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Assessment of drug–drug interactions caused by metabolism-dependent cytochrome P450 inhibition

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ABSTRACT

This study was designed to develop methods for detecting metabolism-dependent reversible, quasi-irreversible, and irreversible cytochrome P450 (CYP) inhibition using pooled human liver microsomes and a liquid chromatography/tandem mass spectrometry (LC–MS/MS) system. Metabolism-dependent inhibition (MDI) was identified based on IC_{50} shifts after pre-incubation of the tested compounds with NADPH. To distinguish reversible MDI from mechanism-based inhibition (MBI), *R*-fluoxetine and ticlopidine were used as positive inhibitors for reversible MDI and MBI of CYP2C19, respectively. *R*-fluoxetine and ticlopidine inhibited CYP2C19 activity, as determined using *S*-mephenytoin as a substrate, and caused 8.7- and 2.3-fold IC_{50} shifts, respectively, after pre-incubation. Inhibition of CYP2C19 by *R*-fluoxetine, but not ticlopidine, was markedly reversed by ultracentrifugation, and two or three ultracentrifugations were not more effective than one, indicating that ultracentrifugation only once may be sufficient to reverse the reversible MDI. To distinguish between quasi-irreversible and irreversible inhibition, diltiazem and mifepristone were used as quasi-irreversible and irreversible inhibitors of CYP3A4, respectively, and CYP3A4 activity was measured using midazolam and testosterone as substrates. After pre-incubation, CYP3A4 IC_{50} shifts caused by diltiazem and mifepristone were greater than 2.5- and 3.7-fold, respectively. Incubation with 2 mM potassium ferricyanide for 10 min reversed the MDI of CYP3A4 by diltiazem, but not mifepristone. Increases in potassium ferricyanide concentration and incubation time reduced the recovery of CYP3A4 activity. The established methods were confirmed using three CYP3A4 inhibitors including diltiazem, mifepristone and amiodarone (a reversible metabolism-dependent inhibitor). We consider these methods to be useful tools for discriminating between reversible MDI and MBI.

1. Introduction

Drug metabolism mediated by hepatic cytochrome P450 (CYP) represents a significant clearance pathway for many drugs. A consequence of the disproportionate ratio between the numbers of enzymes is that for many multidrug therapies, it is common for a patient to receive two or more drugs that are metabolized by the same CYP enzyme. Under these polytherapeutic conditions, patients are at significant risk of suffering from drug–drug interactions (DDIs) as a result of the inhibition and induction of CYP. Inhibition of CYP-mediated drug metabolism is a widespread source of DDIs and may result in serious clinical consequences via either reversible or irreversible means [1,2]. Some fatal adverse drug reactions are mediated by metabolism-dependent DDIs, which have been responsible for the withdrawal of some drugs from the market. To avoid costly failures in drug development, CYP inhibition is now being investigated earlier in the drug discovery process [3].

CYP inhibition can be broadly divided into two categories: direct inhibition by a parent compound and metabolism-dependent inhibition (MDI) by its metabolite(s). Both can cause clinically significant CYP inhibition. For this reason, the US Food and Drug Administration (FDA, [4]) and the European Medical Agency (EMA, [5]) both require an *in vitro* assessment of the ability of drug candidates to cause direct inhibition and MDI of drug-metabolizing CYP enzymes in human liver microsomes (HLMs) [6–9]. MDI can be categorized into three types based on the reversibility of CYP inhibition [10]: reversible inhibition, quasi-irreversible inhibition, and irreversible inhibition. Irreversible inhibition and quasi-irreversible inhibition are jointly referred to as mechanism-based inhibition (MBI). MBI involves the metabolism of the inhibitor to

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a reactive metabolite, which modifies the CYP enzyme and results in irreversible loss of enzyme activity [1]. There are three pathways underlying the inactivation of CYP enzymes by the reactive intermediates: (1) reaction with nucleophilic amino acids in the active site, (2) reaction with the heme nitrogen atoms, and (3) coordination of the heme iron ion to form a metabolic intermediate complex [11].

Many pharmaceutical companies have established MDI assay systems to detect either enzymatic activity loss at a single concentration or IC_{50} shifts caused by pre-incubation with NADPH [12–14]. These systems use pooled human liver microsomes, recombinant CYP isozymes, and primary human hepatocytes with CYP probe substrates and fluorescent substrates. However, a strategy for applying MDI screening to discriminate between a reversible MDI and MBI has not been well established.

This study was designed to develop methods for detecting metabolism-dependent reversible, quasi-irreversible, and irreversible CYP inhibition using pooled HLMs and an liquid chromatography/tandem mass spectrometry (LC–MS/MS) system. The reversibility of CYP isoform inactivation was tested after ultracentrifugation for reversible MDI and after oxidation of CYP with potassium ferricyanide ($K_3Fe(CN)_6$) for quasi-irreversible inhibition. *R*-fluoxetine was used as a positive control for the reversible MDI of CYP2C19 and diltiazem was used as a positive control for the quasi-irreversible inhibition of CYP3A4 [15,16].

2. Materials and methods

2.1. Chemicals and reagents

Testosterone, ticlopidine, (*R*)-fluoxetine, amiodarone, diltiazem, mifepristone, carbamazepine, potassium ferricyanide, and the reduced form of β-nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). *S*-mephenytoin, 4′-hydroxymephenytoin, 1-hydroxymidazolam, 6β-hydroxytestosterone, and pooled human liver microsomes (BD UltraPool HLM 150, lot 38289) were purchased from BD Gentest Co. (Woburn, MA, USA). The manufacturer supplied information regarding protein concentration, CYP content, and enzyme activity. Midazolam was purchased from Bukwang Pharmaceutical Co. (Seoul, Republic of Korea). All other reagents and chemicals were of analytical or HPLC grade.

