SHORT COMMUNICATION

Comparison of the inhibition potentials of icotinib and erlotinib against human UDP-glucuronosyltransferase 1A1

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KEY WORDS
Icotinib; Erlotinib; UGT1A1; Inhibitory effects; Drug–drug interactions (DDIs)

Abstract  UDP-glucuronosyltransferase 1A1 (UGT1A1) plays a key role in detoxification of many potentially harmful compounds and drugs. UGT1A1 inhibition may bring risks of drug–drug interactions (DDIs), hyperbilirubinemia and drug-induced liver injury. This study aimed to investigate and compare the inhibitory effects of icotinib and erlotinib against UGT1A1, as well as to evaluate their potential DDI risks via UGT1A1 inhibition. The results demonstrated that both icotinib and erlotinib are UGT1A1 inhibitors, but the inhibitory effect of icotinib on UGT1A1 is weaker than that of erlotinib. The IC50 values of icotinib and erlotinib against UGT1A1-mediated NCHN-glucuronidation in human liver microsomes (HLMs) were 5.15 and 0.68 μmol/L, respectively. Inhibition kinetic analyses demonstrated that both icotinib and erlotinib were non-competitive inhibitors against UGT1A1-mediated glucuronidation of NCHN in HLMs, with the Ki values of 8.55 and 1.23 μmol/L, respectively. Furthermore, their potential DDI risks via UGT1A1 inhibition were quantitatively predicted by the ratio of the areas under the concentration–time curve (AUC) of NCHN. These findings are helpful for the medicinal chemists to...
design and develop next generation tyrosine kinase inhibitors with improved safety, as well as to guide reasonable applications of icotinib and erlotinib in clinic, especially for avoiding their potential DDI risks via UGT1A1 inhibition.

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1. Introduction

Human uridine-disphosphate glucuronosyltransferase 1A1 (UGT1A1), one of the most important phase II conjugative enzymes, is of particular importance for human health. As the sole physiologically relevant enzyme involved in the metabolic elimination of endogenous bilirubin, UGT1A1 plays key roles in preventing bilirubin accumulation to toxic levels. It is well known that bilirubin, an endogenous toxic metabolite degraded from hemoglobin, can be produced appropriately 250–400 mg each day in human; UGT1A1 inhibition or dysfunction may bring increased risks of bilirubin-related diseases such as hyperbilirubinemia, kernicterus and drug-induced liver injury. Another biological function of UGT1A1 is the metabolism and detoxification of many xenobiotics including clinical drugs (such as etoposide and SN-38), environmental toxicants and chemical carcinogens. Inhibition of UGT1A1 may decrease the metabolic rate and increase the plasma concentration of these xenobiotics, causing severe drug–drug interactions or other undesirable effects. It is noteworthy that many tyrosine kinase inhibitors (TKIs) including erlotinib, nilotinib, pazopanib, lapatinib, regorafenib and sorafenib exhibit strong inhibitory effects against UGT1A1, which is closely associated with their side effects, such as hyperbilirubinemia, liver function impairment and hepatotoxicity.

TKIs are a class of chemotherapy drugs. Such agents are commonly used for the treatment of a variety of cancers, including non-small cell lung cancer (NSCLC), head and neck, colorectal, renal, prostate, breast, and primary brain cancer. In recent years, more than thirty TKIs have been approved for the treatment of certain forms of cancers, and several others are at various stages of clinical studies. In contrast to traditional chemotherapy drugs, most of TKIs have specific effects on target cancerous cells, and thus displayed fewer side effects and high therapeutic index in clinical. However, it has been reported that some TKIs (such as erlotinib, pazopanib, sorafenib and nilotinib) may have caused a broad set of undesirable side effects, including hyperbilirubinemia, liver injury or other safety issues. Taking into account that UGT1A1 is the key enzyme responsible for bilirubin detoxification and that many TKIs have been proved as potent UGT1A1 inhibitors, it is necessary to investigate the inhibitory effects of newly developed TKIs on UGT1A1 and to predict the potential DDI risks of TKIs via UGT1A1 inhibition.

