COMPARISON OF THE DRUG-DRUG INTERACTIONS POTENTIAL OF ERLOTINIB AND GEFITINIB VIA INHIBITION OF UDP-GLUCURONOSYLTRANSFERASES

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Running title page

a) **Running title:** DDI via Inhibition of UGTs by Erlotinib and Gefitinib

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c) **The number of text pages:** 28

The number of tables: 1

The number of figures: 5

The number of references: 40

The number of words in the Abstract: 198

The number of words in the Introduction: 546

The number of words in the Discussion: 1087

d) **Abbreviations used are:** UGT, UDP-glucuronosyltransferase; DDI, drug-drug interactions; 4-MU, 4-methylumbelliferone; 4-MUG, 4-methylumbelliferone-D-glucuronide; HLMs, human liver microsomes; AUC, area under the curve; EGFR, epidermal growth factor receptor; UDPGA, uridine 5'-diphosphoglucuronic acid.
Abstract

We aimed to investigate and compare the effects of erlotinib and gefitinib on UDP-glucuronosyltransferase (UGT) activities, and to quantitatively evaluate their drug-drug interaction (DDI) potential due to UGT inhibition. The inhibitory effects of erlotinib and gefitinib on UGTs were determined using HPLC by measuring the formation rates for 4-methylumbelliferone (4-MU) glucuronide, imipramine N-glucuronide, and bilirubin glucuronides using recombinant human UGT isoforms and human liver microsomes (HLMs) in the absence or presence of erlotinib and gefitinib. Inhibition kinetic studies were conducted. AUC ratios were used to predict the risk of potential DDI in vivo. Erlotinib exhibited selective potent competitive inhibition against 4-MU glucuronidation by UGT1A1, and gefitinib demonstrated a wide range of inhibition against UGT-mediated 4-MU glucuronidation, particularly against UGT1A1, UGT1A7, UGT1A9 and UGT2B7. Erlotinib also exerted potent mixed inhibition against bilirubin glucuronidation in HLMs. We estimated that coadministration of erlotinib at 100 mg/day or higher doses may result in at least a 30% increase in the area under the curve (AUC) of drugs predominantly cleared by UGT1A1. Thus, the co-administration of erlotinib with drugs primarily cleared by UGT1A1 may result in potential DDI. In contrast, gefitinib is unlikely to cause a clinically significant DDI through inhibition of glucuronidation.
Introduction

Erlotinib and gefitinib are potent, reversible, and selective inhibitors of the tyrosine kinase of the epidermal growth factor receptor (EGFR). Both drugs have been approved for the treatment of patients with non-small cell lung cancer, and erlotinib is also indicated for pancreatic cancer. In addition, phase II trials have suggested activity for these agents in a number of other solid tumors (Sequist and Lynch, 2008). Gefitinib and erlotinib share a common chemical backbone structure and exhibit similar oral bioavailability and disposition characteristics in humans after oral administration (Siegel-Lakhai et al., 2005).

Drug-drug interactions (DDI) have received increasing attention over the past few decades. Several DDIs were reported to be associated with erlotinib and gefitinib. Coadministration of erlotinib has been reported to enhance the carboplatin exposure (Patnaik et al., 2006) and increase the serum concentration of phenytoin (Grenader et al., 2007). Drug interactions were also observed in two patients who received both gefitinib and warfarin simultaneously, which resulted in an enhancement of the warfarin effect (Onoda et al., 2005). Combination of gefitinib with capecitabine and radiation in pancreatic and rectal cancer patients (Czito et al., 2006), as well as the combination of gefitinib with 5-fluorouracil, leucovorin, and irinotecan in patients with colorectal cancer (Veronese et al., 2005), are associated with excessive toxicity, suggesting an interaction at a pharmacokinetic or pharmacodynamic level.

Metabolizing enzyme-based DDI constitute the major proportion of clinically important DDI. Gefitinib and erlotinib are eliminated predominantly by oxidation by cytochrome P450 (CYP) enzymes. Both drugs are metabolized primarily by CYP3A4 and CYP3A5. In addition, CYP1A2 is considerably involved in erlotinib metabolism, and CYP2D6 is involved in gefitinib metabolism (Li et al., 2007). Therefore, both
drugs may alter the systemic exposure of some CYPs substrates, implying a potential DDI. However, it is unknown whether DDI by erlotinib and gefitinib are associated with other metabolizing enzymes.

