreversible nature of mechanism based inhibitors leads to larger drug-drug interactions than a typical competitive inhibitor would. Unlike reversible inhibition, mechanism-based inhibitors may be unusually potent because the inactivation is cumulative and the enzymatic activity can only be restored after de novo protein synthesis. Additionally, hepatic accumulation of dasatinib or high intestinal and hepatic exposure during absorption may lead to higher than expected rates of CYP3A4 inactivation.

Findings in the current study that dasatinib is bioactivated through formation of both quinone-imine and imine-methide reactive intermediates indicates that dasatinib is likely to form protein adducts in the liver in addition to the adduction to glutathione shown in vitro. Quinone-imines are known to covalently modify cellular proteins and have been implicated in a number of drug-related adverse effects (Guengerich and MacDonald, 2007; Tang, 2007). This leads to the potential for idiosyncratic hepatotoxicity, sometimes referred to as immune-mediated.
Idiosyncratic hepatotoxicity or drug induced hepatitis. Idiosyncratic reactions are rare occurring in a small number of patients usually between 0.01 and 1% and do not follow a strict dose response. Based upon the hapten hypothesis where drug toxicity is mediated by drug–protein adduct formation (Griem et al., 1998). Injured cells are internalized by phagocytes, such as Kupffer cells and dendritic cells where they are processed and adducted peptides are presented by major histocompatibility complex class II to helper T cells. B cells producing autoantibodies or antibodies against haptenized protein mediate antibody-dependent toxicity (Walgren et al., 2005; Masubuchi and Horie, 2007).

It is widely recognized that additional signals are needed to initiate an immune response. This has lead to the “danger hypothesis”. The danger hypothesis suggests that MHC-II peptide presentation by the antigen presenting cell alone does not cause a physiological T-cell response (Matzinger, 1998). The danger hypothesis is that it is not the foreignness but rather the ability of a compound to trigger ‘alarm’ signals that determines whether it will induce an immune response. Hepatotoxic drugs may act as the source of antigen and provide the alarm by damaging the cell and causing necrotic cell death (Curtsinger et al., 1999).

Because dasatinib was able to be bioactivated through hydroxylation and oxidation to form a quinone-imine and through oxidation of the methyl to form an imine-methide intermediate, single modifications to the molecule were not successful in eliminating bioactivation or mechanism-based inhibition. Imine-methide formation requires the presence of a methyl group in the ortho or para position. Removal of the methyl group from these positions successfully eliminated the glutathione adducts resulting from imine-methide formation. Formation of quinone-imine intermediates required dasatinib hydroxylation. In theory quinone-
Imine intermediates could be formed by hydroxyl groups at either the ortho or para position. However, in the analogs that we generated, glutathione adducts appeared to proceed through hydroxylations at the para position as seen by the absence of detectable glutathione adducts in such compounds as 1250, and 1260. Perhaps hydroxylation at the ortho position is sterically hindered. Interestingly, blocking the para position with fluorine or chlorine did not prevent quinone-imine formation. Analogs containing fluorine or chlorine in the para position were found to be dehalogenated and resulted in the same glutathione adducts as their non-fluorinated counterparts. In fact the rates of inactivation were virtually identical and the $K_I$ for the fluorinated analogs were lower implying that they may be more efficiently bioactivated. We propose an oxidative mechanism proceeding via epoxide formation.

In conclusion, we have shown that dasatinib is a mechanism-based inactivator of CYP3A4 and is metabolized to generate reactive quinone-imine and imine-methide intermediates which spontaneously react with glutathione. Additionally, we have demonstrated that dasatinib bioactivation can be prevented by appropriate modification of the chloromethylphenyl ring.
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REFERENCES


FOOTNOTES

Scripps manuscript #19776.
FIGURE LEGENDS

Figure 1. Time- and concentration-dependent inactivation of CYP3A4 by Dasatinib. Six concentrations of dasatinib (0, 2.5, 5, 10, 20, and 40 μM) were incubated with human liver microsomes. Aliquots were removed and assayed for remaining CYP3A4 activity at various time points. Each point represented the mean from three separate replicates that do not differ by more than 10%. The remaining activity vs. incubation time is plotted and the slopes for the individual dasatinib concentrations are fit to the Kitz-Wilson plot (insert). The calculated Kᵢ and kᵢₐct for CYP3A4 inactivation were 6.3 μM and 0.034 min⁻¹, respectively.

