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Allosteric activation of midazolam CYP3A5 hydroxylase activity by icotinib – Enhancement by ketoconazole

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Chemical compounds studied in this article:
ICO, icotinib (PubChem CID: 22024915)
MDZ, midazolam (PubChem CID: 4192)
KTZ, ketoconazole (PubChem CID: 456201)
1′-OH-MDZ, 1′-hydroxy-midazolam (PubChem CID: 107917)
4-OH-MDZ, 4-hydroxy-midazolam (PubChem CID: 124449)
TST, testosterone (PubChem CID: 6013)
NIF, nifedipine (PubChem CID: 4458)
Phenacetin (PubChem CID: 4754)
Bupropion (PubChem CID: 444)
Amodiaquine (PubChem CID: 2165)
Diclofenac (PubChem CID: 3033)
S-mephenytoin (PubChem CID: 107921)
Dextromethorphan (PubChem CID: 5360696)
CYP3cide (PubChem CID: 71315139)
Propranolol (PubChem CID: 4946)

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ABSTRACT

Icotinib (ICO), a novel small molecule and a tyrosine kinase inhibitor, was developed and approved recently in China for non-small cell lung cancer. During screening for CYP inhibition potential in human liver microsomes (HLM), heterotropic activation toward CYP3A5 was revealed. Activation by icotinib was observed with CYP3A-mediated midazolam hydroxylase activity in HLM (~40% over the baseline) or recombinant human CYP3A5 (rhCYP3A5) (~70% over the baseline), but not in the other major CYPs including rhCYP3A4. When co-incubated with selective CYP3A4 inhibitor CYP3cide or monoclonal human CYP3A4 inhibitory antibody in HLM, the activation was extended to ~60%, suggesting CYP3A5 might be the isozyme involved. Further, the relative activation was enhanced to ~270% in rhCYP3A5 in the presence of ketoconazole. The activation was substrate and pathway dependent and observed only in the formation of 1′-OH-midazolam, and not 4-OH-midazolam, 6β-OH-testosterone, or oxidized nifedipine. The activation requires the presence of cytochrome *b5* and it is only observed in the liver microsomes of dogs, monkeys, and humans, but not in rats and mice. Kinetic analyses of 1′-OH-midazolam formation showed that ICO increased the V_{max} values in HLM and rhCYP3A5 with no significant changes in K_m values. By adding CYP3cide with ICO to the incubation, the V_{max} values increased 2-fold over the CYP3cide control. Addition of ketoconazole with ICO alone or ICO plus CYP3cide resulted in an increase in V_{max} values and decrease in K_m values compared to their controls. This phenomenon may be attributed to a new mechanism of CYP3A5 heterotropic activation, which warrants further investigation.

1. Introduction

Icotinib (icotinib hydrochloride tablets, Betta Pharma Co., Hangzhou, Zhejiang, China, Fig. 1) is a novel quinazoline compound and oral epidermal growth factor receptor (EGFR) inhibitor that has proven to be a potent blocker of tyrosine kinase [1,2]. Due to its superior pharmacological activity, icotinib was approved by the

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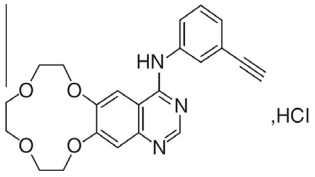

Fig. 1. The chemical structure of Icotinib hydrochloride.

Chinese food and drug administration (CFDA) to treat non-small cell lung cancer (NSCLC) and has been marketed since 2011 [3,4]. Despite icotinib being a prescription drug, investigation of its pharmacokinetic drug-drug interaction (DDI) has not been extensively conducted. The Chinese drug label of icotinib contains limited information on icotinib-related DDI potential and the enzymes responsible for its metabolism.

Chen et al. [5] have conducted a reaction phenotyping study using three different methods (rhCYP × abundance, relative activity factor-RAF, and chemical inhibitors), and their results suggest that the relative contributions of CYP450 toward the hepatic metabolism of icotinib were 13–25% for CYP1A2, 8–25% for CYP2D6, 0–28% for CYP2C9, 13–28% for CYP2C19, and 36–53% for CYP3A4/5. The CYP relative contribution data were then applied to a physiologically based pharmacokinetic (PBPK) model to predict the corresponding DDI when icotinib was treated as a victim. With major metabolic contribution observed from CYP3A4 (~50%), the AUC ratios under concomitant administration of ketoconazole (KTZ) and rifampin were predicted to be 3.22 and 0.55-fold of the controls, respectively. Their work provides useful knowledge of the combination of icotinib with a perpetrator drugs in anticancer chemotherapy.

On the other hand, the DDI risk of icotinib itself as a perpetrator has not been well studied. Considering that icotinib belongs to a group of drugs that share a common quinazoline ring backbone structure and are used to treat advanced or metastatic NSCLC, it is possible that the characteristic CYP inhibition property may exist in many quinazoline containing EGFR-tyrosine kinase inhibitors (TKI) including icotinib. In addition, it has been reported that this class of compounds may have the potential to cause stimulation of CYP3A4 activity besides inhibition [6–8]. Hence, the purpose of this study was to quantitatively examine the inhibitory effects (both reversible and time-dependent) of icotinib on seven major CYP isoforms (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4/5) using human liver microsomes (HLM). The potential of icotinib to activate CYP3A was also investigated, along with the substrate-, species-, and pathway-dependency of the activation as well as the effects of CYP3A modulators, such as human monoclonal CYP3A4 inhibitory antibodies (MAB-3A4), CYP3cide, and ketoconazole. The enzyme kinetic characterization of the heterotropic effects by icotinib in HLM and rhCYP3A5 was also investigated.