UDP-glucuronosyltransferase (UGT) enzymes catalyze the conjugation of various endogenous substances and exogenous compounds. The human UGT superfamily is comprised of 2 families (UGT1 and UGT2). At least 19 human UGTs have been identified to date based on sequence homologies (Mackenzie et al., 2005). UGT-catalyzed glucuronidation reactions are responsible for the metabolism of approximately 35% of all drugs metabolized by phase II enzymes. Approximately one-seventh of the drugs prescribed in the United States in 2002 are cleared by UGTs (Williams et al., 2004). UGT-mediated drug interactions can potentially occur for many drugs. In fact, several significant DDI have been clinically observed. Erlotinib has been suggested to be an inhibitor of UGT1A1 (EMEA). However, the effects of erlotinib and gefitinib on UGT activity have not been fully characterized. Understanding the effects of erlotinib and gefitinib on UGT activities is important to ensure their safe administration and develop new coadministration therapies with both drugs.

The aim of this study was to investigate and compare the effects of erlotinib and gefitinib on the activities of human UGTs. Using a panel of recombinant human UGT isoforms, we found potent inhibition of erlotinib against UGT1A1. Gefinitib, on the other hand, demonstrated a wide range of inhibition against not only UGT1A1 but also UGT1A7, UGT1A9, and UGT2B7. The effects of erlotinib and gefitinib on bilirubin glucuronidation were investigated in human liver microsomes (HLMs). The potential for DDI in vivo was also quantitatively predicted and compared using AUC ratios.
Methods

Chemicals. Erlotinib (OSI-774) was purchased from Biaffin GmbH & Co KG (Kassel, Germany). Gefitinib and indinavir were purchased from Toronto Research Chemicals, Inc. (North York, Canada). Bilirubin, 4-methylumbelliferone (4-MU), 4-methylumbelliferone-β-D-glucuronide (4-MUG), β-glucuronidase (from Escherichia coli), alamethicin (from Trichoderma viride), Tris-HCl, imipramine, p-nitrophenol, androsterone, diclofenac, phenylbutazone, ascorbic acid, hecogenin, 7-hydroxycoumarin and uridine 5′-diphosphoglucuronic acid (UDPGA) (trisodium salt) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of HPLC grade or of the highest grade commercially available.

Human liver microsomes and recombinant human UGTs. Pooled human liver microsomes (HLM) and a panel of recombinant human UGT supersomes (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) expressed in baculovirus-infected insect cells were purchased from BD Gentest Corp. (Woburn, MA, USA). Pooled HLMs were derived from 22 donors (90% Caucasian, 5% Hispanic and 5% African-American; 15 men and 7 women). The median age was 48 years with a range of 10 to 70.

Inhibition of 4-MU glucuronidation assay. 4-MU, a nonselective substrate of UGTs, was used as probe substrate for all UGTs except UGT1A4. Incubations with each individual enzyme were conducted using conditions previously described (Uchaipichat et al., 2004) with a slight modification. A typical incubation mixture (200 μl total volume) contained recombinant UGTs (final concentration: 0.25, 0.15, 0.05, 0.05, 0.05, 0.05, 0.5, 0.25, 0.75 and 0.5 mg/ml for UGT1A1, UGT1A3,
UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15 and UGT2B17, respectively), 5 mM UDPGA, 5 mM MgCl₂, 50 mM Tris-HCl buffer (pH 7.4), 50 μg/mg protein alamethicin, and 4-MU in the absence or presence of different concentrations of inhibitors. Incubations with 4-MU were performed at the concentration corresponding to the apparent $K_m$ or $S_{50}$ value reported for each isoform (110, 1200, 110, 15, 750, 30, 80, 1200, 350, 250 and 2000 μM 4-MU for UGT1A1, UGT1A3, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17, respectively) (Uchaipichat et al., 2006b). Known UGT inhibitors were used as positive controls: diclofenac for UGT1A1, UGT1A6, UGT1A7, and UGT1A9; androsterone for UGT1A3, UGT2B7 and UGT2B15, and phenybutazone for UGT1A8 and UGT1A10, respectively (Uchaipichat et al., 2004; Uchaipichat et al., 2006a). There is no positive control reported available for UGT2B4 and UGT2B17. The negative controls are incubations without UDPGA. These tested chemicals and inhibitors were dissolved in DMSO. The final concentration of DMSO in the incubation system was 1% (v/v). Alamethicin was dissolved in 90/10 incubation buffer/ethanol, and the final concentration of ethanol was less than 0.1%. Microsomes were pre-incubated with alamethicin on ice for 15 min prior to incubation. There was a 5 min preincubation step at 37°C before the reaction was started by addition of UDPGA. Incubation times were 120 min for UGT1A1, UGT1A10, UGT2B4, UGT2B15, and UGT2B17, 75 min for UGT1A3, and 30 min for UGT1A6, UGT1A7, UGT1A8, and UGT1A9. The reactions were quenched by adding 100 μl acetonitrile and internal standard (7-hydroxycoumarin, 100 μM). Rate of product formation for each isoform was linear with respect to protein concentration and incubation time. The incubation mixtures were then centrifuged at 20,500 ×g for 15 min to obtain the supernatant. Aliquots (20 μl) were
then analyzed by HPLC (Hitachi High Technologies America, Schaumburg, IL). Chromatographic separation was achieved using a C18 column (3.9×300 mm I.D., 10μm particle size) (Sigma-Aldrich, St. Louis, MO) at a flow rate of 1 ml/min and UV detection at 316 nm. The mobile phase consisted of 10 mM KH₂PO₄, pH 2.7 (A) and acetonitrile (B). The following gradient was applied at a flow rate of 1 ml/min: 0-4 min 80% A and 20% B, 4.1-8 min 50% A and 50% B, 8.1-12 min, 30% A and 70% B. The metabolites were quantified using a standard curve made by combining 4-MUG stock and incubation buffer and processing as described above. All experiments were performed in two independent experiments in duplicate.

