Arch Pharm Res Vol 30, No 4, 469-474, 2007

Archives of Pharmacal Research
http://apr.psk.or.kr

Enzyme Kinetic Study of a New Cardioprotective Agent, KR-32570 using Human Liver Microsomes and Recombinant CYP Isoforms

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(Received March 28, 2006)

KR-32570 (5-(2-Methoxy-5-chlorophenyl)furan-2-ylcarbonyl)guanidine) is a new cardioprotective agent for preventing ischemia-reperfusion injury. Human liver microsomal incubation of KR-32570 in the presence of NADPH resulted in the formation of two metabolites, hydroxy-KR-32570 and O-desmethyl-KR-32570. In this study, a kinetic analysis of the metabolism of two metabolites from KR-32570 was performed in human liver microsomes, and recombinant CYP1A2, and CYP3A4. The metabolism for hydroxy- and O-desmethyl-KR-32570 formation from KR-32570 by human liver microsomes was best described by a Michaelis-Menten equation and a Hill equation, respectively. The Cl_{int} values of hydroxy- and O-desmethyl-KR-32570 formation were similar to each other (0.03 vs 0.04 μL/min/pmol CYP, respectively). CYP3A4 mediated the formation of hydroxy-KR-32570 from KR-32570 with Cl_{int} = 0.24 μL/min/pmol CYP3A4. The intrinsic clearance for O-desmethyl-KR-32570 formation by CYP1A2 was 0.83 μL/min/pmol CYP1A2. These findings suggest that CYP3A4 and CYP1A2 enzymes are major enzymes contributing to the metabolism of KR-32570.

Key words: KR-32570, Enzyme kinetics, Microsomes, LC/MS/MS

INTRODUCTION

Since the excessive activation of Na^+/H^+ exchanger isoform-1 (NHE-1) has been known to play an important role in the progression of ischemica/reperfusion injury (Karmazyn *et al.*, 1999), many efforts have been devoted to develop a potent and selective NHE-1 inhibitor as cardioprotective drug. Several monocyclic acylguanidines such as cariporide (Baumgarth *et al.*, 1997) and zoniporide (Guzman-Perez *et al.*, 2001) have been reported as the selective and potent NHE-1 inhibitors. Later, several compounds based on a bicyclic acylguanidines have been designed with the aim to discover more potent and highly water-soluble inhibitor. KR-32570 (5-(2-Methoxy-5-chlorophenyl)furan-2-ylcarbonyl)guanidine) has been identified as a potent inhibitor of NHE-1 (IC_{50} = 0.081 μM), the enzyme responsible for the excessive Ca^{2+} influx during ischemia and reperfusion (Kim *et al.*, 2005; Lee *et al.*, 2005a, 2005b). It is currently being evaluated in preclinical studies as a new cardioprotective agent for ischemia and reperfusion injury. In isolated perfused rat hearts subjected to 30-min ischemia/30-min reperfusion, KR-32570 significantly improved cardiac contractile function and severe contracture in conjuction with causing a marked reduction in lactate dehydrogenase release (Lee *et al.*, 2005a). Furthermore, KR-32570 greatly limited the infarct size in the *in vivo* rat myocardial infarction model. Although the preclinical studies are still under way, KR-32570 appeared to be relatively non-toxic that the oral LD_{50} value of KR-32570 in rats was greater than 1,000 mg/kg. Taken together, KR-32570 would be a good candidate for cardiovascular diseases with a relatively low

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toxicity.

The low bioavailability of KR-32570 in rats was due to considerable gastrointestinal first-pass effect of oral dose. KR-32570 was oxidized to six metabolites in human liver microsomes by hydroxylation and demethylation: hydroxy-KR-32570, *O*-desmethyl-KR-32570, and hydroxy-*O*-desmethyl-KR32570. These phase I metabolites were further metabolised to their glucuronide conjugates (Kim *et al.*, 2006). In addition, the specific cytochrome P450 (CYP) isoforms responsible for KR-32570 oxidation to two major metabolites, *O*-desmethyl-KR-32570 and hydroxy-KR-32570 were identified using the combination of correlation analysis, chemical inhibition in human liver microsomes and metabolism by expressed recombinant CYP isoforms. The results show that CYP3A4 contributes to the oxidation of KR-32570 to hydroxy-KR-32570, and CYP1A2 plays the predominant role in *O*-demethylation of KR-32570 (Kim *et al.*, 2006).

The present study was in support of early drug discovery and developments efforts, and experiments were conducted for enzyme kinetic study of the predominant metabolites formation from KR-32570 using human liver microsomes. This study further examined the roles of particular human CYP enzymes in the formation of major metabolites, *O*-desmethyl-KR-32570 and hydroxy-KR-32570. Such information may be of considerable clinical impact in regard to potential drug interactions and interindividual variations of drug metabolism.

