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Rapid Communication

Metabolic Pathway of Icotinib *In Vitro*: The Differential Roles of CYP3A4, CYP3A5, and CYP1A2 on Potential Pharmacokinetic Drug-Drug Interaction

TianHong Zhang [1], KeRong Zhang [2], Li Ma [3], Zheng Li [1], Juan Wang [1], YunXia Zhang [1], Chuang Lu [4], Mingshe Zhu [3, 5, *], XiaoMei Zhuang [1, *]

[1] *Key Laboratory of Toxicology and Medical Countermeasures, Beijing Institute of Pharmacology and Toxicology, Beijing, China*
[2] *AB Sciex, Beijing, China*
[3] *Pharmacetucial Candidate Optimization, Bristol-Myers Squibb, Princeton, New Jersey 08540*
[4] *Sanofi Company, Waltham, Massachusetts 02451*
[5] *MassDefect Technologies, Princeton, New Jersey 08540*

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ABSTRACT

Icotinib is the first self-developed small molecule drug in China for targeted therapy of non–small cell lung cancer. To date, systematic studies on the pharmacokinetic drug-drug interaction of icotinib were limited. By identifying metabolite generated in human liver microsomes and revealing the contributions of major cytochromes P450 (CYPs) in the formation of major metabolites, the aim of the present work was to understand the mechanisms underlying pharmacokinetic and pharmacological variability in clinic. A liquid chromatography/UV/high-resolution mass spectrometer method was developed to characterize the icotinib metabolites. The formation of 6 major metabolites was studied in recombinant CYP isozymes and human liver microsomes with specific inhibitors to identify the CYPs responsible for icotinib metabolism. The metabolic pathways observed *in vitro* are consistent with those observed in human. Results demonstrated that the metabolites are predominantly catalyzed by CYP3A4 (77%~87%), with a moderate contribution from CYP3A5 (5%~15%) and CYP1A2 (3.7%~7.5%). The contribution of CYP2C8, 2C9, 2C19, and 2D6 is insignificant. Based on our observations, to minimize drug-drug interaction risk in clinic, coprescription of icotinib with strong CYP3A inhibitors or inducers must be weighed. CYP1A2, a highly inducible enzyme in the smoking population, may also represent a determinant of pharmacokinetic and pharmacological variability of icotinib, especially in lung cancer patients with smoking history.

Introduction

Non–small cell lung cancer (NSCLC) accounts for nearly 85% of all lung cancer cases, yet its treatment remains a challenge.[1] Icotinib, a novel epidermal growth factor receptor tyrosine kinase inhibitors (TKIs) originally developed by Beta Pharma, Inc. (Zhejiang, China), has been approved in China to treat NSCLC.[2] Owing to its superior efficacy, icotinib has won first prize at the national Sci-Tech Progress Convention in 2016.

For anti-cancer agents, drug-drug interaction (DDI) is susceptible to occur as cancer patients are often on multiple medications with an average of 6.8 drugs at any given time. Although increasing frequency of identified DDI by coprescription of drugs that induce or inhibit metabolic pathways of TKIs has been reported,[3] studies on the metabolism-related pharmacokinetic DDI of icotinib were very limited to date due to it is relatively new to the market. An assessment of the inhibitory potential of icotinib to cytochromes P450 (CYPs) in human liver microsomes (HLM) showed that it had mild inhibition of the 7 major CYPs with IC_{50} values all greater than 20 μM, which is far greater than its clinical plasma free concentration.[4] Icotinib was also found to stimulate CYP3A-mediated midazolam metabolism that is 1′-hydroxymidazolam formation in HLM.[4] A similar phenomenon was previously observed with gefitinib and erlotinib,[5] both of which are TKIs of epidermal growth

Abbreviations used: CYP, cytochrome P450; HRMS, high-resolution mass spectrometer; LC, liquid chromatography; MS, mass spectrometry; Thio-TEPA, N,N′,N″-triethylenethiophosphoramide.

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* *Correspondence to:* Xiaomei Zhuang (Telephone: +13-683600728; Fax: +13-68225893) and Mingshe Zhu (Telephone: 609-694-3946).

E-mail addresses: mingshe.zhu@yahoo.com (M. Zhu), xiaomeizhuang@163.com (X. Zhuang).

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factor receptor. An earlier study demonstrated that the metabolism of icotinib was dependent primarily on CYP3A4.[6] Since there is substantial overlap in the substrate specificity of CYP3A4 and CYP3A5, the present study was designed to investigate whether CYP3A5 might have a role in the metabolism of icotinib. This was of particular interest because CYP3A5 is polymorphically expressed in special populations and, if involved in icotinib metabolism, it could contribute to pronounced pharmacokinetic and pharmacological variability. The other aim of the current work was to elucidate the metabolites of icotinib in HLM and to identify the human CYP isoforms responsible for the metabolic pathways of icotinib for a better understanding of the mechanisms underlying pharmacokinetic and pharmacological variability in clinic by using recombinant CYPs and HLM with specific CYP inhibitors.

Materials and Methods

Metabolites Generation and Identification in HLM

Icotinib (20 μM) was incubated in HLM (1 mg/mL) for 45 min. The incubations (0.5 mL) contained phosphate buffer (100 mM, pH 7.4), $MgCl_2$ (5 mM), and nicotinamide adenine dinucleotide phosphate (NADPH) (1 mM). Metabolic reactions were initiated at 37°C by addition of NADPH after 5 min preincubation. Negative and positive controls were conducted: the absence of NADPH or the presence of CYP probes instead of icotinib, respectively. The CYP probes are phenacetin, diclofenac, S-mephenytoin, dextromethorphan, and midazolam for CYP1A2, 2C9, 2C19, 2D6, and 3A4/5, respectively.