**Inhibition of imipramine N-glucuronidation assay.** Imipramine was used as probe substrate for UGT1A4. Imipramine N-glucuronidation activity was determined as published previously (Nakajima et al., 2002). Imipramine was incubated in the absence or presence of different concentrations of inhibitors. Hecogenin, a known UGT1A4 inhibitor, was used as positive control. Incubation was performed for 90 min using a protein concentration of 0.5 mg of protein/ml recombinant UGT1A4 or pooled HLMs. HPLC separation was achieved using a C18 column (3.9×300 mm I.D., 10μm) (Sigma-Aldrich, St. Louis, MO) at a flow rate of 1 ml/min and UV detection at 254 nm. The mobile phase consisted of 30:70 acetonitrile: 10 mM KH₂KO₄ buffer (pH 2.6) (v:v). For the quantification of imipramine N-glucuronide, it was assumed that the glucuronide of imipramine had the same molar absorbance as its aglycone, as no imipramine monoglucuronide standards were available. The quantification of the glucuronide was accomplished using a standard curve for imipramine. All experiments were performed in two independent experiments in duplicate.
Inhibition of bilirubin glucuronidation assay. Bilirubin glucuronidation activity was determined with a slight modification of a previously published method (Udomuksorn et al., 2007). Bilirubin was incubated in the absence or presence of different concentrations of erlotinib or gefitinib (0-100 µM). Indinavir, a known inhibitor of bilirubin glucuronidation, was used as positive control. Reactions were performed for 20 min in 0.5 mg/ml pooled HLMs. HPLC separation was achieved using a SymmetryShield™ RP8 column (3.0×150 mm I.D., 5 µm particle size) (Waters, Milford, MA) with a flow rate of 1 ml/min and UV detection at 450 nm. Initial mobile-phase composition was 78% 100 mM ammonium acetate buffer at pH 4.85 (mobile phase A) and 22% acetonitrile (mobile phase B). The proportion of acetonitrile was increased to 44% over 20 min, then further increased to 90% over 1 min and then held constant for 7 min before returning to the starting composition. Authentic standards of each of the bilirubin glucuronides were not available for use, so a bilirubin standard curve (0.1–5 µM) was used to quantify glucuronide formation.

Inhibition kinetics analysis. Inhibition constant ($K_i$) values were determined using various concentrations of 4-MU or bilirubin in the presence or absence of erlotinib and gefitinib. $K_i$ values were calculated by nonlinear regression using the equations for competitive inhibition (eq. 1), noncompetitive inhibition (eq. 2), or mixed inhibition (eq. 3), (Copeland, 2000)

$$v = \frac{V_{max}S}{(K_m(1+I/K_i)+S)} \quad (1)$$

$$v = \frac{V_{max}S}{(K_m+S)(1+I/K_i)} \quad (2)$$

$$v = \frac{V_{max}S}{(K_m+S)(1+I/\alpha K_i)} \quad (3)$$

where $v$ is the velocity of the reaction; $S$ and $I$ are the substrate and inhibitor concentrations, respectively; $K_i$ is the inhibition constant describing the affinity of the
inhibitor for the enzyme; $K_m$ is the substrate concentration at half of the maximum velocity ($V_{\text{max}}$) of the reaction. The type of inhibition was determined from the fitting of data to the enzyme inhibition models. Goodness of fit to kinetic and inhibition models was assessed from the F statistic, $r^2$ values, parameter standard error estimates and 95% confidence intervals. Kinetic constants are reported as the mean value ± standard error of the parameter estimate. IC$_{50}$ values (concentration of inhibitor that reduces enzyme activity by 50%) were determined by the Data Analysis Toolbox of Microsoft Excel software (Microsoft Inc, Redmond, WA).

