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A Comparison of the *In Vitro* Inhibitory Effects of Thelephoric Acid and SKF-525A on Human Cytochrome P450 Activity

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Abstract

Thelephoric acid is an antioxidant produced by the hydrolysis of polyozellin, which is isolated from *Polyozellus multiplex*. In the present study, the inhibitory effects of polyozellin and thelephoric acid on 9 cytochrome P450 (CYP) family members (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) were examined in pooled human liver microsomes (HLMs) using a cocktail probe assay. Polyozellin exhibited weak inhibitory effects on the activities of all 9 CYPs examined, whereas thelephoric acid exhibited dose- and time-dependent inhibition of all 9 CYP isoforms (IC_{50} values, 3.2-33.7 μM). Dixon plots of CYP inhibition indicated that thelephoric acid was a competitive inhibitor of CYP1A2 and CYP3A4. In contrast, thelephoric acid was a noncompetitive inhibitor of CYP2D6. Our findings indicate that thelephoric acid may be a novel, non-specific CYP inhibitor, suggesting that it could replace SKF-525A in inhibitory studies designed to investigate the effects of CYP enzymes on the metabolism of given compounds.

Key Words: Thelephoric acid, Polyozellin, SKF-525A, Cytochrome P450, Non-specific inhibitor

INTRODUCTION

Cytochrome P450s (CYPs) are a family of enzymes that play key roles in the metabolism of xenobiotics and endogenous compounds. Of the 35 CYPs known in man, three sub-families, CYP1, CYP2, and CYP3, appear to be responsible for the metabolism of drugs (Spatzenegger and Jaeger, 1995). Adverse medicinal drug-drug and herbal-drug interactions have been implicated in the drug-dependent inhibition and induction of CYPs (Guengerich, 1997). Thus, being able to predict the contribution made by each CYP in metabolic reactions that take place in human liver microsomes is an important part of the drug development process (Emoto *et al*., 2003). In order to make these predictions, the initial investigatory steps involve determining inhibitory activity against CYPs using non-specific CYP inhibitors.

SKF-525A (2-diethylaminoethyl-2,2-diphenylvalerate-HCl, proadifen) is a widely used non-specific CYP inhibitor used to investigate the contributions made by CYP-related metabolism (Chung *et al*., 2000; Emoto *et al*., 2003; Lee *et al*., 2007). However, it does not strongly inhibit all CYP family members (Emoto *et al*., 2003; Franklin and Hathaway, 2008). In particular, SKF-525A has been reported to weakly inhibit recombinant human CYP1A2 and CYP2E1 within the concentration range of 100-1,200 μM (Emoto *et al*., 2003). Furthermore, in rat liver and recombinant human CYP2C9 microsomes, SKF-525A has been shown to strongly inhibit CYP2C-dependent activity (Schenkman *et al*., 1972; Chang *et al*., 1995; Huang and Waxman, 2001), whereas in human microsomes, it strongly inhibits CYP2D6-, CYP2C19-, and CYP3A-catalyzed reactions (Ono *et al*., 1996). Another non-specific CYP inhibitor, ABT (1-aminobenzotriazole), displays inhibitory activity comparable to that of SKF-525A, but only against CYP2C9 and CYP2C19 (Emoto *et al*., 2003). Thus, a novel, non-specific inhibitor is required to enable the accurate assessment of drug metabolism by CYPs. We have previously reported novel, non-specific inhibitors by screening chemically synthesized compounds or isolated compounds from plants or herbs for CYP inhibition (Jahng *et al*., 2012; Kim *et al*., 2013; Song *et al*., 2013).