2. Materials and methods

2.1. Chemical and enzyme sources

Icotinib hydrochloride (ICO) was purchased from MedChem Express (Shanghai, China) with a purity greater than 98%. Midazolam (MDZ), 1′-OH-MDZ, 4-OH-MDZ, testosterone, 6β-OH-testosterone, phenacetin, acetaminophen, diclofenac, S-mephenytoin, 4-OH-diclofenac, 4-OH-mephenytoin, bupropion, OH-bupropion, amodiaquine, N-desethyamodiaquine, dextromethorphan, dextrorphan, ketoconazole, aconitine, and CYP3cide were all purchased from Sigma-Aldrich (St. Louis, MO). Nifedipine, oxidized nifedipine, human liver microsomes (150-donor pool, mixed sex), male murine liver microsomes, male rat liver microsomes, male dog liver microsomes, male cynomolgus monkey liver microsomes, cDNA-expressed CYP3A4 and CYP3A5 enzymes with reductase and with or without cytochrome b_5, and human CYP3A4 inhibitory monoclonal antibodies (MAB-3A4) were purchased from Corning Life Sciences (Tewksbury, MA). The ratio of CYP content: reductase activity:b_5 content (with b_5 rhCYP only) was characterized by the vendor as follows, for rhCYP3A4:0.1 nmole/ml (7.5 mg protein/ml):3200 nmole/(min × mg protein):1200 pmol/mg protein; for rhCYP3A5:0.1 nmole/ml (15 mg protein/ml):4300 nmole/(min × mg protein):1900 pmol/mg protein. All rhCYP3A5 used in the activity studies contains cytochrome b_5 unless specifically indicated in the experimental section. NADPH was purchased from Roche Life Science (Basel-Stadt, Switzerland).

2.2. Inhibition or activation assay of ICO for individual CYPs

To determine the IC_{50} values of icotinib for seven major CYPs in HLM, seven different concentrations of icotinib were used from 0 to 100 μM (0, 0.1, 0.5, 2.5, 10, 25, 50, and 100 μM). Incubations were performed at 37 °C in a shaking water bath. The final concentration of HLM was 0.5 mg/ml and substrate and cofactor concentrations were as follows: phenacetin (30 μM), bupropion (70 μM), amodiaquine (2 μM), diclofenac (5 μM), S-mephenytoin (50 μM), dextromethorphan (5 μM), MDZ (5 μM), and NADPH (1.0 mM) in a 0.1 M sodium phosphate buffer ($MgCl_2$ 3.3 mM, pH 7.4).

All incubations were 100 μl in volume and conducted in 1 ml 96-deep well plate. The reactions were started by the addition of NADPH (1 mM) after the pre-incubation of HLM, the test drug, and the CYP substrate at 37 °C for 5 min. All incubations were stopped after 10 min (except 30 min for S-mephenytoin) by the addition of 400 μl of chilled acetonitrile containing IS (50 ng/ml propranolol). After centrifugation at 18,000×g for 10 min, the supernatant was diluted with 10 volumes of 50% acetonitrile in water and then collected and analyzed using LC-MS/MS. Positive controls, including α-naphthoflavone, Thio-TEPA, monteleukast, sulfaphenazole, benzylnirvanol, quinidine, and ketoconazole were included simultaneously in the study. Stock solutions of the test drugs were prepared in DMSO. The final concentration of DMSO in incubations was less than 0.1% (v/v). The IC_{50} values for ICO were calculated for individual P450 enzymes by quantitating the metabolite formation for each probe substrate based on their respective standard curves: acetaminophen (CYP1A2), OH-bupropion (CYP2B6), N-desethyamodiaquine (CYP2C8), 4-OH-diclofenac (CYP2C9), 4-OH-mephenytoin (CYP2C19), dextrorphan (CYP2D6), and 1′-OH-MDZ (CYP3A4/5). The percentage activity remaining at various ICO concentrations was calculated by comparison to the vehicle control (0 μM ICO). GraphPad Prism version 5.0 was used to calculate the IC_{50} with a sigmoidal module (La Jolla, CA).

A time-dependent inhibition shift assay was performed using a similar experimental setup as the CYP inhibition assay, with two curves for each assay. A 30 min preincubation of ICO in HLM with or without NADPH was performed prior to the addition of substrate and NADPH in both groups. The incubation was carried out for another 10 min (30 min for S-mephenytoin). The remaining steps of sample processing and LC-MS/MS analyses were the same as described in the above section.

2.3. Heterotropic activation assay of ICO for CYP3A4/5 in HLM and supersomes®

Discrete assays for monitoring the heterotropic activation of CYP3A4/5 enzymatic activity in HLM or Supersomes® of rhCYP3A4 and rhCYP3A5 with or without cytochrome *b5* were performed using a similar procedure to the one described above. After a