In Vitro Assessment of Time-Dependent Inhibitory Effects on CYP2C8 and CYP3A Activity by Fourteen Protein Kinase Inhibitors

Anne M. Filppula, Pertti J. Neuvonen, and Janne T. Backman

Department of Clinical Pharmacology, University of Helsinki, Helsinki, Finland (A.M.F., P.J.N., J.T.B.) and HUSLAB, Helsinki University Central Hospital, Helsinki, Finland (P.J.N., J.T.B.)
Menten constant; $K_{\text{obs}}$, initial inactivation rate constant; P450, cytochrome P450.
Abstract

Previous studies have shown that several protein kinase inhibitors are time-dependent inhibitors of cytochrome P450 3A (CYP3A). We screened fourteen kinase inhibitors for time-dependent inhibition of CYP2C8 and CYP3A. Amodiaquine N-deethylation and midazolam 1'-hydroxylation were used as marker reactions for CYP2C8 and CYP3A activity, respectively. A screening, IC_{50}-shift, and mechanism-based inhibition were assessed with human liver microsomes. In the screening, bosutinib isomer 1, crizotinib, dasatinib, erlotinib, gefitinib, lestaurtinib, nilotinib, pazopanib, saracatinib, sorafenib, and sunitinib exhibited an increased inhibition of CYP3A after a 30-min preincubation with NADPH, as compared to no preincubation. Axitinib and vandetanib tested negative for time-dependent inhibition of CYP3A and CYP2C8, and bosutinib was the only inhibitor causing time-dependent inhibition of CYP2C8. The inhibitory mechanism by bosutinib was consistent with weak mechanism-based inhibition, and its inactivation variables K_{i} and k_{inact} were 54.8 µM and 0.018 1/min. As several of the tested inhibitors were reported to cause mechanism-based inactivation of CYP3A4 during the progress of this work, detailed experiments with these were not completed. However, lestaurtinib and saracatinib were identified as mechanism-based inhibitors of CYP3A. The K_{i} and k_{inact} of lestaurtinib and saracatinib were 30.7 µM and 0.040 1/min, and 12.6 µM and 0.096 1/min, respectively. Inhibition of CYP2C8 by bosutinib was predicted to have no clinical relevance, whereas therapeutic lestaurtinib and saracatinib concentrations were predicted to increase the plasma exposure to CYP3A-dependent substrates by ≥2.7-fold. The liability of kinase inhibitors to affect CYP450 enzymes by time-dependent inhibition may have long-lasting consequences and result in clinically relevant drug-drug interactions.
Introduction

In recent years, several therapeutic protein kinase inhibitors have been approved for cancer treatment and numerous are under investigation. These drugs target specific mutated or overexpressed protein kinase receptors, which are associated with cancer initiation or progression. Since cancer patients are typically treated with many medications concurrently, a careful assessment of the drug-drug interaction potential of these novel cancer drugs is important.

Recently, several protein kinase inhibitors such as dasatinib, erlotinib, gefitinib, imatinib, and lapatinib have been demonstrated to affect the drug-metabolizing enzyme cytochrome P450 (CYP) 3A4 by mechanism-based inhibition in vitro (Li et al., 2009; Li et al., 2010; Dong et al., 2011; Filppula et al., 2012; Kenny et al., 2012). Unlike reversible inhibition, mechanism-based inhibition leads to a permanent inactivation of the enzyme, so that enzyme activity can be regained only by synthesis of new enzyme. In vitro, this inhibition type is characterized by a concentration-, NADPH- and time-dependence, because the inhibitor is first metabolized to an intermediate, which then binds covalently to the metabolizing enzyme (Silverman, 1995; VandenBrink and Isoherranen, 2010). In the clinics, these characteristics can manifest in a slow onset and a long-lasting inhibition, making a careful assessment of this inhibition mechanism and its clinical consequences particularly important. For instance, imatinib 400 mg daily has increased the area under the plasma concentration-time curve (AUC) of the CYP3A4 substrate simvastatin on average by 3.5-fold, with individual changes for the subjects ranging from no increase to >10-fold increase in AUC (O’Brien et al., 2003). This interaction cannot be explained based on published values for direct CYP3A4 inhibition by imatinib (Filppula et al., 2012).
Besides altering the pharmacokinetics of concomitantly administered drugs, mechanism-based inhibitors may also affect their own metabolism by time-dependent autoinhibition. As most protein kinase inhibitors are reported to undergo metabolism by CYP3A4 (Di Gion et al., 2011; Scheffler et al., 2011; Pajares et al., 2012), variability in the expression of this enzyme together with a possible autoinhibition may be one explanation for the large interindividual variability (25-80%) observed in the clearance of protein kinase inhibitors (Sparreboom and Verweij, 2009; Di Gion et al., 2011; Pajares et al., 2012). Moreover, autoinhibition of CYP3A4 could increase the relative importance of other enzymes in the metabolism of these drugs.

For imatinib, autoinhibition of CYP3A4 has been suggested to increase the role of CYP2C8 in its pharmacokinetics during long-term medication (Filppula et al., 2013). Also other protein kinase inhibitors such as erlotinib, nilotinib, pazopanib, and sunitinib are metabolized by CYP2C8 to various degrees (http://www.accessdata.fda.gov/drugsatfda_docs/nda/2007/022068s000_ClinPharmR.pdf, http://www.accessdata.fda.gov/drugsatfda_docs/nda/2009/022465s000_MedR.pdf, http://www.accessdata.fda.gov/drugsatfda_docs/nda/2006/021938_S000_Sutent_BioPharmR.pdf)(Rakhit et al., 2008). During recent years, CYP2C8 has gradually gained increased attention as clinically relevant CYP2C8 substrates and inhibitors have been identified (Backman et al., 2002; Niemi et al., 2003a; Niemi et al., 2003b; Jaakkola et al., 2005; Niemi et al., 2006; Tornio et al., 2007; Karonen et al., 2010), functional CYP2C8 single nucleotide polymorphisms have been characterized and its crystal structure has been resolved (Dai et al., 2001; Bahadur et al., 2002; Schoch et al., 2004). These studies have shown that the active sites of CYP2C8 and CYP3A4 are similar in size, but differ in shape, which likely explains why CYP2C8 and CYP3A4 often have common
substrates but yield different metabolite profiles (Totah and Rettie, 2005; Aquilante et al.,
2013).