In this study, we investigated the *in vitro* inhibitory effects of polyozellin and thelephoric acid in order to develop a novel

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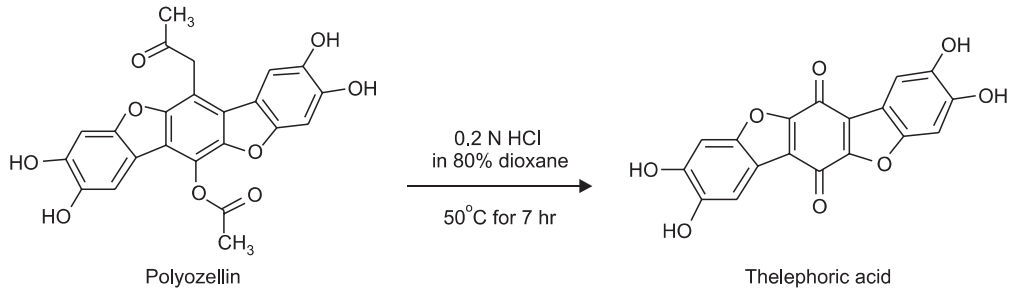

Fig. 1. Preparatory procedure and chemical structure of thelephoric acid.

CYP inhibitor to be used in drug metabolism screenings. Polyozellin is a terphenylquinone pigment isolated from *Polyozellus multiplex*, and thelephoric acid, which is prepared from polyozellin, is an antioxidant that inhibits *in vitro* prolyl endopeptidase activity (Hwang *et al*., 1997; Kwak *et al*., 1999). In the present study, we investigated whether polyozellin or thelephoric acid specifically inhibit the activities of 9 CYPs and compared the results obtained with those of SKF-525A.

MATERIALS AND METHODS

Chemicals

Polyozellin was isolated from *Polyozellus multiplex* (Black chanterelle), and thelephoric acid was prepared from polyozellin (Fig. 1). Pooled human liver microsomes (HLMs) were obtained from BD Gentest (Woburn, MA, USA). Glucose-6-phosphate, *B*-NADPH, and glucose-6-phosphate dehydrogenase were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and used as received.

Thelephoric acid preparation

Polyozellin (160 mg) was isolated from the fruiting bodies of *Polyozellus multiplex* and added to 20 mL of 0.2 N HCl in 80% dioxane. The resulting mixture was then heated in a water bath at 50°C for 7 hours. The dark residue obtained by evaporation was repeatedly washed with MeOH and water, and the remaining black powder was dried in a vacuum desiccator and determined to be 109 mg of >98% pure thelephoric acid.

Screening for inhibitory effects on CYP activities

To investigate the inhibitory effects of polyzellin and thelephoric acid on the activities of CYPs, we used the cocktail assay described in the FDA guidance document for drug interaction studies (2006). In a total volume of 0.1 mL of 0.1 M potassium phosphate buffer (pH 7.4), the incubation mixture consisted of the following: 0.1 mg of HLMs, polyozellin or thelephoric acid (at concentrations ranging from 2-50 μM), cocktail probe substrates, a NADPH-generating system (NGS) composed of 0.1 M glucose-6-phosphate, 10 mg/mL *B*-NADPH, and 1.0 U/mL glucose-6-phosphate dehydrogenase. The probe substrates used are in listed in Table 1. The reaction mixture was pre-incubated for 5 min at 37°C prior to initiating the reaction by the addition of 0.15 mL NGS. After incubation for 60 min, the reaction was stopped by the addition of 100 μl of acetonitrile containing 0.1% formic acid and 10 μg/mL of terfenadine in methanol (internal standard). After mixing and centrifuging at 13,000 g for 10 min, a 10-μl aliquot was injected onto a C_{18} column for LC-MS/MS analysis.