**Predicted concentrations of erlotinib and gefitinib at UGT catalytic sites.** The concentrations of erlotinib and gefitinib in humans were estimated according to the equations for the average systemic plasma concentration after repeated oral administration ([I]$_{av}$) (eq. 4), the maximum systemic plasma concentration after repeated oral administration ([I]$_{\text{max}}$) (eq. 5), the maximum unbound systemic plasma concentration after repeated oral administration ([I]$_{\text{max,u}}$) (eq. 6), the maximum hepatic input concentration ([I]$_{\text{in}}$) (eq. 7), and the maximum unbound hepatic input concentration ([I]$_{\text{in,u}}$) (eq. 8) (Ito et al., 2004),

$$[I]_{av} = \frac{(D/\tau)}{(CL/F)}$$  \hspace{1cm} (4)

$$[I]_{\text{max}} = \frac{([I]_{av} \cdot k \cdot \tau)}{(1-\exp(-k \cdot \tau))}$$  \hspace{1cm} (5)

$$[I]_{\text{max,u}} = \frac{([I]_{av} \cdot k \cdot \tau \cdot f_u)}{(1-\exp(-k \cdot \tau))}$$  \hspace{1cm} (6)

$$[I]_{\text{in}} = [I]_{av} + k_a F_a D/Q_h$$  \hspace{1cm} (7)

$$[I]_{\text{in,u}} = f_u ([I]_{av} + k_a F_a D/Q_h)$$  \hspace{1cm} (8)

where D and $\tau$ represent dose and dosing interval of inhibitors used in the *in vivo* interaction study, respectively; $k$ is the elimination rate constant; $k_a$ is the absorption rate constant; $F_a$ is the fraction absorbed from the gut into the portal vein; $Q_h$ is the...
hepatic blood flow rate, and \( f_u \) is the unbound fraction. The values of \( k_a \), \( F_a \) and \( f_u \) for erlotinib and gefitinib were obtained from the literature (EMEA; Frohna et al., 2006; Li et al., 2006; Rudin et al., 2008; Schaiquevich et al., 2008; Siegel-Lakhai et al., 2005). \( Q_h \) was assumed to be 1610 ml/min (Ito et al., 2004).

Calculation of AUCi/AUC ratio. The magnitudes of inhibitory interactions of erlotinib and gefitinib were estimated as the ratio of the area under the plasma concentration–time curve in the presence and absence of the inhibitor (AUCi/AUC). This ratio was predicted using the formula (eq. 9) for oral administration of a high hepatic clearance drug (Ito et al., 2004):

\[
\frac{AUC_i}{AUC} = \frac{1}{(f_m/(1+[I]/K_i)+(1-f_m))}
\]

where \( AUC_i \) and \( AUC \) are the AUC in the presence and absence of inhibitor, respectively; \( K_i \) is inhibitor constant (obtained from in vitro experiments); \( f_m \) is the fraction metabolized by the inhibited enzyme; \([I]\) is the inhibitor concentration at the enzyme active site.

In view of the general assumption that only unbound drug is available for interaction with the enzyme active site, and the consideration that the aim of DDI research is to exclude the highest risk, we used in this study the maximum unbound hepatic input concentration (\([I]_{in,u}\)) as the inhibitor concentration at the active site of the UGTs except for UGT1A7. As UGT1A7 is present only in the esophagus, stomach, and lung (Strassburg et al., 1997), we used the maximum unbound systemic plasma concentration after repeated oral administration (\([I]_{max,u}\)) as the inhibitor concentration at the enzyme active site in extrahepatic tissues. Since the fraction metabolized by the UGT isoforms inhibited by erlotinib or gefitinib (\(f_m\)) of the coadministered drug is unknown, we arbitrarily selected 0.1 to 1 to calculate the AUC/AUC ratio.
Results

Inhibition of UGTs activities by erlotinib and gefitinib in recombinant human UGTs

The IC₅₀ values of the inhibitors of each UGT isoform were comparable with previously published data (Uchaipichat et al., 2004; Uchaipichat et al., 2006a). No glucuronidation of erlotinib and gefitinib was found in the course of the incubations.