In the present study, we first screened fourteen kinase inhibitors for their potential to inhibit CYP2C8 and CYP3A by NADPH- and time-dependent inhibition. Inhibitors that caused an increased inhibition following preincubation with NADPH, as compared to no preincubation, and which had not previously been identified as time-dependent inhibitors, were further investigated in IC<sub>50</sub> and mechanism-based inhibition experiments. We then carried out static predictions to estimate the potential clinical relevance of our in vitro findings.
Materials and Methods

Chemicals and Microsomes.

Human liver microsomes (HLM) were obtained from BD Biosciences (Woburn, MA). Axitinib, bosutinib, bosutinib isomer 1, crizotinib, dasatinib, erlotinib, gefitinib, lestaurtinib, nilotinib, pazopanib, saracatinib, sorafenib, sunitinib, and vandetanib were purchased from LC Laboratories (Woburn, MA). Crizotinib, dasatinib and montelukast were from Sequoia Research Products Ltd. (Pangbourne, UK), and amodiaquine dihydrochloride dihydrate, ammonium formate and β-NADPH from Sigma Aldrich (St Louis, MO). N-desethylamodiaquine hydrochloride and N-desethylamodiaquine-d5 were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada), ketoconazole from Janssen Biotech (Olen, Belgium), midazolam from Hoffmann-La Roche & Co. Ltd. (Basel, Switzerland) and 1′-hydroxymidazolam from SPI-Bio (Montigny Le Bretonneux, France). Triazolam was obtained from the Upjohn Company (Kalamazoo, MI), and acetonitrile and methanol from Rathburn Chemicals Ltd. (Walkerburn, Scotland). Other chemicals were from Merck (Darmstadt, Germany).

Incubation Conditions.

Amodiaquine N-deethylation and midazolam 1′-hydroxylation were used as marker reactions for CYP2C8 and CYP3A activity, respectively. The incubations contained HLM (0.1 mg/ml protein; if not otherwise indicated) in 0.1 M sodium phosphate buffer (pH 7.4). Amodiaquine and midazolam concentrations were 2 or 10 µM depending on experiment, as described in detail below. To evaluate the inhibitory effects of axitinib, bosutinib, bosutinib isomer 1, crizotinib, dasatinib, erlotinib, gefitinib, lestaurtinib, pazopanib,
nilotinib, saracatinib, sorafenib, sunitinib, and vandetanib (Supplementary Figure S1) on CYP2C8 and CYP3A activities, inhibitor concentrations between 0.01 and 500 µM (depending on inhibitor tested) were added to the incubations mixtures.

In direct inhibition incubations, inhibitor or solvent control and substrate were premixed with microsomes and buffer for 3 min before addition of 1.0 mM NADPH, which initiated the reactions. In time-dependent inhibition incubations, the inhibitor or buffer control was preincubated with HLM for up to 30 min with NADPH, followed by addition of substrate to initiate the reaction. The final incubations were performed in a shaking water bath (37°C) for 2 min. Reactions were stopped by moving 100 µl sample to 300 µl mobile phase B solution containing N-desethylamodiaquine-d5 and triazolam as internal standards. Samples were thereafter handled as previously described (Filppula et al., 2012).

Amodiaquine, ketoconazole, midazolam, and montelukast were dissolved in methanol, bosutinib, bosutinib isomer 1, crizotinib, lestaurtinib, saracatinib, and vandetanib in ethanol, and all other inhibitor compounds in DMSO. In the preliminary screening and IC₅₀ experiments, the final solvent concentration in all incubations (including controls) with bosutinib, bosutinib isomer 1, crizotinib, lestaurtinib, saracatinib, and vandetanib was 1%, whereas in all incubations (including controls) for the other inhibitors that was 0.2%. In mechanism-based experiments with bosutinib, lestaurtinib and saracatinib, the solvent concentration was ≤1%. Due to solubility limitations, bosutinib concentrations >150 µM and vandetanib concentrations >180 µM could not be tested. Incubations were performed in duplicates (controls in triplicates), and the incubation time was within the linear range for the rates of metabolite formation.

Measurement of Drug Concentrations.
Sample handling and determination of N-desethylamodiaquine and 1'-hydroxymidazolam concentrations using an Agilent 1100 series high-performance liquid chromatography system (Agilent Technologies, Waldbronn, Germany) coupled to a SCIEX API 2000 tandem mass spectrometer (MDS Sciex, Concord, ON, Canada) were conducted as previously described (Filppula et al., 2012). The lower limit of quantification was 0.001 µM for N-desethylamodiaquine and 0.002 µM for 1'-hydroxymidazolam. The interday coefficient of variation for N-desethylamodiaquine was 16% at 0.001 µM, 9.4% at 0.01 µM and 6.7% at 0.1 µM and for 1'-hydroxymidazolam it was 15% at 0.002 µM, 11% at 0.02 µM and 7.4% at 0.2 µM. Due to technical issues, samples from the experiment with competitive inhibitors and part of the mechanism-based experiment for bosutinib were analyzed by use of an API 3000 liquid chromatography tandem mass spectrometry system (MDS Sciex) using the same method as with the API 2000 system. With the API 3000 system, the interday coefficient of variation for N-desethylamodiaquine was 13% at 0.001 µM, 11% at 0.01 µM and 2.0% at 0.1 µM and for 1'-hydroxymidazolam it was 13% at 0.002 µM, 11% at 0.02 µM and 3.4% at 0.2 µM.

Screening and IC_{50} Shift Experiments.