Table 1. Concentration-dependent inhibitory effects of thelephoric acid on CYPs in HLMs

hCYP isoforms	IC_{50} (μM)		
	Thelephoric acid		SKF-525A
	Pre-incubation 0 min	Pre-incubation 15 min	
CYP1A2	3.2	2.1	>50
CYP2A6	5.0	2.1	>50
CYP2B6	8.5	2.6	0.9
CYP2C8	24.6	6.9	26.5
CYP2C9	27.5	10.0	33.4
CYP2C19	4.9	2.8	2.1
CYP2D6	3.4	2.5	3.5
CYP2E1	4.5	2.8	>50
CYP3A4	11.3	3.2	20

To determine the inhibitory effects of thelephoric acid on the activities of the 9 CYPs, HLMs (0.1 mg) were incubated with thelephoric acid (0.1 μM, 0.25 μM, 1 μM, 2.5 μM, 10 μM, and 25 μM) at 37°C for 60 min after pre-incubation for 0 min or 15 min, respectively. On the other hand, the inhibitory effects of SKF-525A on the activities of the CYPs were determined after pre-incubation for 20 min. The data shown are the means of duplicate experiments.

Mechanism of inhibition of CYP1A2, CYP2D6, and CYP3A4 by thelephoric acid

To investigate the mechanism of CYP inhibition by thelephoric acid, 0.2 mg of HLMs was pre-incubated with thelephoric acid (0.5-10 μM) in 0.1 M potassium phosphate buffer (pH 7.4) for 0 min, 5 min, 10 min, or 20 min in the presence of NGS. Cocktail chemical substrates were then added and incubated for 60 min at 37°C.

To investigate the inhibition of CYP1A2, CYP2D6, and CYP3A4 by thelephoric acid, 0.1 mg of HLMs was incubated with thelephoric acid at 0 μM, 1 μM, or 5 μM and the probe substrates in 0.1 M potassium phosphate buffer (pH 7.4) for 60 min. The following probe substrates were used: 20 μM, 40 μM, or 80 μM of phenacetin for CYP1A2; 1.25 μM, 2.5 μM, or 5.0 μM dextromethorphan for CYP2D6; and 0.625 μM, 1.25 μM, or 2.5 μM midazolam for CYP3A4.