As shown in Fig 1, erlotinib (100 μM) inhibited UGT1A1 activity, reducing 4-MU glucuronidation by 88.3% (P<0.01). The inhibition by erlotinib was also observed against UGT1A3, UGT2B7, UGT1A9, UGT1A7, and UGT2B15, reducing 4-MU glucuronidation activities by 42.3%, 32.8%, 31.9%, 27.4% and 18.1% at 100 μM, respectively.

Similarly, gefitinib had an inhibitory effect against UGT1A1 activity, reducing glucuronidation by 79.1% at 100 μM. However, it exhibited a slightly broader inhibition profile than erlotinib. At 100 μM, gefitinib inhibited the activities of UGT1A7, UGT1A9, and UGT2B7 by 61.6%, 55.5%, and 70.9%, respectively. The inhibition was also observed against UGT2B15 (47.9%), 1A4 (39.8%) and 1A3 (18.8%) at 100 μM.

In addition, erlotinib and gefitinib exhibited a stimulation of UGT1A4 and UGT2B17 catalytic activity by 67.3% and 81.5% at 100 μM, respectively.

Inhibition kinetic analysis in recombinant UGTs

Kinetic experiments were performed to further characterize the inhibition of UGT activities by erlotinib and gefitinib. Erlotinib and gefitinib strongly inhibited the formation of 4-MUG by UGT1A1. The representative Lineweaver-Burk plots for the inhibition of 4-MUG formation by erlotinib and gefitinib (Fig. 2A and 3A) and
analysis of the parameters of the enzyme inhibition model suggested that the inhibition types were competitive. Based on nonlinear regression analysis and Dixon plots presented in Fig. 2B and Fig. 3B, erlotinib and gefitinib showed competitive inhibition against the formation of 4-MUG with $K_i$ of $0.64 \pm 0.06 \, \mu M$ and $2.42 \pm 0.31 \, \mu M$ in recombinant UGT1A1, respectively.

Gefitinib was found to be a strong competitive inhibitor of UGT1A7 with a $K_i$ of $5.11 \pm 0.43 \, \mu M$ (Fig. 3C & 3D). It also exerted intermediate mixed inhibition against UGT1A9 with $K_i$ of $1.41 \pm 0.16 \, \mu M$ and $\alpha K_i$ of $44.10 \pm 1.55 \, \mu M$ (Fig. 3E & 3F), as well as intermediate competitive inhibition against UGT2B7 with $K_i$ of $39.48 \pm 4.17 \, \mu M$ (Fig. 3G & 3H).

Inhibition of bilirubin glucuronidation activity by erlotinib and gefitinib in HLMs

The kinetic studies were first performed using pooled HLMs. The apparent kinetic parameters $K_m$ and $V_{max}$ of bilirubin glucuronidation were estimated to be $1.11 \pm 0.25 \, \mu M$ and $460.20 \pm 22.57 \, \text{pmol/min/mg protein}$, respectively.

Inhibition experiments were then conducted in HLMs. The IC$_{50}$ value of indinavir was $110.6 \, \mu M$, which is comparable with previously published data (Zhang et al., 2005). Erlotinib exhibited potent inhibition against bilirubin glucuronidation with an IC$_{50}$ of $4.19 \pm 0.24 \, \mu M$ at a bilirubin concentration of $1 \, \mu M$. Further kinetic experiments showed mixed inhibition by erlotinib. The $K_i$ was $2.97 \pm 1.09 \, \mu M$, and $\alpha K_i$, a measure of the affinity of ES for I, was $7.78 \, \mu M$. However, the effect of gefitinib was found surprisingly to be much weaker than that of erlotinib, and the IC$_{50}$ was more than $100 \, \mu M$ (Fig 4).
The calculated concentrations of erlotinib and gefitinib in blood

The oral doses and pharmacokinetic parameters of erlotinib and gefitinib were obtained from previous publications (EMEA; Ito et al., 2004; Siegel-Lakhai et al., 2005; Frohna et al., 2006; Li et al., 2006; Rudin et al., 2008; Schaiquevich et al., 2008). The calculated blood concentrations of erlotinib and gefitinib after oral administration are listed in Table 1. Comparison of the calculated $[I]_{\text{max}}$ with the reported $C_{\text{max}}$ after administration of oral doses of erlotinib or gefitinib showed that the $[I]_{\text{max}}$ values fell within the reported concentration range, with the exception of the $[I]_{\text{max}}$ at 700mg/day gefitinib, which is higher than expected.