Icotinib (purity > 98%) was purchased from Biochempartner Co., Ltd. (Shanghai, China). Erlotinib (purity > 99%) was purchased from Roche Co., Ltd. (Shanghai, China). NCHN was chemically synthesized and its glucuronidation product NCHN-O-glucuronicide (NCHNG) was biosynthesized by authors as previously reported. Uridine-5′-diphosphoglucuronic acid (UDPGA) was obtained from Sigma–Aldrich (St. Louis, MO, USA). UGT1A1 was obtained from BD Gentest (Woburn, MA, USA). HLMs, derived from 50 donors, were obtained from BioreclamationIVT (Baltimore, USA). The tissues were from both males (n = 25) and females (n = 25); the median age was 58 years with the age range

2. Materials and methods

2.1. Chemicals and reagents

Icotinib (purity > 98%) was purchased from Biochempartner Co., Ltd. (Shanghai, China). Erlotinib (purity > 99%) was purchased from Roche Co., Ltd. (Shanghai, China). NCHN was chemically synthesized and its glucuronidation product NCHN-O-glucuronicide (NCHNG) was biosynthesized by authors as previously reported. Uridine-5′-diphosphoglucuronic acid (UDPGA) was obtained from Sigma–Aldrich (St. Louis, MO, USA). UGT1A1 was obtained from BD Gentest (Woburn, MA, USA). HLMs, derived from 50 donors, were obtained from BioreclamationIVT (Baltimore, USA). The tissues were from both males (n = 25) and females (n = 25); the median age was 58 years with the age range

Figure 1 Chemical structures of icotinib and erlotinib.
of 17 to 89; the ethnicity were 2% Caucasian, 10% Hispanic, 4% Black, 2% Asian and 2% others. BioreclamationIVT maintained strict adherence to all applicable ethical guidelines and regulations and the tissues were directly from non-profit organization that have provided assurance as to legal compliance. BioreclamationIVT does not traffic in human tissues and has the utmost respect and appreciation for all donated tissues. Liver preparations were stored at –80 °C until use. The solvents and other reagents were of analytical reagent grade.

2.2. Inhibition assays of NCHN-4-O-glucuronidation

The inhibitory effects of icotinib and erlotinib against NCHN-4-O-glucuronidation were determined according to previously published methods with a slight modification. A typical incubation mixtures (total volume 200 μL) was consisted of Tris–HCl buffer (pH 7.4, 50 mmol/L), UGT1A1 (0.06 mg/mL) or HLM (0.2 mg/mL), MgCl2 (50 mmol/L), Brij 58 (0.1 mg/mg protein) and NCHN (20 μmol/L for inhibition screening; 15–100 μmol/L for inhibition constant determination) in the presence or absence of different concentrations of icotinib and erlotinib (1, 10, and 100 μmol/L for inhibition screening; 0.2–60 μmol/L for inhibition constant determination). HLMs were pre-incubated with Brij 58 on ice for 20 min before incubation. After 3 min pre-incubation at 37 °C, the reaction was initiated by the addition of 10 μL of UDPGA. NCHN was incubated with UGT1A1 (0.06 mg/mL) for 50 min or HLMs (0.2 mg/mL) for 40 min in the presence or absence of different concentrations of icotinib and erlotinib. Then, the reactions was quenched by adding 200 μL of acetonitrile, the incubation mixtures were then centrifuged under the condition of 20,000 × g, 4 °C for 20 min to obtain the supernatant. Lastly, 200 μL aliquots of the supernatants were diverted into the 96-well plates, the fluorescence intensity of NCHNG were read by Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, USA), under the excitation wavelength of 362 nm and the emission wavelength of 450 nm at a gain setting of 80. The positive control (positive inhibitor, nilotinib) was also carried out under the same conditions. The tested chemicals and inhibitors were all dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the incubation system was 1% (v/v), which has only a minor effect on the catalytic activities of most human UGT enzymes. The concentrations of erlotinib and icotinib presented in the incubations were corrected for binding to HLMs according to the method of equilibrium dialysis.