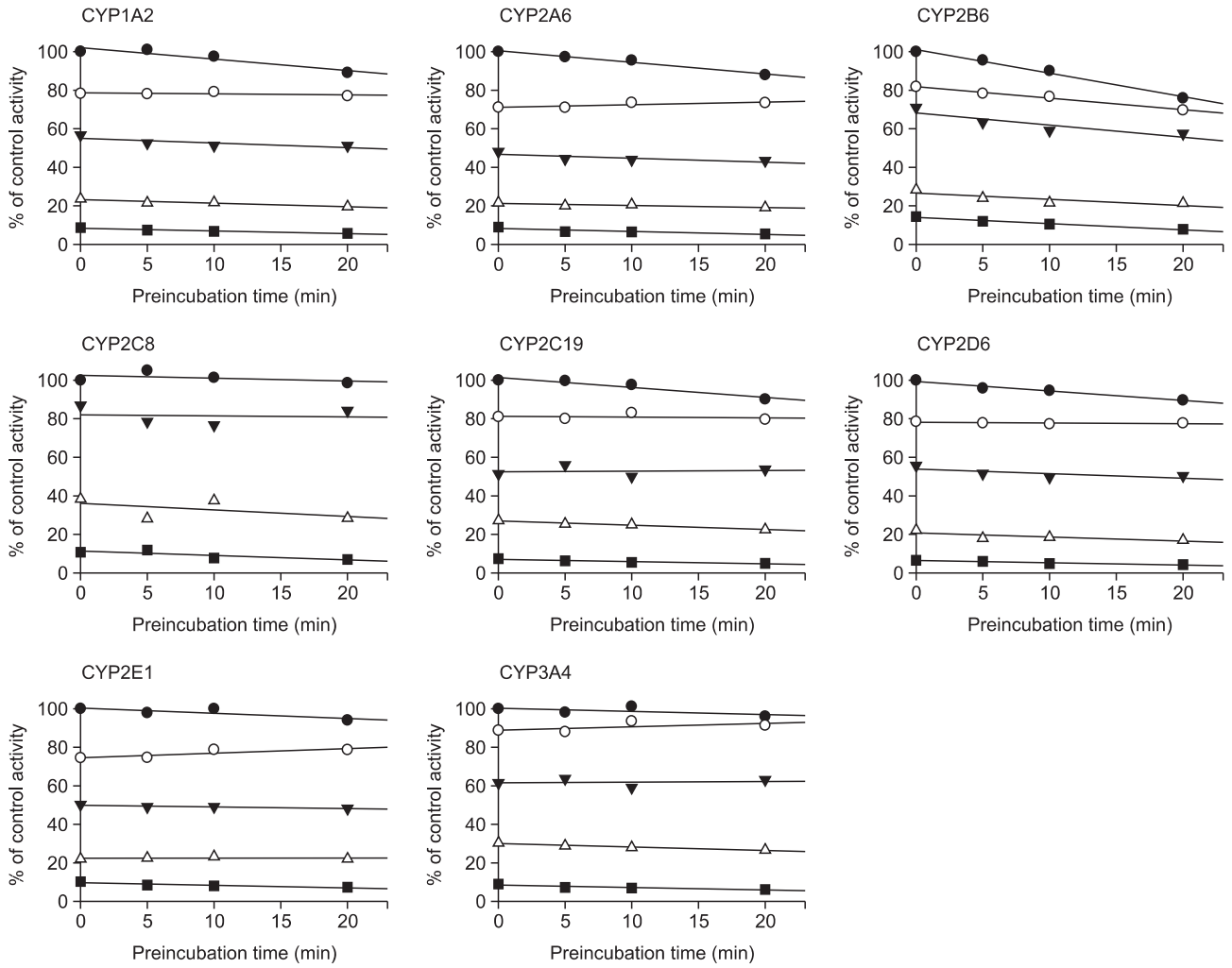

Fig. 2. Time- and concentration-dependent inhibition of human liver microsomal CYPs. Microsomal reaction mixtures were pre-incubated for 15 min without (●) or with thelephoric acid at 0.5 μM (○), 1 μM (▼), 2.5 μM (Δ), or 10 μM (■). The results shown are the means of duplicate experiments.

Kinetic analysis

IC_{50} values (the concentration of inhibitor causing a 50% inhibition of enzyme activity) were obtained by plotting percent activity versus log[I] concentration. Kinetic parameters were estimated by curve fitting using SigmaPlot (version 10.0, Systat Software, Inc.). All results were analyzed in duplicate.

Instruments

All measurements were performed using a tandem mass spectrometer in multiple reaction monitoring mode. LC-MS/MS was conducted using an Accela™ LC system and a TSQ Vantage triple quadruple mass spectrometer (Thermo Fisher Scientific Inc., USA) equipped with an HESI-II electrospray ionization source. Chromatographic separation was performed on an Inertsil® ODS-3, 3-μm (2.1×150 mm, GL Science) column with a column temperature of 40°C and gas flow rate of 0.23 mL/min. The mobile phases used were LC-grade water containing 0.1% formic acid (A) and LC-grade acetonitrile containing 0.1% formic acid (B). A gradient of 100% A to 90% B over 15 min was used.

RESULTS

Evaluations of drug metabolism by CYP enzymes are routinely performed during drug development, and SKF-525A and ABT are widely used to non-specifically inhibit CYP enzymes *in vitro*. However, although SKF-525A inhibits the activities of CYPs following metabolic-intermediate (MI) complex formation, it does not inhibit the activities of all CYPs in relation to drug metabolism (Emoto *et al*., 2003; Franklin and Hathaway, 2008). Therefore, a novel, non-specific inhibitor is required to enable the accurate assessment of drug metabolism by CYPs.