Inhibitor concentrations causing approximately 50% and 20-30% direct inhibition of CYP2C8 and CYP3A activity, or buffer control were 1) simultaneously incubated with 2 µM substrate (direct inhibition), or 2) first preincubated with NADPH for 30 min, after which 2 µM substrate was added to the mixture (metabolism-dependent inhibition). Compounds inhibiting CYP2C8 and/or CYP3A activity in a time-dependent manner, and which had not previously been identified as time-dependent inhibitors, were further investigated in IC_{50} shift experiments. In addition, as no IC_{50} values for CYP2C8 and CYP3A inhibition by axitinib had been published at the time of the study, we also
conducted IC\textsubscript{50} incubations for axitinib. The IC\textsubscript{50} experiments were carried out similarly as the screening, but inhibitor concentrations tested ranged from 0.01-500 µM, causing enzyme inhibition from 0 to nearly 100%. In the screening, bosutinib isomer 1 was also tested but it was not included in further experiments as it is not under investigation as a therapeutic drug.

**Mechanism-Based Inhibition and Follow-Up Experiments.**

Inhibitors that caused a decrease of ≥1.5-fold for IC\textsubscript{50} with a 30-min preincubation, as compared to no preincubation (Grimm et al., 2009), were selected for characterization of time-dependent inhibition. Briefly, various inhibitor concentrations were preincubated with 0.5 mg/ml HLM and NADPH for up to 30 min. At determined time points, an aliquot of 15 µl of the preincubation mixture was moved to another tube containing NADPH and 10 µM amodiaquine or midazolam in buffer in a final incubation volume of 300 µl. Accordingly, the protein and inhibitor concentrations had been diluted 20-fold and a substrate concentration higher than its Michaelis-Menten constant (K\textsubscript{m}) was used to minimize direct inhibition by the inhibitors.

To determine the effect of a competitive inhibitor on the time-dependent inactivation of CYP2C8 by bosutinib and of CYP3A by lestaurtinib and saracatinib, preincubation mixtures were prepared as above. However, for bosutinib, preincubations were carried out with or without montelukast (0.5 and 5 µM) as CYP2C8 inhibitor, both in the absence (control) and presence of bosutinib (150 µM). Similarly, for lestaurtinib and saracatinib, preincubation tubes contained ketoconazole (0.1 and 1 µM) as CYP3A inhibitor, without (control) or with lestaurtinib (30 µM) or saracatinib (10 µM). Mixtures were then preincubated for 30 min; aliquots were transferred to incubation tubes (20-fold dilution)
and the residual amodiaquine deethylation (for bosutinib) or midazolam 1'-hydroxylation activity (for lestaurtinib and saracatinib) was measured as previously described.

To evaluate the effect of dialysis on the inhibitory effects of bosutinib, lestaurtinib and saracatinib, HLM (0.5 mg/ml) were preincubated with or without inhibitor (150 µM bosutinib, 200 µM lestaurtinib or 64 µM saracatinib) and NADPH for 30 min. The preincubation mixtures were then immediately dialysed against 0.1 M sodium phosphate buffer (pH 7.4) three times for 2 h in a volume of 2 l at 4°C, and followed by a 20-fold dilution and measurement of CYP2C8 or CYP3A marker reactions as explained above.

Data Analysis.

IC₅₀ values were determined by nonlinear regression analysis using SigmaPlot (version 11.0; Systat Software, Inc., San Jose, CA). For estimation of metabolism-dependent inactivation constants, preincubation time-dependent loss of CYP2C8 or CYP3A activity in the absence of the inhibitor was accounted for by adjusting the observed metabolism rate with reference to the respective control (vehicle) incubation at each preincubation time. The initial rate constant of cytochrome P450 (P450) inactivation (K(obs)) by each inhibitor concentration was determined by linear regression analysis of the natural logarithm of the initial linear portion of the plotted percentage of activity remaining versus preincubation time (Kitz and Wilson, 1962). Thereafter, the K(obs) values were used to determine the maximum inactivation rate (k_inact) and the inhibitor concentration needed to cause half of k_inact (Kᵢ). Preliminary estimates of Kᵢ and k_inact were obtained from a double-reciprocal plot of K(obs) (y-axis) versus inhibitor concentration [I] (x-axis)(Kitz and Wilson, 1962). Final Kᵢ and k_inact values were then estimated by nonlinear regression using the following equation: K(obs) = k_inact × [I] / (Kᵢ + [I]) (Kitz and Wilson, 1962; Jones et al., 1999).
Drug-Drug Interaction Predictions.

Obtained $K_i$, $k_{\text{inact}}$ and direct IC$_{50}$ values were used for predictions of the potential clinical impact of the inhibition. For substrates that are metabolized in both intestine and liver, the following equation can be used to simultaneously evaluate the potential clinical impact of both competitive (direct) and mechanism-based inhibition (Fahmi et al., 2008):

$$\frac{AUC_{po,i}}{AUC_{po}} = \frac{1}{[A \times B] \times f m_{\text{P450i}} + (1 - f m_{\text{P450i}})} \times \frac{1}{[C \times D] \times (1 - F_G) + F_G}$$

where $AUC_{po,i}$ and $AUC_{po}$ are the areas under the concentration-time curve of the substrate in the presence and absence of an inhibitor, respectively, $fm_{\text{P450i}}$ expresses the fraction of the substrate dose cleared by a specific P450 enzyme and $F_G$ describes the intestinal bioavailability of the substrate. $A$ and $C$ are mechanism-based inhibition components in the liver and intestine, respectively, and $B$ and $D$ are competitive inhibition components in the liver and intestine, respectively:

$$A, C = \frac{k_{\text{deg}}}{k_{\text{deg}} + \frac{k_{\text{inact}} \times [I]}{K_I + [I]}}$$

$$B, D = \frac{1}{1 + \frac{[I]}{K_I}}$$

$k_{\text{deg}}$ is the rate constant of hepatic or intestinal P450 degradation in the absence of the inhibitor. In our predictions, a half-life of 22 h ($k_{\text{deg}}$ corresponds to 0.00053 1/min) was used for CYP2C8 (Backman et al., 2009). The CYP3A inhibition observed in our incubations was assumed to be completely due to inhibition of CYP3A4, and 36 h and 23 h were used as the CYP3A4 half-life in the liver and intestine, respectively (corresponding to $k_{\text{deg}}$ values of 0.00032 and 0.00050 1/min, respectively)(Fromm et al.,
DMD #57695

