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.)

ABSTRACT

Previous studies have shown that several protein kinase inhibitors are time-dependent inhibitors of cytochrome P450 (CYP) 3A. We screened 14 kinase inhibitors for time-dependent inhibition of CYP2C8 and CYP3A. Amodiquine N-deethylolation and midazolam 1'-hydroxylation were used as marker reactions for CYP2C8 and CYP3A activity, respectively. A screening, IC\textsubscript{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 with 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, inhibitor concentration that supports half-maximal rate of inactivation (K\textsubscript{i}) and maximal inactivation rate (k\textsubscript{kinact}), were 54.8 \textmu M 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\textsubscript{i} and k\textsubscript{kinact} of lestaurtinib and saracatinib were 30.7 \textmu M and 0.040 1/min, and 12.6 \textmu M 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 \textgeq 2.7-fold. The liability of kinase inhibitors to affect CYP 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. Because 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, 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, 400 mg imatinib 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 (Spurreboom 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...
In the present study, we first screened 14 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 with no preincubation, and which had not previously been identified as time-dependent inhibitors, were further investigated in IC50 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 (4-[3,5-dichloro-4-methoxyphenyl]amino)-6-methoxy-7-[3-(4-methyl-1-piperazinyl)propoxy]-3-quinolinicarbonitrile), etoposide, gefitinib, lestatutinib, nilotinib, pazopanib, saracatinib, sorafenib, sunitinib, and vandetanib were purchased from LC Laboratories (Woburn, MA). Crizotinib, dasatinib, and montelukast were from Sequoia Research Products (Pangbourne, U.K.), 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 (North York, ON, Canada), ketoconazole from Janssen Biotech (Olen, Belgium), amodiaquine dihydrochloride dihydrate, ammonium formate, and triazolam as internal standards. Samples were thereafter handled as previously described (Filppula et al., 2013).

Amodiaquine, ketoconazole, midazolam, and montelukast were dissolved in methanol, bosutinib, bosutinib isomer 1, crizotinib, lestatutinib, saracatinib, and vandetanib in ethanol, and all other inhibitor compounds in dimethylsulfoxide.

In the preliminary screening and IC50 experiments, the final solvent concentration in all incubations (including controls) with bosutinib, bosutinib isomer 1, crizotinib, lestatutinib, saracatinib, and vandetanib was 1%, whereas in all incubations (including controls) for the other inhibitors it was 0.2%. In mechanism-based experiments with bosutinib, lestatutinib, 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'-hydroxy-midazolam it was 13% at 0.002 μM, 11% at 0.02 μM, and 3.4% at 0.2 μM. Screening and IC50-Shift Experiments. Inhibitor concentrations causing approximately 50% and 20–30% direct inhibition of CYP2C8 and CYP3A activity or buffer control were simultaneously incubated with 2 μM substrate (direct inhibition) or 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 IC50-shift experiments. In addition, as no IC50 values for CYP2C8 and CYP3A inhibition by axitinib had been published at the time of the study, we also conducted IC50 incubations for axitinib. The IC50 experiments were carried out similarly as the screening, but inhibitor concentrations tested ranged from 0.01 to 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 IC50 with a 30-min preincubation, as compared with 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 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 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 lestatutinib and saracatinib, preincubation mixtures were prepared as above. However, for bosutinib, preincubutions 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 lestatutinib and saracatinib, preincubation tubes contained ketoconazole (0.1 and 1 μM) as CYP3A inhibitor, without (control) or with lestatutinib (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 lestatutinib and saracatinib) was measured, as explained above.

To evaluate the effect of dialysis on the inhibitory effects of bosutinib, lestatutinib, 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 dialyzed 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 CYP3A4 marker reactions, as explained above.