In the present study, we investigated the inhibitory effects of polyzollin and thelephoric acid on the activities of 9 CYPs and compared them to those of SKF-525A in HLMs. The inhibitory effects of polyozellin and thelephoric acid on the activities of common drug-metabolizing human CYP isoforms in pooled HLMs were determined using a cocktail probe assay. Assays with polyozellin concentrations ranging from 0-50 μM that were incubated at 37°C for 60 min and only and weak inhibitory effects, whereas thelephoric acid strongly inhibited

all CYP-catalyzed probed enzymes in the following concentration-dependent manner: CYP1A2-catalyzed phenacetin *O*-deethylase, CYP2A6-catalyzed coumarin 7-hydroxylase, CYP2B6-catalyzed bupropion hydroxylase, CYP2C8-catalyzed amodiaquine *N*-deethylase, CYP2C9-catalyzed diclofenac 4′-hydroxylase, CYP2C19-catalyzed omeprazole 5-hydroxylase, CYP2D6-catalyzed dextromethorphan *O*-demethylase, CYP2E1-catalyzed chlorzoxazone 6-hydroxylase, and CYP3A4-catalyzed midazolam 1-hydroxylase, respectively (Supplemental Table 1).

SKF-525A is the most commonly used non-specific CYP inhibitor in drug metabolism studies. To evaluate the inhibitory effects of thelephoric acid, we compared the IC_{50} values of thelephoric acid and SKF-525A with respect to the inhibition of the 9 CYPs in HLMs (Table 1). The IC_{50} values of thelephoric acid were determined after assays were incubated at 37°C for 60 min with and without pre-incubation with NADPH for 10 min. The IC_{50} values of SKF-525A were determined after pre-incubating HLMs for 20 min with NADPH because SKF-525A inhibits CYPs by forming MI complexes (Franklin and Hathaway, 2008). The IC_{50} values of thelephoric acid were within the range of 3.2-33.7 μM (Table 1), whereas SKF-525A displayed only weak inhibitory effects on CYP-dependent activities within this concentration range. Thelephoric acid strongly inhibited the activities of all 9 tested CYP-catalyzed enzyme reactions, whereas SKF-525A exhibited selective inhibition. This experiment demonstrated that thelephoric acid is a more non-specific inhibitor of the 9 CYPs than SKF-525A in HLMs.

In addition, the IC_{50} shifts caused by the presence of NADPH and pre-incubation of all 9 CYP isoforms with thelephoric acid were investigated (Table 1). All IC_{50} values of samples pre-incubated with NADPH for 15 min were shifted to lower IC_{50} values. The IC_{50} value of thelephoric acid with CYP3A4 decreased 3.5-fold, and the thelephoric acid IC_{50} values with the CYP2C family also decreased 1.7-3.5 fold by pre-incubation with NADPH. Thelephoric acid was a stronger inhibitor of CYP2B6 after pre-incubation with NADPH.

We investigated the mechanism by which thelephoric acid inhibited the 9 CYP isoforms (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) in pooled HLMs. CYP inhibition was determined by pre-incubating microsomal mixtures with NADPH at 37°C for 0-15 min. When HLMs were pre-incubated with NADPH, thelephoric acid strongly and dose-dependently inhibited the activities of 8 CYPs, with the exception of CYP2B6. These 8 CYPs were slightly inhibited in a time-dependent manner at high thelephoric acid concentrations (2.5 μM and 10 μM), whereas CYP2B6-catalyzed bupropion hydroxylation was slightly inhibited in a time-dependent manner throughout this concentration range (Fig. 2).

In order to determine the mode of CYP inhibition by thelephoric acid in HLMs, we conducted a kinetic study on three CYP isoforms, CYP1A2, CYP2D6, and CYP3A4, because CYP1A2 and CYP2D6 had the lowest IC_{50} values and CYP3A4 is known to be the major drug metabolizer. All three CYPs were strongly inhibited by thelephoric acid. Dixon plots were obtained for CYP1A2-catalyzed phenacetin *O*-deethylation, CYP2D6-mediated dextromethorphan *O*-demethylation, and CYP3A4-catalyzed midazolam 1-hydroxylation. The patterns of inhibition by thelephoric acid for these selected CYPs were competitive for CYP1A2 and CYP2D6, but non-competitive for CYP3A4 (Fig. 3).