In Vitro *Incubation Optimization*

Icotinib metabolism was investigated using a range of human microsomal protein concentrations (0.2-1 mg/mL), incubation periods (5-90 min), and icotinib concentrations (0.5-5 μM), to select an optimal incubation condition for a linear production of major metabolites. The final conditions chosen were 5-μM icotinib, 0.5-mg/mL HLM, and 30 min incubation. The formation of each metabolite under this condition was in a linear range (Fig. S1).

Identification of CYP-Mediated Metabolic Pathway of Icotinib in Recombinant CYPs and HLM

Individual expressed CYP isoforms were firstly used to identify the metabolic pathway of icotinib. Icotinib (5 μM) was incubated with recombinant human CYP1A2, 2B6, 2C8, 2C9, CYP2C19, 2D6, 3A4, and 3A5 (20 pmol/mL) in triplicate. After a 5-min preincubation, metabolic reactions were initiated by the addition of NADPH (1 mM) and stopped after 30 min by the addition of 0.4 mL of acetonitrile containing 0.2-μM aconitine as internal standard. Metabolite formation was compared with control reactions without NADPH being added.

Similar incubations were performed in HLM (0.5 mg/mL) with absence (control) or presence of selective CYP chemical inhibitors. The final concentrations of the selective CYP inhibitors were 1 μM for α-naphthoflavone (CYP1A2), montelukast (CYP2C8), sulfaphenazole (CYP2C9), benzylnirvanol (CYP2C19), quinidine (CYP2D6), CYP3cide (CYP3A4), ketoconazole (CYP3A4 and 3A5), and 50 μM for N,N′,N″-triethylenethiophosphoramide (Thio-TEPA) (CYP2B6). An additional 10-min preincubation was applied to the time-dependent inhibitors Thio-TEPA and CYP3cide before the addition of icotinib.

Analytical Methods

The metabolite profiling was elucidated with high-resolution mass spectrometry (liquid chromatography [LC]/UV/high-resolution mass spectrometer [HRMS]) (TripleTOF 5600; AB Sciex). The quantification of icotinib and its 6 major metabolites (M4, M6, M7, M8, M11, and M16) was achieved with a LC/mass spectrometry (MS)/MS system (LC-20AD, Shimadzu equipped with API 5000; AB Sciex). The ratios of parent drug and each metabolite to IS were used for semiquantification of each metabolite by assuming the parent, and metabolites have similar ionization efficiencies. The information of chemicals and reagents and detailed experimental procedures are presented in the Supplemental Section.

Results

Identification of Icotinib Metabolites Formed in HLM

LC/UV profiles of icotinib metabolites formed in the HLM displayed 6 major metabolites (M4, M6, M7, M8, M11, and M16) (Fig. S2A). These metabolites were detected and characterized by LC/HRMS (Fig. S2B). The chemical structures of M6, M7, M8, M11, and M16 were determined based on the interpretation of accurate mass spectra and compared with the icotinib metabolites previously identified in human urine and feces by nuclear magnetic resonance.[7] The major metabolic pathways of icotinib in HLM are proposed in Figure 1, which includes hydroxylation of the aromatic ring to M11, oxidation of the alkyne moiety to M4, and oxidation-mediated ring open of the crown ether moiety to a few intermediates that undergo ether oxidation to carbolic acid metabolites of M6, M7, and M8 or reduction to alcoholic metabolite of M16. Several minor metabolites (M1, M2, M3, M5, M9, M10, and M12-M15) in HLM showed weak signals in the LC/UV profile (Fig. S3) and were discovered and structurally characterized by LC/HRMS (Table 1). MS/MS spectra and proposed structures of icotinib metabolites formed in HLM were presented in Figure S4. A majority of these minor metabolites are secondary metabolites or analogues derived from the 6 major metabolites. Icotinib is a chemical apology of erlotinib, and the current results show that the major biotransformation pathways and associated metabolite structures of icotinib are very similar to that of erlotinib.[8] Regarding the metabolites contribution to the DDI liability, our study has shown none of the major metabolites exceeded 25% of the total UV peak area. Our previous work[4] also identify that icotinib is not a time-dependent inhibitor for all 7 major CYPs, thus those identified metabolites being reactive metabolites are also unlikely.

Biotransformation is the major elimination route of icotinib in humans.[7] Therefore, a CYP phenotyping experiment that monitors the formation of the 6 major metabolites in HLM should provide valuable information on the roles of CYPs in the major metabolic pathways and, in turn, the potential DDI liability of icotinib as a victim drug.

Identification of CYP-Dependent Metabolic Pathways of Icotinib in HLM

The semiquantification of 6 major metabolites generated in HLM was developed and validated according to the study by Svedberg et al.[8] (see Supplemental Material). The formation of 6 major metabolites in 8 rCYP or HLM with/without CYP inhibitors was studied to identify the CYPs that are responsible for icotinib metabolic pathways. The formation of each metabolite of icotinib was NADPH-dependent, and the contribution of CYP3A4 was the highest, followed by CYP3A5 and CYP1A2. When the relative