2.3. Inhibition kinetics analyses

Inhibition kinetic analysis was performed in both HLMs and recombinant human UGT1A1. IC50 values were determined using the same substrate concentration in the presence of different inhibitor concentrations. Ki values and the inhibition kinetic types (competitive inhibition, noncompetitive inhibition, uncompetitive inhibition and mixed inhibition) were determined by using various concentrations of NCHN and multiple concentrations of icotinib or erlotinib. IC50 values and Ki values were all estimated by the nonlinear regression analysis of Graphpad Prism 6.0 (San Diego, CA, USA). The following equations for competitive inhibition Eq. (1), noncompetitive inhibition Eq. (2), or mixed inhibition Eq. (3) were used to calculate the Ki values.

\[ v = \frac{(V_{max}S)}{(K_m + S)} + \frac{1}{K_i} \]  \hspace{1cm} (1)

\[ v = \frac{(V_{max}S)}{(K_m + S) + S]} + \frac{1}{K_i} \]  \hspace{1cm} (2)

\[ v = \frac{(V_{max}S)}{(K_m + S) + (1 + 1/K_i)} \]  \hspace{1cm} (3)

where v is the velocity of the reaction; Ki is the inhibition constant describing the affinity of the inhibitor for the enzyme; S and I are the substrate and inhibitor concentrations, respectively; Vmax is the maximum velocity; K_m is the Michaelis constant (substrate concentration at 0.5 Vmax). Goodness-of-fit parameters were employed to identify the most appropriate inhibition kinetic types.

2.4. Prediction of the DDI potential in vivo

The magnitudes of inhibitory potential mediated by icotinib and erlotinib were estimated by the ratio of AUC in the presence and absence of the inhibitor. This ratio was predicted by using the formula Eq. (4):
reports that CYPs were the major metabolism enzymes for erlotinib and icotinib. The inhibition of erlotinib and icotinib against UGT1A1 in HLMs is not time-dependent, implying that erlotinib and icotinib cannot trigger irreversible inhibition against UGT1A1 (data not shown).

3.2. Inhibition kinetic analyses for icotinib and erlotinib against NCHN-O-glucuronidation in UGT1A1

Inhibition kinetic assays were further performed to characterize the inhibition types and the inhibition kinetic constants of icotinib and erlotinib against UGT1A1. As shown in Fig. 3 and Supplementary information Fig. S2, both Lineweaver-Burk and Dixon plots demonstrated that icotinib functioned as a noncompetitive inhibitor against NCHN-O-glucuronidation in recombinant human UGT1A1. The $K_i$ value of icotinib against NCHN-O-glucuronidation in recombinant human UGT1A1 was determined as 10.04 μmol/L (Table 1). In contrast, erlotinib was found to be a more potent noncompetitive inhibitor against UGT1A1. The $K_i$ value of erlotinib against NCHN-O-glucuronidation in human UGT1A1 was determined as 1.72 μmol/L (Table 1). These findings demonstrated that both erlotinib and icotinib displayed the same inhibition type (noncompetitive inhibition) against UGT1A1-mediated NCN-4-O-glucuronidation, while icotinib exhibited relatively weak inhibitory effect ($K_i$ value of 10.04 μmol/L versus 1.72 μmol/L) against UGT1A1 compared to erlotinib.