1996; Greenblatt et al., 2003; Rowland Yeo et al., 2011). As no direct $K_i$ values were determined in the present study, we used direct $IC_{50}/2$ instead. Predictions were carried out for clinically relevant unbound plasma concentrations of the inhibitors. Intestinal inhibitor concentrations were calculated assuming no binding of the inhibitor to enterocytic proteins ($f_{u,gut} = 1$).

First, predictions considering direct and mechanism-based inhibition of hepatic CYP2C8 and CYP3A4 by bosutinib, lestaurtinib and saracatinib were carried out for substrates with fraction metabolized by CYP2C8 or CYP3A4 varying from 0.5 to 1. These predictions were related to steady state unbound trough ($C_{\text{trough,}\,u}$), peak ($C_{\text{max,}\,u}$) and hepatic inlet peak ($C_{\text{max,}\,u,\text{hep,inlet}}$) concentrations of the inhibitors in plasma during standard dosing regimen (Supplementary Table S1). The unbound hepatic inlet peak concentrations were calculated according to (Ito et al., 1998):

$$C_{\text{max,}\,u,\text{hep,inlet}} = f_u \times \left( C_{\text{max}} + \frac{\text{dose} \times k_a \times f_a}{Q_h} \right)$$

where $f_u$ represents the plasma unbound fraction, $k_a$ is the absorption rate constant, $f_a$ is the fraction absorbed, and $Q_h$ expresses the hepatic blood flow (20.7 ml/min/kg) (Houston and Galetin, 2008). $f_a$ was assumed to be 0.8 (Kenny et al., 2012). $f_u$, $k_a$ and dose for each inhibitor are described in Supplementary Tables S1-S2. In addition, predictions based on direct inhibition of CYP2C8 and CYP3A4 by axitinib and of CYP2C8 by gefitinib were conducted using the described equations (Supplementary Tables S1-S2).

Then, the reduction in intestinal CYP3A4 activity after administration of bosutinib, lestaurtinib and saracatinib was calculated as follows:

$$\text{Remaining intestinal CYP3A4 activity} = C \times D$$
using intestinal inhibitor concentrations $[I]_G$. These concentrations were estimated using the following equation (Rostami-Hodjegan and Tucker, 2004):

$$[I]_G = \frac{dose \times k_a \times f_a}{Q_{ent}}$$

where $Q_{ent}$ represents the enteroic blood flow (248 ml/min) (Obach et al., 2007) (Supplementary Table S2).

Finally, CYP3A4 inhibitor interactions with midazolam were predicted with the assumption that $f_{\text{mCYP3A4}}$ and $F_G$ of midazolam are 0.94 and 0.51 (Galetin et al., 2006; Gertz et al., 2010), respectively.
Results

Screening of Time-Dependent Inhibition of CYP2C8 in HLM Incubations.

In the screening, all inhibitors tested caused direct CYP2C8 inhibition at different concentrations. However, axitinib, nilotinib, and sorafenib were identified as potent direct inhibitors of CYP2C8, causing a ~50% decrease in CYP2C8 activity at low concentrations (0.15 µM, 0.2 µM, and 0.7 µM, respectively) (Fig. 1, Supplementary Fig. S2 and Supplementary Table S3). At the concentrations tested, bosutinib, gefitinib, and saracatinib caused a moderate or small time- and NADPH-dependent increase in inhibition of CYP2C8 activity (Fig. 1, Supplementary Fig. S2), and were further tested in IC₅₀ experiments. Compared to no preincubation, inhibition of CYP2C8 decreased after a 30-min preincubation with NADPH in incubations with axitinib, dasatinib, erlotinib, lestaurtinib, nilotinib, pazopanib, sorafenib, and sunitinib, suggesting that these inhibitors had been extensively metabolized during the preincubation to metabolites that did not affect CYP2C8 activity. Unlike in incubations with bosutinib, preincubation of bosutinib isomer 1 with NADPH did not increase CYP2C8 inhibition. In addition, preincubation of crizotinib with NADPH did not alter its inhibition of CYP2C8, as compared to direct inhibition.

Screening of Time-Dependent Inhibition of CYP3A in HLM Incubations.

As previously recognized in the literature (Supplementary Table S4), the inhibition of CYP3A activity by crizotinib, dasatinib, erlotinib, gefitinib, nilotinib, pazopanib, sorafenib, and sunitinib increased after preincubation for 30 min with NADPH (Fig. 1, Supplementary Fig. S1). As new findings, lestaurtinib and saracatinib also affected CYP3A in this way, and they were therefore further tested in IC₅₀ experiments. In
addition, unlike bosutinib, preincubation of bosutinib isomer 1 with NADPH increased the inhibition of CYP3A. With the exception of erlotinib, gefitinib, and vandetanib, all inhibitors tested affected CYP3A by direct inhibition. Vandetanib (180 µM) did not inhibit CYP3A at all, while erlotinib and gefitinib markedly stimulated the formation of 1'-hydroxymidazolam in direct inhibition experiments (Fig. 1, Supplementary Fig. S2).

IC_{50} Experiments.

Preincubation of bosutinib with NADPH resulted in a 2.6-fold decrease in its IC_{50} value for CYP2C8 inhibition to 16.9 µM (Fig. 2), and it was therefore further tested in mechanism-based experiments. In contrast to preliminary findings, gefitinib and saracatinib did not increase the inhibition of CYP2C8 following preincubation. Following preincubation, the IC_{50} value for CYP3A inhibition by lestaurtinib decreased 2.2-fold to 2.1 µM. Likewise, after preincubation the IC_{50} of saracatinib was markedly decreased, by 26-fold to 1.8 µM.