Data Analysis. IC₅₀ values were determined by nonlinear regression analysis using SigmaPlot (version 11.0; Systat Software, 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 CYP enzyme inactivation (Kₐ) 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 Kₐ values were used to determine the maximum inactivation rate (kₐ) and the inhibitor concentration needed to cause half of kₐ [inhibitor concentration that supports half-maximal rate of inactivation (Kᵢ)]. Preliminary estimates of Kᵢ and kₐ were obtained from a double-reciprocal plot of Kₐ (y-axis) versus inhibitor concentration [I] (x-axis) (Kitz and Wilson, 1962). Final Kᵢ and kₐ values were then estimated by nonlinear regression using the following equation: kₐ = Kᵢ × 1(1 + Kᵢ) (Kitz and Wilson, 1962; Jones et al., 1999).

Drug-Drug Interaction Predictions. Obtained Kᵢ, kₐ, and direct IC₅₀ 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):

\[
\frac{AUC_{\text{pred}}}{AUC_{\text{po}}} = \frac{1}{[A \times B] \times f_{\text{mP}}} + \frac{1}{[C \times D] \times (1 - F_G) + F_G}
\]

where AUCₚₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑ廨ide 1 2. In addition, predictions based on direct inhibition of CYP2C8 and CYP3A4 by axitinib and of CYP2C8 by gefitinib were conducted using the described equations (Supplemental Tables 1 and 2). Then, the reduction in intestinal CYP3A4 activity after administration of bosutinib, lestaurtinib, and saracatinib was calculated as follows:

Remaining intestinal CYP3A4 activity = C × D

using intestinal inhibitor concentrations [Iₕ]. These concentrations were estimated using the following equation (Rostami-Hodjegan and Tucker, 2004):

\[
[I]ₕ = \frac{dose \times k_a \times f_s}{Q_{out}}
\]

where Qₗ represents the enterocyte blood flow (248 ml/min) (Obach et al., 2007) (Supplemental Table 2).

Finally, CYP3A4 inhibitor interactions with midazolam were predicted with the assumption that fmCYP3A4 and F_G of midazolam are 0.94 and 0.51 (Galetin et al., 2006; Gerz 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, 0.2, and 0.7 μM, respectively) (Fig. 1, Supplemental Fig. 2, Supplemental Table 3). At the concentrations tested, bosutinib, gefitinib, lestaurtinib, and saracatinib caused a moderate or small time- and NADPH-dependent increase in inhibition of CYP2C8 activity (Fig. 1, Supplemental Fig. 2) and were further tested in IC₅₀ experiments. Compared with 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 with direct inhibition.

Screening of Time-Dependent Inhibition of CYP3A in HLM Incubations. As previously recognized in the literature (Supplemental Table 4), 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, Supplemental Fig. 2). 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 did not increase CYP3A inhibition. In addition, preincubation of crizotinib with NADPH did not alter its inhibition of CYP2C8, as compared with direct inhibition.

IC₅₀ Experiments. Preincubation of bosutinib with NADPH resulted in a 2.6-fold decrease in its IC₅₀ 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 \( \mu \text{M} \). Likewise, after preincubation, the IC$_{50}$ of saracatinib was markedly decreased, by 26-fold to 1.8 \( \mu \text{M} \).

**Mechanism-Based Inhibition of CYP2C8 and CYP3A.** The inhibition of CYP2C8 activity by bosutinib and of CYP3A activity by lestaurtinib and saracatinib was concentration-, NADPH-, and preincubation time-dependent (Fig. 3). With the nonlinear regression method, apparent inactivation variables $K_I$ and $k_{\text{inact}}$ for CYP2C8 by bosutinib were estimated to 54.8 \( \mu \text{M} \) and 0.018 1/min. For lestaurtinib, the $K_I$ and $k_{\text{inact}}$ for CYP3A inhibition were 30.7 \( \mu \text{M} \) and 0.040 1/min, and for saracatinib 12.6 \( \mu \text{M} \) and 0.096 1/min. The $k_{\text{inact}}$ values imply that approximately 2% of CYP2C8 and 4% and 10% of CYP3A are 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 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 as the contributing mechanism. Using bosutinib plasma-unbound peak and estimated unbound hepatic inlet peak concentrations of 0.021 and 0.23 \( \mu \text{M} \), respectively (Daud et al., 2012), the predicted maximal AUC increase was small for substrates completely metabolized by CYP2C8 (≤1.2-fold). Also, 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 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.