3.3. Inhibition kinetic analyses for icotinib and erlotinib against NCHN-O-glucuronidation in HLM

To further validate whether icotinib and erlotinib could inhibit UGT1A1 in complex biological systems, the inhibition kinetic for icotinib and erlotinib against UGT1A1-mediated NCHN-O-glucuronidation were performed in pooled HLMs. As shown in Fig. 4, Table 1 and supplementary information Fig. S2 both Lineweaver-Burk and Dixon plots demonstrated that icotinib and erlotinib displayed noncompetitive inhibition type against UGT1A1-mediated NCHN-O-glucuronidation in HLMs, which was in accordance with the inhibition types of icotinib and erlotinib against NCHN-O-glucuronidation in recombinant human UGT1A1. The $K_i$ values of icotinib and erlotinib against NCHN-O-glucuronidation by UGT1A1 in HLMs were determined as 8.55 and 1.23 μmol/L, respectively. These results demonstrated that erlotinib exhibited more potent inhibitory effects on UGT1A1 in HLMs (about 7-fold for NCHN-O-glucuronidation) than that of

Table 1: Inhibition kinetic parameters of icotinib and erlotinib against UGT1A1-mediated NCHN-4-O-glucuronidation in both recombinant human UGT1A1 and HLMs.

| Enzyme source | Icotinib | Erlotinib |
|---------------|----------|-----------|
|               | IC$_{50}$ (μmol/L) | $K_i$ (μmol/L) | Inhibition type | IC$_{50}$ (μmol/L) | $K_i$ (μmol/L) | Inhibition type |
| UGT1A1        | 8.76 ± 0.78 | 10.04     | Noncompetitive | 0.69 ± 0.02 | 1.72 | Noncompetitive |
| HLM           | 5.15 ± 0.28 | 8.55      | Noncompetitive | 0.68 ± 0.01 | 1.23 | Noncompetitive |

Figure 2: Dose-dependent inhibition curves of icotinib and erlotinib against UGT1A1-mediated NCHN-4-O-glucuronidation in recombinant human UGT1A1 (A and B) and HLMs (C and D).
icotinib, which agreed well with the results obtained from UGT1A1. All these findings demonstrated that both erlotinib and icotinib are noncompetitive inhibitors against UGT1A1-mediated NCHN-O-glucuronidation, while icotinib displayed relatively weak inhibition against UGT1A1.

3.4. Quantitative prediction of DDI risks of icotinib and erlotinib

The inhibition potentials of icotinib and erlotinib against human UGT1A1 and the clinical DDI risk in vivo were evaluated by estimating the changes in AUC of NCHN predominantly metabolized by UGT1A1. Following oral administration of icotinib (125 mg × 3 daily) and erlotinib (150 mg daily), the maximum plasma concentration of icotinib and erlotinib in human was 4.79 and 6.06 μmol/L, respectively. The $C_{\text{max,u}}$ of erlotinib and icotinib were calculated as 0.55 and 0.53 μmol/L, respectively. The corrected $K_{\text{i,u}}$ and uncorrected $K_i$ values of icotinib and erlotinib against UGT1A1-mediated NCHN-O-glucuronidation in HLMs were much closed to each other, due to the negligible binding of icotinib and erlotinib to proteins in HLMs. The predicted AUC ratio and the increased percent were listed in Table 2. The AUC of NCHN would be increased by 5%–43% following administration of erlotinib (150 mg daily), while the AUC of NCHN was slightly increased by 1%–6% following administration of icotinib (125 mg × 3 daily). These results suggested that icotinib was unlikely to cause a significant DDI through inhibition of UGT1A1, while erlotinib exhibited much higher DDI potentials.