Mechanism-Based Inhibition of CYP2C8 and CYP3A.

The inhibition of CYP2C8 activity by bosutinib and of CYP3A activity by lestaurtinib and saracatinib were concentration-, NADPH- and preincubation time-dependent (Fig. 3). With the nonlinear regression method, apparent inactivation variables K_i and k_{inact} for CYP2C8 by bosutinib were estimated to 54.8 µM and 0.018 1/min. For lestaurtinib, the K_i and k_{inact} for CYP3A inhibition were 30.7 µM and 0.040 1/min, and for saracatinib 12.6 µM and 0.096 1/min. The k_{inact} values imply that approximately 2% of CYP2C8, and 4 and 10% of CYP3A is inactivated each minute when saturating concentrations of bosutinib, lestaurtinib and saracatinib, respectively, are incubated with HLM.
In subsequent experiments, ketoconazole reduced the lestaurtinib- and saracatinib-induced inactivation of CYP3A, in a concentration-dependent manner (Table 1). Montelukast reduced the inactivation of CYP2C8 by bosutinib only slightly. However, dialysis of preincubated HLM-bosutinib mixture for $3 \times 2$ h at 4°C did not abolish the inhibitory effect of bosutinib on amodiaquine deethylation (Table 2). Similarly, dialysis of preincubated HLM-lestaurtinib and HLM-saracatinib solution did not abolish the inhibitory effects of lestaurtinib and saracatinib on midazolam 1'-hydroxylation.

**Prediction of In Vivo Drug Interactions Due to Inhibition of CYP2C8 or CYP3A.**

The predicted fold increase in the AUC of a victim drug with different fractions metabolized by CYP2C8 or CYP3A4 is shown in Fig. 4, when reversible and mechanism-based inhibition of hepatic CYP2C8 or CYP3A4 are the contributing mechanisms. Using bosutinib plasma unbound peak and estimated hepatic inlet concentrations of 0.015 and 0.23 µM, respectively (Daud et al., 2012), the predicted maximal AUC increase was small for substrates completely metabolized by CYP2C8 ($\leq 1.2$-fold). Also, predictions based on direct CYP2C8 inhibition only (calculated using direct IC$_{50}$ values) by unbound peak concentrations of axitinib, gefitinib, lestaurtinib and saracatinib, resulted in minimal AUC increases ($\leq 1.1$-fold; Supplementary Table S2).

For lestaurtinib, predictions based on its calculated plasma unbound $C_{\text{max}}$ (0.28 µM) after doses of 80 mg BID (Supplementary Table S2)(http://aml17.cardiff.ac.uk/files/aml17_protocolv2.pdf), yielded a predicted AUC increase of 2.7 for a substrate completely metabolized by hepatic CYP3A4. The corresponding increase after dosing of saracatinib 175 mg once daily (unbound $C_{\text{max}}$ of 0.082 µM)(Baselga et al., 2010) was predicted at 3.2. When using unbound hepatic inlet concentrations, lestaurtinib and saracatinib were predicted to cause maximal AUC
increases of 2.8- and 5.5-fold, respectively. Furthermore, lestaurtinib and saracatinib were predicted to completely reduce (>99%) the activity of intestinal CYP3A4 at intestinal inhibitor concentrations of 9.8 and 4.3 µM, respectively (data not shown).

For midazolam, when considering inhibition of both intestinal and hepatic CYP3A4, the maximal AUC increase was predicted to 4.7- and 5.5-fold after coadministration with lestaurtinib and saracatinib, respectively, when unbound peak concentrations and the above doses of the inhibitors were used (Supplementary Table S2).

Predictions based on direct inhibition of hepatic CYP3A4 by axitinib and bosutinib, and of CYP2C8 by gefitinib resulted in minor effects on the concentrations of a substrate completely metabolized by CYP3A or CYP2C8 (≤1.1-fold AUC increase)(Supplementary Table S2). However, intestinal axitinib and bosutinib concentrations of 0.28 and 31 µM, respectively, were predicted to reduce the activity of intestinal CYP3A4 by 8 and 90% (data not shown).
Discussion

In the present study, fourteen protein kinase inhibitors were initially screened for their potential time-dependent inhibitory effects on CYP2C8 and CYP3A activity. As compared to no preincubation, one inhibitor (bosutinib) exhibited increasing CYP2C8 inhibition and twelve inhibitors (bosutinib isomer 1, crizotinib, dasatinib, erlotinib, gefitinib, lestaurtinib, nilotinib, pazopanib, saracatinib, sorafenib and sunitinib) caused increasing inhibition of CYP3A, following preincubation with NADPH. Eight of these inhibitors were recently reported to be time-dependent CYP3A inhibitors (Li et al., 2009; Li et al., 2010; Dong et al., 2011; Kenny et al., 2012; Mao et al., 2013), and were not further investigated. As novel findings, however, lestaurtinib and saracatinib were identified as irreversible mechanism-based inhibitors of CYP3A, and bosutinib as a weak irreversible mechanism-based inhibitor of CYP2C8. Consistent with criteria for mechanism-based inhibition (Silverman, 1995), these conclusions are based on the following findings: the inhibitory effect on amodiaquine deethylation by bosutinib and on midazolam 1'-hydroxylation by lestaurtinib and saracatinib were dependent on preincubation time, inhibitor concentration and NADPH; the presence of a competitive inhibitor decreased the inhibition; and dialysis was unable to restore enzyme activity.