MATERIALS AND METHODS

Chemicals and reagents

KR-32570 and its metabolite, *O*-desmethyl-KR-32570 (5-(2-Hydroxy-5-chlorophenyl)furan-2-ylcarbonyl)guanidine), were synthesized by the Korea Research Institute of Chemical Technology (Taejeon, Korea) with a purity > 99.0%. β-nicotinamide adenine dinucleotide phosphate (β-$NADP^+$), EDTA, $MgCl_2$, glucose-6-phosphate (G6P), and glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Solvents were HPLC grade (Fisher Scientific CO., Pittsburgh, PA, U.S.A.) and the other chemicals were of the highest quality available. Pooled human liver microsomes (coded H161), single donor human liver microsomes, and cDNA-expressed CYP isoforms (Supersomes®) were purchased from BD Gentest (Woburn, MA, U.S.A.). The manufacturer supplied information regarding protein concentration, CYP content, and enzyme activity (data available at www.gentest.com).

In vitro metabolism of KR-32570 by human liver microsomes

Preliminary experiments showed that the formation of two major metabolites from KR-32570 was linear with respect to both time over 60 min and liver microsomal protein concentration (0.125-1.5 mg/mL) at 37°C. Thus a 20 min incubation time and a 0.25 mg/mL microsomal protein concentration were selected.

In kinetic experiments, eight concentrations of KR-32570 (0-200 μM in incubation) were incubated in duplicate with three different individual human liver microsomes or cDNA-expressed CYP isoforms. The incubation mixture containing human liver microsomes (0.25 mg protein/mL) or cDNA-expressed CYP isoforms (5 pmol for CYP1A2, and 10 pmol for CYP3A4), which was reconstituted in 100 mM phosphate buffer (pH 7.4), was preincubated for 10 min at 37°C. The reaction was initiated by adding the NADPH-generating system (including 1.3 mM NADP, 3.3 mM glucose 6-phosphate, 3.3 mM $MgCl_2$, and 1.0 U/mL glucose-6-phosphate dehydrogenase), and the reaction mixtures (final volume 250 μL) were incubated for 20 min at 37°C in a shaking water bath. In all experiments, KR-32570 was dissolved in methanol, and the final concentration of organic solvent did not exceed 0.5%. The reaction was terminated by the addition of 100 μL of acetonitrile (including 5 μM dextrorphan as internal standard) on ice. The mixtures were centrifuged at 20,000 g for 5 min at 4°C, and aliquots of the supernatant were injected into an LC/MS/MS system.

LC/MS/MS analysis of KR-32570 and its metabolites

For the quantitation of KR-32570 and its metabolites, a tandem quadrupole mass spectrometer (API 3000 LC/MS/MS, Applied Biosystems, Foster City, CA, U.S.A.), coupled with an Agilent 1100 series HPLC system (Agilent, Wilmington, DE, U.S.A.) was used. The separation was performed on a Luna C18 column (2 mm i.d. × 100 mm, 3 μm, Phenomenex, Torrance, CA, U.S.A.) using the mobile phase that consisted of acetonitrile and water (20:80, v/v) at a flow rate of 0.2 mL/min. The column temperature was 40°C. For identification of the metabolites, mass spectra were recorded by electrospray ionization with a positive mode. The turboion spray interface was operated at 5500 V and 375°C. The operating conditions were optimised by flow injection of an analyte and were determined as follows: nebulizing gas flow, 1.23 L/min; auxiliary gas flow, 4.0 L/min; curtain gas flow, 1.44 L/min; orifice voltage, 10 V; ring voltage 350 V; collision gas (nitrogen) pressure, 3.58×10^{-5} Torr. Quadruples Q1 and Q3 were set on unit resolution. Multiple-reaction-monitoring (MRM) mode using specific precursor/product ion transitions was employed for the quantification. Detection of the ions was performed by monitoring the transitions of *m/z* 294 → 179 for KR-32570, *m/z* 310 → 195 for hydroxy-KR-32570, *m/z* 280 → 165 for *O*-desmethyl-KR-32570, and *m/z* 258 → 157 for

dextrorphan (IS). The analytical data were processed by Analyst software (version 1.2).