Quantitative prediction of DDI risk (AUC/AUC)

The magnitudes of the potential inhibitory interactions of erlotinib and gefitinib with UGTs were evaluated by estimating the ratio of the AUC in the presence and absence of the inhibitor (AUC$_i$/AUC$_o$).

The calculated results were shown as AUC ratio isolines plotted against $f_m$ by UGT isoform and oral doses of erlotinib or gefitinib in Fig 5. When the dose of erlotinib is more than 140 mg/day and the $f_m$ of coadministered drug metabolized by UGT1A1 is more than 0.9, the AUC of coadministered drug will increase more than 40%; when the dose is more than 110 mg/day and the $f_m$ is more than 0.8, or the dose is more than 100 mg/day and the $f_m$ is 1, AUC will increase more than 30%. However, for gefitinib, even when administered at the highest dose (700 mg/day) and an $f_m$ of 1, the AUC ratio is less than 1.3 for the substrates of each UGT isoform inhibited.

Here we assumed the $f_m$ of bilirubin is 1, as UGT1A1 is the dominant isoform involved in bilirubin glucuronidation (Bosma et al., 1994). When the oral dose of erlotinib is 150 mg/day, the AUC of bilirubin will increase more than 10%.

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Discussion

Our data offer in vitro evidence that erlotinib is a potent competitive inhibitor of UGT1A1. UGT1A1 inhibition is also the mechanism by which atazanavir and indinavir cause hyperbilirubinemia (Zhang et al., 2005). Reduced glucuronidation rates are associated with the risk for severe toxicity during irinotecan treatment (Innocenti et al., 2004). Since the reduction in UGT1A1 activity varies depending on the substrate and incubation system (Udomuksorn et al., 2007), we also investigated the effects of erlotinib and gefitinib on bilirubin glucuronidation in HLMs in the current study. The inhibition potential is similar to that of atazanavir, but much more potent than that of indinavir. It is consistent with the observation that erlotinib can cause hyperbilirubinemia (Jakacki et al., 2008), similar to that observed with atazanavir and indinavir (Zhang et al., 2005). Therefore, inhibition of UGT1A1 activity by erlotinib can decrease the conjugation of bilirubin, which can be clinically significant.

UGT1A1 is also responsible for the metabolism of several other endogenous and exogenous substrates, including 15% drugs that have glucuronidation as a clearance mechanism of the top 200 drugs in the United States in 2002 (Williams et al., 2004). The inhibition of UGT1A1 is particularly important if a drug has a narrow therapeutic index, such as etoposide and irinotecan (Kawato et al., 1991; Wen et al., 2007). Interestingly, gefitinib exhibited very weak inhibition against bilirubin glucuronidation in HLMs, although it potently inhibited 4-MU glucuronidation in recombinant UGT1A1. This finding confirms the observation that the reduction of UGT1A1 activity might vary with the substrate (Udomuksorn et al., 2007), and also offers new experimental evidence for the opinion that UGT1A1 has two or more binding sites for xenobiotics and endobiotics (Rios and Tephly, 2002).
In contrast with erlotinib, gefitinib exhibited competitive inhibition against not only UGT1A1 but also UGT1A7, UGT1A9 and UGT2B7. The latter enzyme is involved in the metabolism of 35% drugs involved in glucuronidation of the top 200 prescribed drugs in the United States in 2002 (Williams et al., 2004). UGT1A9 and UGT1A7 are also involved in the glucuronidation of a number of drugs (Kiang et al., 2005). In addition, UGT1A1, UGT1A9, and UGT2B7 are expressed in both human liver and some extrahepatic tissues including the gastrointestinal tract, whereas UGT1A7 is present only in the esophagus, stomach, and lung (Kiang et al., 2005). UGTs in the gastrointestinal tract may contribute significantly to the first-pass metabolism of orally administered drugs that undergo glucuronidation. Our results showed that gefitinib might affect the glucuronidation and first-pass metabolism of more orally administered drugs than erlotinib.

We observed an increase of UGT1A4 activity in the presence of erlotinib and UGT2B17 activity in the presence of gefitinib. Enzyme activation is not an uncommon event in enzymology, however, the underlying mechanisms are unclear. One possibility is the existence of the enzyme in multimeric form, so that the binding of one molecule to one subunit may increase the affinity of the other subunit(s) for another molecule. Williams et al. found that UGT1A1-catalyzed estradiol-3-glucuronidation is stimulated by 17α-ethynylestradiol, anthraflavic acid, and 2-amino-1-methyl- 6-phenylimidazo [4,5-b] pyridine in human liver microsomes. They also proved that the observed activation is not an artifact of the in vitro systems examined but true behaviors of UGT1A1 at least in vitro (Williams et al., 2002). However, further studies will need to be performed to evaluate whether this in vitro phenomenon also occurs in vivo.