The obtained IC$_{50}$ value for direct CYP3A inhibition by lestaurtinib (4.7 µM) is in good agreement with previously reported $K_i$ values (4.3-5.2 µM) (http://issx.confex.com/issx/15na/webprogram/Paper11788.html, http://aml17.cardiff.ac.uk/files/aml17_protocolv2.pdf). However, preincubation with NADPH decreased the IC$_{50}$ 2.2-fold and in mechanism-based experiments the $K_i$ and $k_{inact}$ of lestaurtinib were determined to 31 µM and 0.040 1/min. Among the inhibitors tested, saracatinib caused the greatest, a 26-fold, reduction in IC$_{50}$ for CYP3A following preincubation, and its $K_i$ and $k_{inact}$ for CYP3A inhibition were 12.6 µM and 0.096 1/min. In
our CYP2C8 experiments, bosutinib inhibited CYP2C8 with a direct IC$_{50}$ of 43 µM and preincubation with NADPH reduced the IC$_{50}$ 2.6-fold. Furthermore, the inhibition of CYP2C8 by bosutinib proceeded in a time-, concentration- and NADPH-dependent manner, suggesting that it affects CYP2C8 by mechanism-based inhibition. Apparent $K_i$ and $k_{inact}$ values for CYP2C8 inactivation by bosutinib were estimated to 54.8 µM and 0.018 1/min. Our findings differ from those of a recent study, where bosutinib did not exhibit time-dependent inhibitory effects on paclitaxel 6α-hydroxylation, another CYP2C8 marker reaction (Wang et al., 2014). In addition, in this recent study, the inhibition of paclitaxel p-3'-hydroxylation, which was used as a probe for CYP3A4 activity, increased following preincubation with NADPH as compared to no preincubation. As our results for CYP3A inhibition by bosutinib seem to be in reasonably good agreement with those of the manufacturer of bosutinib (no time-dependent inhibition, $K_i$ of 27 µM) (http://www.accessdata.fda.gov/drugsatfda_docs/nda/2012/203341Orig1s000ClinPharmR.pdf), possible explanations for this discrepancy could be differences in experimental conditions, e.g. the substrate used and/or uncertainties concerning the inhibitor compound. The bosutinib isomer 1 was originally unintentionally sold to the research community (also to us) as authentic bosutinib by several vendors (http://www.pkcpharma.com/TwoOrMoreBosutinibs.html)(Levinson and Boxer, 2012; Beeharry et al., 2013; Braun and Schweizer, 2014). The bosutinib results of the recent study correspond well with the inhibitory characteristics of bosutinib isomer 1 in our study. It should also be noted that this recent study observed weak mechanism-based inhibition of CYP3A4 by axitinib (Wang et al., 2014). In our study, detailed time-dependent inhibition studies were not carried out with axitinib, because preincubation reduced its IC$_{50}$ CYP3A inhibition by less than 1.5-fold, which is a common criterion for time-dependent inhibition studies (Grimm et al., 2009). Thus, our results are in line with previous literature (Supplementary Tables S3 and S4).
When considering previous and present results, it seems that many kinase inhibitors affect P450 enzymes, in particular CYP3A, by mechanism-based inhibition. In this inhibition type, the inhibitor is first metabolized to a reactive intermediate, which inactivates the metabolizing enzyme by binding covalently to it (Silverman, 1995). For instance, dasatinib is proposed to be bioactivated through hydroxylation and oxidation of its chloromethylphenyl ring, forming reactive quinone-imines and imine-methides, which may inactivate CYP3A4 (Li et al., 2009). For imatinib, its piperazine and p-toluidine structures have been suggested to undergo hydroxylation and desaturation to imine and imine-methide intermediates, respectively (Li et al., 2014). Quinone-iminies and imine-methides are reactive electrophiles, which besides binding to P450 enzymes, may also adduct to other cellular proteins and initiate immune-mediated idiosyncratic reactions (Kalgutkar et al., 2007). Both bosutinib and saracatinib contain a piperazine ring, which following bioactivation could be responsible for the inactivation of CYP2C8 and CYP3A observed in our study. Interestingly, while bosutinib exhibited time-dependent inhibitory effect on CYP2C8 but not on CYP3A, bosutinib isomer 1 displayed opposite effects on these enzymes in the screening. However, as numerous structural elements have been suggested to cause mechanism-based inhibition (Kalgutkar et al., 2005), more studies are needed to identify the bioactivation pathways of the mechanism-based inhibitors tested in the present study.

Due to its irreversibility and time- and concentration-dependent nature, mechanism-based inhibition may result in slow onset, cumulative and long-lasting drug-drug interactions in vivo. In this study, we used static predictions considering both direct and mechanism-based inhibition to estimate the total drug-drug interaction risk of our in vitro findings. For bosutinib, interaction predictions based on unbound peak bosutinib concentrations resulted in insignificant AUC increases for CYP2C8 substrates. When
considering inhibition of hepatic CYP3A4, unbound peak lestaurtinib and saracatinib concentrations were predicted to increase the AUC of a sensitive CYP3A4 substrate by up to 2.7- and 3.2-fold, respectively. In addition, intestinal concentrations of these inhibitors were estimated to completely inactivate CYP3A4 in the intestine (>99%). Thus, when intestinal CYP3A4 inhibition was included in the predictions, lestaurtinib and saracatinib were predicted to increase midazolam exposure by 4.7- and 5.5-fold, respectively. Using unbound hepatic inlet inhibitor concentrations, the interaction magnitudes were even higher (up to 8.4-fold). Thus, the predicted inhibition potency of lestaurtinib and saracatinib seems to be higher than that generally observed for kinase inhibitors, which affect CYP3A4 by mechanism-based inhibition (typically AUC increases of <2-fold) (Supplementary Table S5) (Kenny et al., 2012). However, some of these interaction studies have used a single inhibitor dose, which might have underestimated the maximal interaction magnitude, as the effect of mechanism-based inhibition can proceed with time and increasing inhibitor concentration. For instance, during multiple dosing, if the dosing frequency of inhibitor is higher than the synthesis rate of new enzyme, the result can be an accumulating enzyme inactivation with increasing time and doses. Lestaurtinib and saracatinib are currently in phase II/III (www.clinicaltrials.gov), and no official drug-drug interaction studies have been reported for these compounds yet.