Data analysis

Results are expressed as means ± S.D. of estimates obtained from three different liver microsome preparations in duplicate experiments. The apparent kinetic parameters of KR-32570 biotransformation (K_m and V_{max}) were determined by fitting a one-enzyme Michaelis-Menten equation, Hill equation model ($V = V_{max} \cdot [S]^n/(K_m^n + [S]^n)$), or substrate inhibition model ($V = V_{max} / (1 + K_m/[S] + [S]/K_{si})$) without considering weighting factor. The calculated parameters were the maximum rate of formation (V_{max}), the Michaelis constant (K_m), the intrinsic clearance ($Cl_{int} = V_{max}/K_m$), and Hill coefficient (n). Calculations were performed using WinNonlin software (Pharsight, Mountain View, CA, U.S.A.).

RESULTS

Kinetics of KR-32570 metabolism in human liver microsomes

Kinetic analysis of KR-32570 metabolite formation rates was performed in three different human liver microsomes preparations. The rates of formation of metabolites were proportional to incubation times up to 60 min and protein concentrations up to 1.5 mg/mL at 20 min. The kinetic profiles of KR-32570 metabolism to hydroxy-KR-32570 and *O*-desmethyl-KR-32570 in human liver microsomes are shown in Fig. 2. In contrast to hydroxy-KR-32570 showing simple Michaelis-Menten kinetic, the formation rates of *O*-desmethyl-KR-32570 revealed sigmoidal saturation curves that were fit to a Hill equation (Fig. 2, left panel). The Eadie-Hofstee plots of *O*-desmethyl-KR-32570 formation showed concave relationships (Fig. 2, right panel), indicating negative (n = 0.48) cooperativity. The kinetic parameters (mean ± S.D.) estimated from the

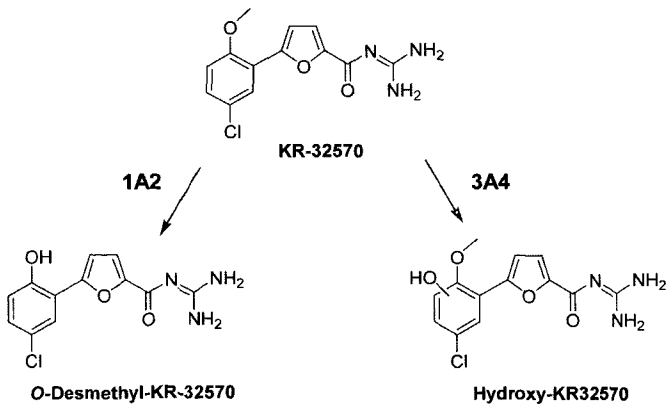

Fig. 1. The major metabolic pathway of KR-32570 in human liver microsomes

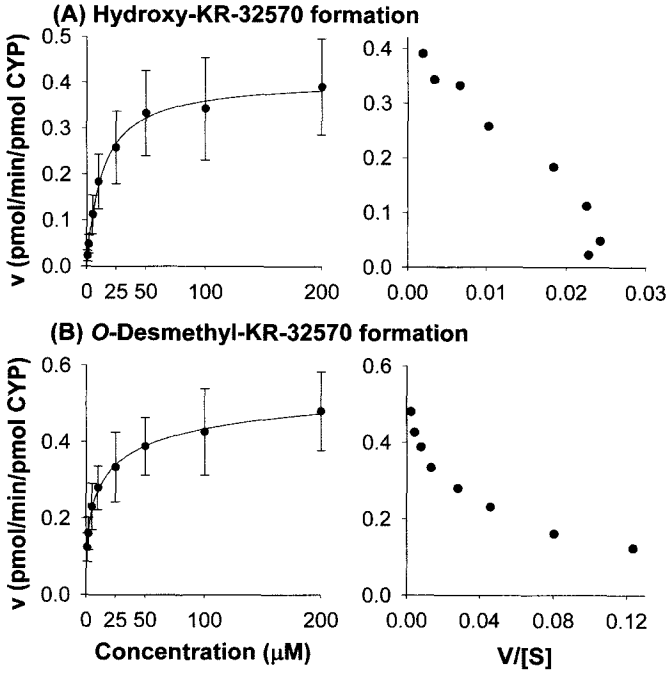

Fig. 2. Kinetics for the formation rate of hydroxy- (A) and *O*-desmethyl-KR-32570 (B) from KR-32570 in human liver microsomes. Left panel, rate of formation of hydroxy- and *O*-desmethyl-KR-32570 versus KR-32570 concentration curves when the kinetic data were fit to a Michaelis-Menten and Hill equation, respectively. Right panel, the corresponding Eadie-Hofstee plots. Each point represents the average obtained from three different human liver microsomes.