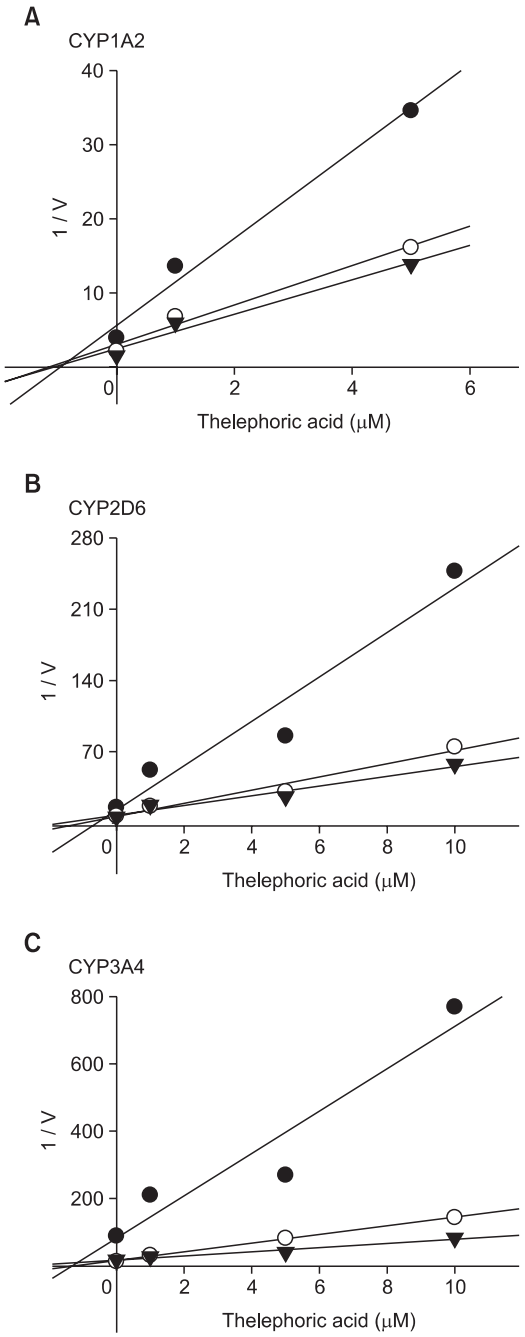

Fig. 3. Dixon-plots for thelephoric acid inhibition of CYP1A2-catalyzed phenacetin *O*-deethylation with 20 μM (●), 40 μM (○), or 80 μM (▼) of phenacetin (A), CYP2D6-catalyzed dextromethorphan *O*-demethylation with 1.25 μM (●), 2.5 μM (○), or 5.0 μM (▼) dextromethorphan (B), and CYP3A4-catalyzed midazolam1-hydroxylation (C) with 0.625 μM (●), 1.25 μM (○), or 2.5 μM (▼) midazolam in pooled HLMs. Reaction mixtures containing thelephoric acid at 0-10 μM were incubated for 60 min. The results shown are the means of duplicate experiments.

DISCUSSION

In the present study, we compared the inhibitory effects of thelephoric acid and SKF-525A on the *in vitro* activities of

CYPs. The study demonstrated that thelephoric acid strongly inhibits the 9 CYP isoforms examined (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) in HLMs competitively and/or noncompetitively *in vitro*. Thelephoric acid exhibited potent inhibitory effects on CYP1A2-, CYP2A6-, CYP2D6- and CYP2E1-catalyzed activities at concentrations less than 5 μM of their IC_{50} in HLMs. Based on weak IC_{50} shifts and dose-dependent inhibition, thelephoric acid was confirmed to be a competitive inhibitor of CYP1A2, CYP2A6 and CYP2E1. The pattern of the Dixon plot for CYP1A2-catalyzed phenacetin *O*-deethylation indicated the competitive inhibition of thelephoric acid. In our study, thelephoric acid inhibition of CYP1A2-, CYP2A6- and CYP2E1-catalyzed reactions was at least 16-fold greater than by SKF-525A, although the inhibitory effect of thelephoric acid on CYP2D6 was comparable to that of SKF-525A. The weak inhibition of SKF-525A on these three CYPs has been previously reported (Emoto *et al*., 2003).