4. Discussion

Recently, TKIs have proven to be an effective therapy to treat certain forms of cancers including non-small cell lung cancer, pancreatic cancer, and chronic myeloid leukaemia due to their selective and potent inhibition of tumour cells in vitro. Unfortunately, the use of majority of TKIs are reported to be associated with serious toxic effects on a number of vital organs including the liver. Erlotinib is a TKI with a boxed label warning due to hepatotoxicity, thus needs to be carefully monitored for patients, which is recommended by FDA. It is noteworthy that erlotinib has been reported to exhibit strong inhibitory effects on UGT1A1 and may result in potential DDI. As the first home-grown anticancer drug developed by Chinese pharmaceutical company (Zhejiang Beta Pharma, Inc., Zhejiang, China), icotinib is structurally similar with erlotinib, implying that icotinib may act on the same targets as erlotinib. It is well known that erlotinib is a potent inhibitor of UGT1A1, while strong inhibition of UGT1A1 may lead to hepatotoxicity including hyperbilirubinemia, kernicterus and drug-induced liver injury. Thus, it is necessary to carefully investigate the inhibitory effects of erlotinib derivatives (such as icotinib) on UGT1A1. However, until now, the inhibitory potency of icotinib on UGT1A1 has not been well investigated, and the differences in UGT1A1 inhibition and potential risk between icotinib and erlotinib have not been clearly evaluated. In these cases, this study focused on the investigation and comparison of the inhibitory effects of icotinib and erlotinib on human UGT1A1 and evaluated their potential DDI risk due to UGT1A1 inhibition.

In this study, the inhibitory effects of icotinib and erlotinib against human UGT1A1 were carefully investigated and compared
to each other. The results clearly demonstrated that both icotinib and erlotinib are UGT1A1 inhibitors as we assumed, but icotinib exhibited relatively weak inhibitory effect against UGT1A1 compared to erlotinib. The inhibition potency (IC50) of erlotinib on UGT1A1 in both recombinant UGT1A1 and HLMs is less than 1 μmol/L, which is more potent than that of icotinib in these enzyme sources (45 μmol/L). Furthermore, inhibition kinetic analysis was performed to characterize and evaluate the inhibition types and inhibition constants of icotinib and erlotinib. The results clearly demonstrated that both compounds functioned as non-competitive inhibitors against NCHN-O-glucuronidation in both recombinant human UGT1A1 and HLMs, while the Ki values of icotinib is relatively greater than that of erlotinib. All these findings suggested that erlotinib is a potent inhibitor against UGT1A1, but icotinib is a moderate inhibitor against UGT1A1.

From the view of chemical structure, both icotinib and erlotinib have a same quinazoline skeleton, but the side-chain of icotinib has a feature of closed ring structure, which makes it to be more hydrophobic31,32. The similar structure makes icotinib and erlotinib act on the same biological targets including EGFR and UGT1A1, but the subtle difference in chemical structure may change the potency of these two agents towards the same target. In this study, we compare the inhibitory potency of icotinib and erlotinib against UGT1A1, our results shown that both compounds display the same inhibition types against UGT1A1, but the subtle difference in chemical structure between them leads to the alteration in inhibitory effects of these two agents on UGT1A1. In contrast to erlotinib, icotinib displayed relatively low affinity towards UGT1A1, the Ki value of icotinib (8.55 μmol/L in HLMs) is about 7-fold than that of erlotinib (1.23 μmol/L in HLMs). All

| Inhibitor | Enzyme source | E_\text{h}^{a} | f_{\text{unp}}^{b} | K_{\text{ci}}^{c} (\mu\text{mol/L}) | C_{\text{max,ci}}^{d} (\mu\text{mol/L}) | AUC ratios^{e} | AUC increased (%) |
|-----------|---------------|----------------|----------------|-------------------------------|----------------------------------|----------------|------------------|
| Icotinib  | HLM           | 0.1–0.9        | 1              | 8.55                          | 0.53                             | 1.01–1.06      | 1%–6%            |
| Erlotinib | HLM           | 0.1–0.9        | 1              | 1.23                          | 0.55                             | 1.05–1.43      | 5%–43%           |