The quantitative prediction of DDI risk indicated that the coadministration of
erlotinib at clinical doses could result in a significant increase of AUC of drugs primarily cleared by UGT1A1, suggesting that erlotinib can induce clinically significant DDI with co-administered UGT1A1 substrates. It is noteworthy that our calculation is based only on the inhibition of hepatic UGT1A1 by erlotinib, and abundant extrahepatic UGT1A1 exists. In addition, the pharmacokinetic parameters used to calculate concentrations are mean values of the parameters reported, but inter-individual variability is high. The diplotype of two polymorphic loci in the ABCG2 promoter involving –15622C/T and 1143C/T is associated with higher C\text{max} of erlotinib (Rudin et al., 2008). Furthermore, the bioavailability of erlotinib can be increased to almost 100% with the intake of food (Smith, 2005). Moreover, in vitro data tend to underestimate inhibition of drug glucuronidation in vivo (Uchaipichat et al., 2006a). Consequently, the actual effects of erlotinib might be more potent than those calculated here, in particular, for some individuals with higher blood concentrations of erlotinib.

Compared with erlotinib, gefitinib may significantly affect a drug that is metabolized by a single UGT and is a high-capacity, low affinity substrate. However, the majority of UGT substrates are glucuronidated by multiple UGT enzymes (Williams et al., 2004), thereby making it difficult to determine whether interactions arising from multiple UGTs inhibition by gefitinib exist when using in vitro inhibition data.

It is noteworthy that although there are a few studies predicting the magnitude of inhibitory drug interactions in vivo (Uchaipichat et al., 2006b; Zhang et al., 2005), the extrapolation from in vitro data to in vivo drug interactions should be taken with caution. Protein binding, active uptake and efflux transporters in tissues may affect the estimation of unbound drug concentrations at the interaction site. In addition, as
the lipid composition varies between membranes from different sources and is a determinant of membrane fluidity, thickness, shape, surface curvature and the ability to form lipid rafts, it is conceivable that the kinetic properties of single recombinant UGT may differ (Miners et al., 2006). Another potential problem is that, the enzymatically generated glucuronidated products are eliminated in vivo, but this does not occur in an in vitro incubation system, and the accumulation of products in the reaction medium may inhibit enzyme activity (Kiang et al., 2005). Furthermore, many studies have indicated UGTs are oligomeric enzymes. Although there are some evidences and experimental data showing that hetero-oligomerization could not be the major reason or basis for the complex substrate specificity of most UGTs (Finel and Kurkela, 2008), hetero-oligomerization might somewhat affect substrate specificity or the region-selectivity of the tested enzyme. Moreover, UGT1A1, UGT1A7, UGT1A9, and UGT2B7 are also expressed in gastrointestinal tract, and drug-drug interactions via inhibition of these UGTs may also take place in the small intestine. The concentrations of substrates in the intestine may be different from the concentrations used here to predict the AUC ratio.

The present findings shed light on the mechanisms underlying clinically significant DDI associated with erlotinib, and also provide the basis for further clinical studies investigating the DDI potential between tyrosine kinase inhibitors with UGT substrates, such as etoposide and SN-38 (the active metabolite of irinotecan).
Acknowledgements

We thank Dr. R. Stephanie Huang for valuable suggestions.
References


Cantarini MV and Lennernas H (2007) Pharmacokinetics of gefitinib in humans: the influence


Czito BG, Willett CG, Bendell JC, Morse MA, Tyler DS, Fernando NH, Mantyh CR, Blobel GC,

toxicity with gefitinib, capecitabine, and radiation therapy in pancreatic and rectal cancer:

EMEA (European Medicines Agency)


Metab 9:70-76.


Evaluation of the absolute oral bioavailability and bioequivalence of erlotinib, an inhibitor of
the epidermal growth factor receptor tyrosine kinase, in a randomized, crossover study in

due to erlotinib. Lung Cancer 57:404-406.

Innocenti F, Undevia SD, Iyer L, Chen PX, Das S, Kocherginsky M, Karrison T, Janisch L, Ramirez J,

Rudin CM, Vokes EE and Ratain MJ (2004) Genetic variants in the


Uchaipichat V, Mackenzie PI, Elliot DJ and Miners JO (2006a) Selectivity of substrate (trifluoperazine) and inhibitor (amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbutazone, quinidine, quinine, and sulfinpyrazone) "probes" for human udp-glucuronosyltransferases.