2.2. In vitro CYP inhibition

2.2.1. MDI assay

All incubations were performed at 37 °C in a shaking water bath using 8-well tube strips placed in an 8 × 12 rack. Five metabolism-dependent CYP inhibitors including ticlopidine, *R*-fluoxetine, amiodarone, diltiazem, and mifepristone were used. The solvent concentration for the inhibitors (acetonitrile and DMSO) was 1% (v/v) for pre-incubation. Pre-incubation mixtures with a total volume of 200 μL per well contained 0.1 M potassium phosphate buffer (pH 7.4), seven different concentrations of the individual inhibitors and 1 mg/mL HLMs in the absence of 1 mM NADPH or in the presence of 1 mM NADPH were incubated for 30 min. The inhibitor concentrations were chosen based on the IC_{50} values in the literature [17]. Following 10-fold dilution from the pre-incubation concentration, the final 5-min incubation was performed in the presence of 1 mM NADPH and CYP substrates, which included *S*-mephenytoin for CYP2C19 and midazolam and testosterone for CYP3A4. CYP substrate concentrations (approximately equal to the respective K_m values) were characterized as described previously [17]. Aliquots (100 μL) were quenched in ice-cold acetonitrile (100 μL) containing carbamazepine (50 nM) as an internal standard and then centrifuged (1000*g*, 20 min). The resulting supernatants were transferred to 96-well plates for LC–MS/MS analysis.

2.2.2. Reversibility of reversible MDI

The reversibility of reversible MDI was investigated by ultracentrifugation at a single concentration. *R*-fluoxetine (30 μM) and ticlopidine (5 μM) were used for the reversible MDI and MBI of CYP2C19, respectively. Amiodarone (30 μM), diltiazem (30 μM), and mifepristone (30 μM) were used for the reversible, quasi-irreversible and irreversible MDI of CYP3A4, respectively. The primary incubation reactions, in total volumes of 200 μL per well, consisted of 0.1 M potassium phosphate buffer (pH 7.4), 2 mg/mL HLMs, individual inhibitor and 1 mM NADPH. To evaluate the potential for MDI, the inhibitors were incubated at 37 °C for 30 min. Re-isolation of microsomes was performed by ultracentrifugation of the pre-incubation mixtures at 100,000*g* for 60 min at 4 °C. The microsomal pellet was washed twice with 0.1 M potassium phosphate buffer (pH 7.4). After ultracentrifugation, the primary incubation mixtures were diluted 10-fold by transferring 20-μL aliquots to 180 μL of 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM NADPH and CYP substrate (100 μM *S*-mephenytoin for CYP2C19; 5 μM midazolam and 50 μM testosterone for CYP3A4) Consequently, final microsomal protein concentration in reaction mixture was 0.2 mg protein/mL. The incubation was continued for 5 min to measure residual CYP activity. The reactions were terminated by the addition of 200 μL acetonitrile containing an internal standard (carbamazepine). Precipitated protein was pelleted by centrifugation (1000*g* for 20 min at 4 °C). The resulting supernatants were transferred to 96-well plates. Metabolite formation was determined by LC–MS/MS. The protein concentration of the resuspended microsomes was determined in a 96-well plate using a BCA protein assay kit (Pierce Chemical, Rockford, IL). The recovery of microsomal protein following ultracentrifugation and re-suspension was 95.7%.

2.2.3. Reversibility of quasi-irreversible inhibition

The reversibility of quasi-irreversible inhibition was investigated by oxidation with potassium ferricyanide. Diltiazem and mifepristone were used for quasi-irreversible and irreversible CYP3A4 inhibition, respectively. Midazolam and testosterone were used for the CYP3A4 activity measurement as substrates. All incubations were performed at 37 °C in a shaking water bath using 8-well tube strips placed in an 8 × 12 rack. To decide the conditions for the oxidation of CYP by potassium ferricyanide, concentration- and time-dependent recovery of CYP3A4 activity was determined in HLMs treated with 30 μM diltiazem for 30 min in the presence of NADPH. Potassium ferricyanide (0, 0.1, 0.4, 2, or 10 mM) was added to the pre-incubated mixtures for 10 min to determine concentration-dependent recovery of CYP3A4 activity inhibited by diltiazem, and 2 mM potassium ferricyanide was added for 0, 5, 10, 15, or 30 min to determine time-dependent recovery.

To test the reversibility of quasi-irreversible CYP3A4 inhibition, pre-incubation mixtures with a total volume of 200 μL per well contained 0.1 M potassium phosphate buffer (pH 7.4), seven different concentrations of the individual inhibitors, 1 mM NADPH, and 2 mg/mL HLMs were incubated for 30 min and the primary incubates (200 μL) were added to 200 μL of 4 mM potassium ferricyanide for 10 min at 37 °C. After the 10 min reaction, each reaction mixture was diluted 5-fold with secondary solution. The secondary mixtures were conducted in 200-μL per well contained 0.1 M potassium phosphate buffer (pH 7.4), HLMs (0.2 mg protein/mL, final), 1 mM NADPH and CYP3A4 substrate (5 μM midazolam or 50 μM testosterone). The incubation was continued for 5 min to measure residual CYP activity. The reactions were terminated by the addition of 200 μL acetonitrile containing internal standard