^{a}E_\text{h} is the hepatic extraction ratio ranging from 0.1 to 0.9 for UGT1A1 substrates.
^{b}The f_{\text{unp}} was set to 1.
^{c}The K_{\text{ci}} values of erlotinib and icotinib is the same to the K_{i} values, due to the negligible binding of erlotinib or icotinib to HLMs (0.2 mg/mL).
^{d}The C_{\text{max}} of icotinib in humans was 4.79 μmol/L after a 125 mg × 3 daily dose of icotinib hydrochloride; The C_{\text{max}} of erlotinib in humans was 6.06 μmol/L after a single 150 mg dose of erlotinib. The unbound C_{\text{max}} of erlotinib or icotinib (C_{\text{max,ci}}) were calculated as C_{\text{max}} \times f_{\text{u}} (f_{\text{u}} was determined as 0.09 and 0.11 for erlotinib and icotinib, respectively).
^{e}Prediction methods as described in the materials and method.
these findings suggested that the subtle change in the side-chains of erlotinib to a closed ring chain can decrease the inhibition on UGT1A1, which could be used to partially explain why icotinib displayed improved safety profile (such as very low incidence of liver function impairment) in contrast to erlotinib.

With the inhibition constant ($K_i$) of these two TKIs in hands, the magnitudes of the potential inhibitory effects of them on UGT1A1 in vivo were also predicted by estimating the changes in AUC ratios. Based on the $C_{\text{max},u}$ and $K_{iu}$ values, the AUC of NCHN would be increased by 5%–43% following administration of erlotinib (150 mg daily), while the AUC of NCHN was slightly increased by 1%–6% following administration of icotinib (125 mg × 3 daily). Thus, the results suggested that erlotinib exhibited much higher DDI potentials than icotinib via UGT1A1 inhibition, which was consistent with the clinical observation that icotinib displayed improved safety profile (such as very low incidence of liver function impairment) in contrast to erlotinib. It should be noted that the inhibitory effects of icotinib or erlotinib on UGT1A1 should be taken with caution in some special populations. It is well-known that UGT1A1 is a highly polymorphic enzyme, and more than one hundred variants have been found. Some polymorphic expression of certain UGT1A1 mutants may result in partial or complete loss of UGT1A1 activity, the exposure of icotinib and erlotinib to these individuals may bring strong effects on UGT1A1-mediated metabolism. Thus, the individuals with UGT1A1 variants that possessing low catalytic activity might be expected to manifest heightened susceptibility to serious toxic effects as a consequence of inhibition of UGT1A1 by icotinib and erlotinib. Furthermore, erlotinib could be metabolized in human primarily by CYP3A4, CYP3A5, CYP1A1 and CYP1A2, while the primarily enzymes for icotinib was CYP3A4 and CYP2C19. Several genetic polymorphisms within these CYPs gene, especially in subjects carrying activity decreasing alleles, might cause high systemic exposure of erlotinib or icotinib. As a result, the concentrations of icotinib or erlotinib in the blood may exceed the maximum concentrations used here in predicting the AUC ratio and thus bring undesirable effects. In these cases, the potential risks of icotinib or erlotinib via UGT1A1 inhibition should be fully considered.

In summary, our results demonstrated that icotinib and erlotinib displayed inhibitory effects on human UGT1A1, while icotinib exhibited relatively weak inhibition against UGT1A1 catalytic activity in HLM compared to erlotinib. In addition, icotinib was unlikely to cause a significant DDI through inhibition of UGT1A1, while erlotinib exhibited much higher DDI potentials. Our findings shed light on the underlying mechanisms of clinically significant hepatotoxicity associated with erlotinib and partially account for the differential hepatic toxicity observed between icotinib and erlotinib. All these findings reveal that the subtle differences in chemical structure between icotinib and erlotinib bring different inhibition potency on UGT1A1, which are very helpful for the medicinal chemists to design and develop next generation TKIs with improved safety. Meanwhile, these findings are very useful for guiding reasonable applications of icotinib and erlotinib in clinic, especially for avoiding the potential DDI risks caused by them via UGT1A1 inhibition.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.apsb.2017.07.004.

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