Figure 2. Chromatographic separation of dasatinib-glutathione adducts generated in incubations with HLM and GSH. Incubation had 40 μM dasatinib, 1 mM NADPH, 5 mM GSH, and 1 mg/ml human liver microsomes (A). Ketoconazole, 1 μM, was added to incubation B. D-2OH-GSH (m/z 825.2 → 347.2) corresponds to a dihydroxylated dasatinib-glutathione adduct. D-OH-GSH (m/z 809.2 → 347.2) corresponds to a hydroxylated dasatinib-glutathione adduct. D-GSH (m/z 793.2 → 347.2) corresponds to a dasatinib-glutathione adduct. IS is 100 nM carbamazepine (m/z 237.0 → 194.0).

Figure 3. Fragmentation pattern of dasatinib and hydroxylated-glutathione adducts. Dasatinib-glutathione adducts were generated in an incubation mixture containing 40 μM dasatinib, 1 mM NADPH, 5 mM GSH, and 1 mg/ml human liver microsomes. The enhanced product ion scan is shown for dasatinib (A), D-OH-GSH (B), and D-2OH-GSH (C). The structure depicted represents one possible regioisomer.
Figure 4. Fragmentation pattern dasatinib-glutathione adduct (a) and (b). Dasatinib-glutathione adducts were generated in an incubation mixture containing 40 μM dasatinib, 1 mM NADPH, 5 mM GSH, and 1 mg/ml human liver microsomes. The enhanced product ion scan is shown for D-GSH (a) and D-GSH (b). The structures depicted are proposed adducts.

Figure 5. Proposed scheme for CYP3A4-mediated metabolic activation of Dasatinib. Dasatinib hydroxylation leads to the formation of a chemically-reactive p-quinone-imine intermediate (major pathway) or hydrogen abstraction of the methyl leads to an o-imine-methide intermediate (minor pathway). These reactive intermediates react with glutathione to form corresponding GSH adducts or may react with CYP3A4 and lead to enzyme inactivation. * labels the proposed sites of glutathione adduction.
Table 1. Kinetic constants for CYP3A4 time-dependent inactivation by dasatinib analogs.

**Dasatinib Analogs**

Modification of piperazine and methylpyrimidine

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<th>R</th>
<th>$K_i$ (μM)</th>
<th>$k_{inact}$ (min$^{-1}$)</th>
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<td></td>
<td>6.3</td>
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<td>984</td>
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Table 2. Kinetic constants for CYP3A4 time-dependent inactivation by dasatinib analogs.

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<th>$k_{inact}$ (min⁻¹)</th>
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<td>Dasatinib</td>
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<td>1622</td>
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<td>N.D. using concentrations up to 100 µM</td>
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Table 3. Glutathione-dasatinib/analog adducts. The bolded modification represents the most intense signal.

<table>
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<tr>
<th>Compound</th>
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No adducts were detected at concentrations up to 40 µM for:
Figure 1.

LN(% Activity Remaining)

Pre-incubation Time (min)

0 10 20 30 35

0 10 20 30 40 50

1/k observed

4.6 4.4 4.2 4 3.8 3.6

% Activity Remaining
Figure 2.
Figure 3.
Figure 5.

Desatinib Bioactivation and Glutathione Adduction

- If X is hydrogen
- If X is chlorine or ammonia

Imine-imine

Oxidation

+ Glutathione

Quinone-imine

+ Glutathione