Another possible consequence of mechanism-based inhibition is autoinhibition of the metabolism of the inhibitor itself. In our study, all inhibitors causing time-dependent CYP3A inhibition undergo metabolism by CYP3A4 (Supplementary Table S6). Therefore, it is possible that these inhibitors may inhibit their own CYP3A4-mediated metabolism during multiple dosing, so that other enzymes become more important than CYP3A4 with time. For instance, saracatinib exhibits nonlinear pharmacokinetics with a
3.8-fold accumulation at 50 mg once daily to 4.8-fold at 250 once daily (Baselga et al., 2010), which might be partly due to inhibition of its own CYP3A4-mediated metabolism and accumulation of parent drug in hepatocytes. Thus, variability in CYP3A4 expression or activity together with mechanism-based CYP3A4 inhibition may partly explain the great variations observed in kinase inhibitor concentrations between patients. In addition, it highlights the potential importance of other enzymes in the metabolism of these kinase inhibitors.

Interestingly, as an additional finding and also observed in previous studies (Li et al., 2007; Dong et al., 2011; Kenny et al., 2012), erlotinib and gefitinib stimulated midazolam 1'-hydroxylation in our direct inhibition experiments. Sorafenib and sunitinib have also been reported to activate the formation of 1'-hydroxymidazolam (Sugiyama et al., 2011; Kenny et al., 2012), but this was not observed in our incubations. The heteroactivation mechanism for these kinase inhibitors has been reported to be substrate-dependent, as it has been observed with midazolam but not with the other CYP3A marker substrates nifedipine and testosterone (Li et al., 2007; Dong et al., 2011; Kenny et al., 2012). It is not clear if the activation by erlotinib and gefitinib occurs via CYP3A4 or CYP3A5, but for sorafenib and sunitinib it is reported to occur via CYP3A5 (Sugiyama et al., 2011). In the present study, we did not differentiate between CYP3A4 and CYP3A5, but the lack of stimulation of midazolam 1'-hydroxylation by sorafenib and sunitinib suggests that CYP3A4 activity was more prominent than that of CYP3A5 in the HLM batch used. The detailed mechanism and clinical relevance of the substrate-dependent heteroactivation by these inhibitors are currently unknown.

In conclusion, our study shows that bosutinib affects CYP2C8 by weak irreversible mechanism-based inhibition, and lestaurtinib and saracatinib are irreversible mechanism-based inhibitors of CYP3A. Furthermore, our findings support recent reports
showing that crizotinib, dasatinib, erlotinib, gefitinib, nilotinib, pazopanib, sorafenib and sunitinib are time-dependent CYP3A inhibitors. Thus, it appears that many members of this novel drug class contain structural elements, which are sensitive to bioactivation reactions forming reactive intermediates. Bioactivation may have consequences in terms of idiosyncratic drug reactions and drug-drug interactions, and it may ultimately also affect the pharmacokinetics of the inhibitor itself. Further studies are needed to elucidate why protein kinase inhibitors affect CYP450 enzymes by mechanism-based inhibition, and its clinical relevance.
Acknowledgments

We thank Jouko Laitila for skillful technical assistance.
Authorship Contributions

Participated in research design: Filppula, Neuvonen, and Backman.

Conducted experiments: Filppula.

Performed data analysis: Filppula, Neuvonen, and Backman.

Wrote or contributed to the writing of the manuscript: Filppula, Neuvonen, and Backman.
References


Footnotes

This work was supported by the Helsinki University Central Hospital Research Fund, Helsinki, Finland. Filppula was also supported by the FinPharma Doctoral Program (FPDP), Helsinki, Finland and the Orion-Farmos Research Foundation, Espoo, Finland.

Reprint requests should be addressed to the corresponding author Prof. Janne T. Backman, Department of Clinical Pharmacology, University of Helsinki and HUSLAB, Helsinki University Central Hospital, P.O. Box 705, FI-00029 HUS, FINLAND (E-mail: janne.backman@helsinki.fi).
Figure legends

Fig. 1.

Effects of protein kinase inhibitors on amodiaquine N-deethylation (CYP2C8 marker reaction) and midazolam 1′-hydroxylation (CYP3A marker reaction) with or without a 30 min preincubation in the presence of NADPH in the screening. The inhibitors are ordered according to their time-dependent inhibitory effect. Erlotinib and gefitinib caused stimulation of midazolam 1′-hydroxylation in direct inhibition experiments (also see Supplementary Fig. S2). The micromolar concentrations of the inhibitors are given. Incubations were conducted in HLM (0.1 mg/ml protein) with 2 µM substrate. Data points are mean + SD values of duplicate incubations. Abbreviations: BOSU, bosutinib; BOSU iso, bosutinib isomer 1; CRIZ, crizotinib; DASA, dasatinib; ERLO, erlotinib; GEFI, gefitinib; LEST, lestaurtinib; NILO, nilotinib; PAZO, pazopanib; SARA, saracatinib; SORA, sorafenib; SUNI, sunitinib. The results of the whole screening are presented in Supplementary Fig. S2.

Fig. 2.

Effects of five protein kinase inhibitors on amodiaquine N-deethylation (CYP2C8 marker reaction) and/or midazolam 1′-hydroxylation (CYP3A marker reaction) with or without a 30 min preincubation in the presence of NADPH. Incubations were conducted in HLM (0.1 mg/ml protein) with 2 µM substrate. Data points are mean ± SD values of duplicate incubations.

Fig. 3.