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predictions based on direct CYP2C8 inhibition only (calculated using direct IC₅₀ values) by unbound peak concentrations of axitinib, gefitinib, lestaurtinib, and saracatinib resulted in minimal AUC increases (≤1.1-fold; Supplemental Table 2).

For lestaurtinib, predictions based on its calculated plasma-unbound Cₘₐₓ (0.28 μM) after doses of 80 mg twice daily (Supplemental Table 2) (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 Cₘₐₓ of 0.082 μM) (Baselga et al., 2010) was predicted to be 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 be 4.7- and 5.5-fold after coadministration with lestaurtinib and saracatinib,
of CYP3A. 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 IC₅₀ value for direct CYP3A inhibition by lestaurtinib (4.7 μM) is in good agreement with previously reported Kᵢ values (4.3–5.2 μM) (http://issx.confex.com/issx/15na/webprogram/Paper11788.html, http://am17.cardiff.ac.uk/files/am17_protocolv2.pdf). However, preincubation with NADPH decreased the IC₅₀ 2.2-fold, and in mechanism-based experiments the Kᵢ 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 IC₅₀ for CYP3A following preincubination, and its Kᵢ 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 IC₅₀ of 43 μM, and preincubation with NADPH reduced the IC₅₀ 2.6-fold. Furthermore, the inhibition of CYP3A by bosutinib proceeded in a time-, concentration-, and NADPH-dependent manner, suggesting that it affects CYP2C8 by mechanism-based inhibition. Apparent Kᵢ 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, in which bosutinib did not exhibit time-dependent inhibitory effects on paclitaxel 6ox-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 with 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, Kᵢ of 27 μM) (http://www.accessdata.fda.gov/drugsatfda_docs/nda/2012/203341Orig1- s000ClinPharmR.pdf), possible explanations for this discrepancy could be differences in experimental conditions, for example, 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.pkpharma.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 IC₅₀ 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 (Supplemental Tables 3 and 4).

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 o-toluidine structures have been suggested to undergo hydroxylation and desaturation to imine and imine-methide intermediates, respectively (Li et al., 2014). Quinone-imines and imine-methides are reactive electrophiles, which, besides binding to P450 enzymes, may also

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**TABLE 1**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Percentage of Control</th>
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<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>Lestautilnib alone</td>
<td>48.5 ± 4.6</td>
</tr>
<tr>
<td>Lestautilnib with ketoconazole (0.1 μM)</td>
<td>53.2 ± 3.3</td>
</tr>
<tr>
<td>Lestautilnib 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>

**TABLE 2**

<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>Lestautilnib prior to dialysis</td>
<td>32.7 ± 3.2</td>
</tr>
<tr>
<td>Lestautilnib 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>

respectively, when unbound peak concentrations and the above doses of the inhibitors were used (Supplemental Table 2).

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 CYP3A4 or CYP2C8 (≤1.1-fold AUC increase) (Supplemental Table 2). 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, 14 protein kinase inhibitors were initially screened for their potential time-dependent inhibitory effects on CYP2C8 and CYP3A activity. As compared with no preincubation, one inhibitor (bosutinib) exhibited increasing CYP2C8 inhibition and 11 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.
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 CYP3A4 observed in our study. Interestingly, whereas bosutinib exhibited time-dependent inhibitory effect on CYP2C8 but not on CYP3A4, 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) (Supplemental Table 5) (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 CYP3A4 inhibition undergo metabolism by CYP3A4 (Supplemental Table 6). 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 whether 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 CYP enzymes by mechanism-based inhibition, and its clinical relevance.
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Authorship Contributions

Participated in research design: Filppula, Neuvonen, Backman.

Conducted experiments: Filppula.

Performed data analysis: Filppula, Neuvonen, Backman.

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

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