Drug Metab Dispos 34:449-456.


Wen ZM, Tallman MN, Ali SY and Smith PC (2007) UDP-glucuronosyltransferase 1A1 is the principal enzyme responsible for etoposide glucuronidation in human liver and intestinal microsomes: Structural characterization of phenolic and alcoholic glucuronides of etoposide and estimation
of enzyme kinetics. Drug Metabolism and Disposition 35:371-380.

Williams JA, Hyland R, Jones BC, Smith DA, Hurst S, Goosen TC, Peterkin V, Koup JR and Ball SE


Williams JA, Ring BJ, Cantrell VE, Campanale K, Jones DR, Hall SD and Wrighton SA (2002)

Differential modulation of UDP-glucuronosyltransferase 1A1 (UGT1A1)-catalyzed estradiol-3-glucuronidation by the addition of UGT1A1 substrates and other compounds to human liver microsomes. Drug Metab Dispos 30:1266-1273.


Footnotes

a) Grant support: Pharmacogenetics of Anticancer Agents Research Group, the National Institutes of Health/National Institute of General Medical Sciences [Grant U01GM61393].

b) This work was partially reported at the American Society for Clinical Pharmacology and Therapeutics (ASCPT) 2009 Annual Meeting, March 18-21, 2009 (Washington).

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Legends for figures

Fig 1. The inhibition of erlotinib and gefitinib on recombinant UGTs activities. 4-MU or imipramine were incubated with pooled HLMs (0.5 mg protein/ml), or recombinant UGTs (0.5 mg protein/ml) at 37 °C in the absence and presence of erlotinib (100 μM) or gefitinib (100 μM), respectively. Data represent the mean of triplicate or quadriplicate determination.

Fig 2. Representative Lineweaver-Burk plots (A) and Dixon plots (B) of the effects of erlotinib on 4-MU glucuronide formation in recombinant UGT1A1. Reactions were performed as described in Materials and Methods. All data points shown represent the mean of duplicate measurements.

Fig 3. Representative Lineweaver-Burk plots and Dixon plots of the effects of gefitinib on 4-MU glucuronide formation in recombinant UGT1A1 (A & B), UGT1A7 (C & D), UGT1A9 (E & F), and UGT2B7 (G & H). Reactions were performed as described in Materials and Methods. All data points shown represent the mean of duplicate measurements.

Fig 4. Kinetics of bilirubin glucuronidation in HLMs (A), the inhibition of erlotinib and gefitinib against bilirubin (1 μM) glucuronidation in HLMs (B), and representative Lineweaver-Burk plots and Dixon plots of the effects of erlotinib on bilirubin glucuronides formation in HLMs (C & D). Reactions were performed as described in Materials and Methods. All data points shown represent the mean of duplicate measurements.

Fig 5. Isolines plots for relationship of AUC ratio against oral dose of erlotinib and
$f_m$ by UGT1A1 (A), as well as the relationship of AUC ratio against oral dose of gefitinib and $f_m$ by UGT1A1(B), UGT1A7(C), UGT1A9(D), or UGT2B7(E) for DDI study.
Table 1 Calculation of possible concentrations of erlotinib and gefitinib

<table>
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<th>Drugs</th>
<th>Dose (mg/day)</th>
<th>Calculated Concentrations (μM)</th>
<th>Reported C\textsubscript{max} (μM)</th>
<th>References</th>
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</table>
Figure 1

Bar graph showing the activity percentage of various UGT enzymes under different conditions. The x-axis represents different enzymes (Ctrl (negative), UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, UGT2B17, HLM (positive Ctrl)). The y-axis represents the activity percentage of Ctrl. The graph includes three conditions: Control, 100 μM erlotinib, and 100 μM gefitinib. The bars represent the mean ± standard deviation.
Figure 4

A

Bilirubin glucuronides formation (pmol/min/mg pro)

0 10 20 30 40
Bilirubin (µM)

B

Bilirubin glucuronides formation (pmol/min/mg pro)

Indinavir
Erlotinib
Gefitinib

Inhibitor Concentration (µM)

C

D

1/(pmol/min/mg pro)

0.01 0.02 0.03 0.04 0.05 0.06
1/(bilirubin, µM)

0.01 0.02 0.03 0.04 0.05
Erlotinib (µM)

Ctrl
erlotinib 1 µM
erlotinib 2 µM
erlotinib 5 µM
erlotinib 7.5 µM

0.5 µM Bilirubin
1 µM Bilirubin
1.5 µM Bilirubin
2 µM Bilirubin