In fact, mostly all CYPs have to be inhibited by a non-specific inhibitor simultaneously to investigate the contribution of only one CYP on drug metabolism. CYP1A2 is one of the major CYPs that metabolizes various clinical drugs, procarcinogens, and endogenous compounds containing planar ring structures that can fit in the narrow and planar active site of CYP1A2 (Zhou *et al*., 2009). CYP2A6 prefers small-molecule substrates, suggesting that its active site is small; it actually has the second smallest active site cavity in CYP family (Yano *et al*., 2005; Di *et al*., 2009). CYP2D6 is one of the primary enzymes that metabolizes compounds containing a basic amine (Sridhar *et al*., 2012). CYP2E1 has the smallest active site cavity and contributes to the metabolism of small molecules, such as ethanol (Porubsky *et al*., 2008). In our study, thelephoric acid was observed to competitively inhibit CYP1A2, CYP2A6, CYP2D6, and CYP2E1, respectively. However, these four CYPs had active sites of different structures, which could not accommodate thelephoric acid. Therefore, thelephoric acid does not share the same substrate-binding site compared to other molecules that fit the classical competitive inhibition model. Instead, the steric hindrance of thelephoric acid may lead to the sharing of a common binding group or induce a conformational change of substrate binding sites within the enzyme.

CYP2B6-, CYP2C8-, CYP2C9-, and CYP2C19-catalyzed reactions were inhibited by thelephoric acid in HLMs. However, although the IC_{50} values of thelephoric acid were reduced 2-fold by pre-incubation with NADPH, this reduction may not have been due to mechanism-based inhibition. Based on the dose-dependent inhibitory effects of thelephoric acid on 5 CYP isoforms, CYP inhibition by thelephoric acid may occur via a mixed mode. Furthermore, thelephoric acid also exhibited potent inhibitory effects on CYP2C19- and CYP2D6-catalyzed reactions, similar to those of SKF-525A. The IC_{50} shift observed when assays were pre-incubated with NADPH may be related to the formation of active metabolites from thelephoric acid metabolism in HLMs, which include various metabolic enzymes, such as CYP, flavin-monooxygenase, and reductases. The results suggested potential inhibitory effects by the metabolic activation of thelephoric acid, but these were not the predominant effects during inhibition. When thelephoric acid was pre-incubated without NADPH in HLMs, time-dependent inhibition was not observed (Fig. 2). The nature of the inhibition of CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP2D6 by thelephoric acid best fit mixed-mode inhibition.

Thelephoric acid also inhibited CYP3A4-catalyzed midazolam 1-hydroxylation in HLMs. The Dixon plot of thelephoric acid with CYP3A4 corresponded to non-competitive inhibition. Thelephoric acid showed strong dose-dependent inhibition and slight time-dependent inhibition of CYP3A4 at a relatively high concentration of 10 μM. Thelephoric acid also inhibited the CYP3A4-catalyzed reaction 6-fold more than SKF-525A after pre-incubating HLMs with NADPH for 20 min. Therefore, these Dixon plots and IC_{50} shifts demonstrate that thelephoric acid is a mixed-mode inhibitor of CYP3A4.

Based on the dose-dependent nature of the inhibitory effects of thelephoric acid, the slight IC_{50} shift caused by pre-incubation, and the shape of its Dixon plot, we conclude that thelephoric acid is a mixed-mode nonspecific inhibitor of CYPs. Our results suggest that thelephoric acid could be used, instead of SKF-525A, as a novel non-specific inhibitor of *in vitro* drug metabolism in HLMS.

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