Preincubation time- and concentration-dependent inhibition of amodiaquine N-deethylation by bosutinib (0-150 µM) and of midazolam 1′-hydroxylation by lestaurtinib (0-200 µM) and saracatinib (0–64 µM) in HLM incubations (0.5 mg/ml). Aliquots were
removed from the preincubation mixtures at indicated time points and diluted 20-fold for measurement of residual CYP2C8 or CYP3A activity. The rate of inactivation of CYP3A activity by each inhibitor concentration ($K_{obs}$) was determined by linear regression analysis of the natural logarithm of the percentage of activity remaining versus preincubation time data (left panel). The $K_i$ and $k_{inact}$ were calculated by nonlinear regression analysis of the $K_{obs}$ versus inhibitor concentration data according to the equation described in Materials and Methods (middle panel) and by linear regression of the double-reciprocal plot of the $K_{obs}$ versus inhibitor concentration (right panel). Data points are mean ± SD values of duplicate (inhibitor) or triplicate (control) incubations.

**Fig. 4.**

Prediction of the effect of bosutinib on the pharmacokinetics of CYP2C8 substrates, and that of lestaurtinib and saracatinib on the pharmacokinetics of CYP3A4 substrates in vivo ($AUC_{po,i}/AUC_{po}$), assuming that intestinal bioavailability is unaffected. The figures illustrate how the effect of the kinase inhibitor on the AUC of the substrate depends on the concentration of inhibitor at the enzyme site and on the fraction of the substrate metabolized by CYP2C8 or CYP3A4 ($fm_{P450}$ varying from 0.5 to 1.0). Predictions were carried out using the equation for both direct and mechanism-based inhibition. During treatment with bosutinib 500 mg daily, its average unbound peak concentration in plasma approximates to 0.021 µM (Daud et al., 2012), and its unbound peak concentration at the hepatic inlet was estimated to 0.23 µM (indicated by the cyan-colored area). The corresponding concentrations for lestaurtinib after treatment with 80 mg twice daily were estimated to 0.28 and 0.28 µM (http://aml17.cardiff.ac.uk/files/aml17_protocolv2.pdf), respectively, and for saracatinib after treatment with 175 mg once daily to 0.082 and 0.16 µM (Baselga et al., 2010),
respectively. In addition, for saracatinib, the unbound steady state trough concentration of 0.040 µM is indicated by the border of the dark cyan area.
Tables

Table 1.

The effect of the competitive CYP2C8 inhibitor montelukast on the preincubation-dependent inactivation of CYP2C8 by bosutinib (150 µM) and that of the CYP3A inhibitor ketoconazole on the preincubation-dependent inactivation of CYP3A by lestaurtinib (30 µM) and saracatinib (10 µM).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bosutinib alone</td>
<td>52.4 ± 1.1</td>
</tr>
<tr>
<td>Bosutinib with montelukast (0.5 µM)</td>
<td>57.9 ± 0.9</td>
</tr>
<tr>
<td>Bosutinib with montelukast (5 µM)</td>
<td>61.8 ± 0.0</td>
</tr>
<tr>
<td>Lestaurtinib alone</td>
<td>48.5 ± 4.6</td>
</tr>
<tr>
<td>Lestaurtinib with ketoconazole (0.1 µM)</td>
<td>53.2 ± 3.3</td>
</tr>
<tr>
<td>Lestaurtinib with ketoconazole (1 µM)</td>
<td>60.4 ± 1.1</td>
</tr>
<tr>
<td>Saracatinib alone</td>
<td>28.5 ± 0.6</td>
</tr>
<tr>
<td>Saracatinib with ketoconazole (0.1 µM)</td>
<td>35.7 ± 2.8</td>
</tr>
<tr>
<td>Saracatinib with ketoconazole (1 µM)</td>
<td>70.1 ± 5.6</td>
</tr>
</tbody>
</table>

Each experiment is compared with its own control, that is, either preincubation without kinase inhibitor or preincubation with competitive inhibitor but without kinase inhibitor. In the experiments, 30 min preincubations including HLM (0.5 mg/ml) and NADPH were carried out with or without competitive inhibitor, both in the absence (controls) and presence of kinase inhibitor, followed by 20-fold dilution for measurement of residual CYP2C8 or CYP3A activity. Values are expressed as mean ± SD of duplicates (controls were carried out as triplicates).
Table 2.

The effect of dialysis on the preincubation-dependent inactivation of CYP2C8 by bosutinib (150 µM), and of CYP3A by lestaurtinib (200 µM) and saracatinib (64 µM).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bosutinib prior to dialysis</td>
<td>61.7 ± 3.4</td>
</tr>
<tr>
<td>Bosutinib with dialysis</td>
<td>60.3 ± 0.0</td>
</tr>
<tr>
<td>Lestaurotinib prior to dialysis</td>
<td>32.7 ± 3.2</td>
</tr>
<tr>
<td>Lestaurotinib with dialysis</td>
<td>17.3 ± 5.9</td>
</tr>
<tr>
<td>Saracatinib prior to dialysis</td>
<td>21.6 ± 1.3</td>
</tr>
<tr>
<td>Saracatinib with dialysis</td>
<td>16.7 ± 3.0</td>
</tr>
</tbody>
</table>

Each experiment is compared with its own control, that is, either preincubation without inhibitor or preincubation without inhibitor followed by dialysis (3 × 2 h at 4°C). In the experiments, HLM (0.5 mg/ml) and NADPH were preincubated with or without inhibitor for 30 min. The samples were immediately dialyzed against sodium phosphate buffer three times for 2 h, followed by a 20-fold dilution and measurement of CYP2C8 or CYP3A4 activity. Values are expressed as mean ± SD of duplicates or more.
Figure 1.

[Bar chart showing CYP2C8 and CYP3A activity with different drugs at varying concentrations.
- CYP2C8: BASA 500, SARA 100, BOSU iso 5, SARA 5, CRIZ 30, BASA 100, BOSU 30, GEFI 15, No preincubation, 30 min preincubation with NADPH.
- CYP3A: BASA 100, GEFI 100, ERLO 10, LEST 5, 80 QTIN, SORA 30, SORI 55, No preincubation, 30 min preincubation with NADPH.
]
Figure 3.
Figure 4.