Table I. Kinetics of KR-32570 metabolism in three individual human liver microsomes

Parameter[a]	Hydroxy-KR-32570	*O*-Desmethyl-KR-32570
V_{max}	0.4 ± 0.1	0.6 ± 0.2
K_m	13.2 ± 1.1	17.1 ± 5.1
Cl_{int}	0.03 ± 0.01	0.04 ± 0.01
n	-	0.48 ± 0.02

Kinetic parameters for the formation of hydroxy- and *O*-desmethyl-KR-32570 were estimated by fitting the velocity vs. substrate concentrations to the Michalis-Menten equation and Hill equation, respectively. Values are means ± S.D. of estimates from three individual human liver microsomal preparations.

[a] V_{max} is espressed as pmol/min/pmol CYP, K_m as μM and Cl_{int} as V_{max}/K_m (μL/min/pmol CYP), n is Hill coefficient.

three human microsomal preparations are shown in Table I. The intrinsic clearance value (0.04 μL/min/pmol CYP) of *O*-desmethyl-KR-32570 formation was similar to that of hydroxy-KR-32570 (0.03 μL/min/pmol CYP).

KR-32570 metabolism in human cDNA-expressed CYP isoforms

Full kinetic analysis was performed for the formation rates of KR-32570 metabolites from KR-32570 by recom-

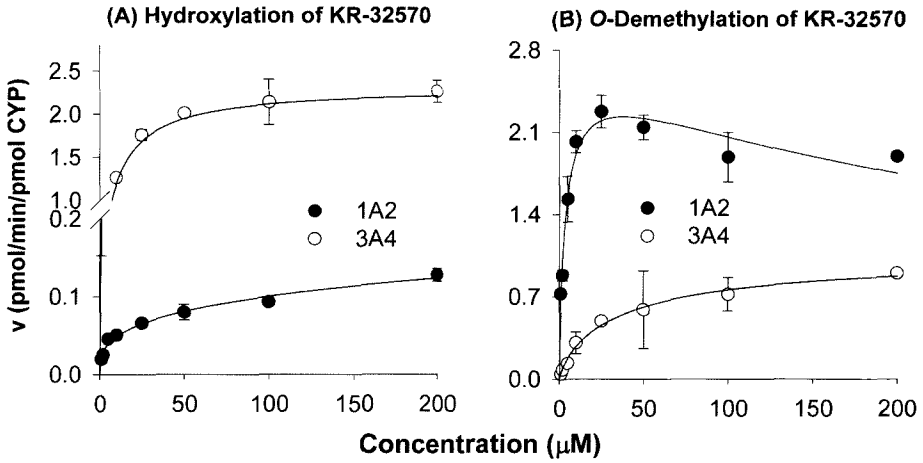

Fig. 3. The formation of hydroxy- (A) and *O*-desmethyl-KR-32570 (B) from KR-32570 in cDNA-expressed CYP1A2 and CYP3A4. Velocity versus substrate curves, where the data were fit to a Hill equation except for *O*-desmethyl-KR-32570 formation by CYP1A2, which was fit to a substrate inhibition equation. Each point represents the mean of duplicate incubations.

Table II. Kinetics of KR-32570 metabolism in cDNA expressed human CYP isoforms

Parameter[a]	Metabolite	CYP1A2	CYP3A4
V_{max}	Hydroxy-KR-32570	0.35	2.32
	O-Desmethyl-KR-32570	2.56	1.10
K_m	Hydroxy-KR-32570	> 200	9.50
	O-Desmethyl-KR-32570	3.08	37.96
Cl_{int}	Hydroxy-KR-32570	-	0.24
	O-Desmethyl-KR-32570	0.83	0.0289
K_{si}	Hydroxy-KR-32570	-	-
	O-Desmethyl-KR-32570	> 200	-
n	Hydroxy-KR-32570	0.40	1.09
	O-Desmethyl-KR-32570	-	0.82

Kinetic parameters were estimated by fitting the velocity vs. substrate concentrations to the Hill equation.

[a] V_{max} is expressed as pmol/min/pmol CYP, K_m as μM and Cl_{int} as V_{max}/K_m (μL/min/pmol CYP). K_{si} represents the inhibition constant for the substrate (μM), and *n* is Hill coefficient.

binant human CYP1A2 and CYP3A4 (Fig. 3). The kinetics for the formation of hydroxy-KR-32570 by CYP1A2 and CYP3A4 was characterised by the Hill equation. The formation rates of *O*-demethyl-KR-32570 by CYP1A2 versus KR-32570 concentration fit better to a substrate inhibition model. The formation rate of *O*-desmethyl-KR-32570 versus KR-32570 concentrations was characterized by an initial rapid increase at lower concentrations followed by a progressive decline at higher substrate concentrations (Fig 3B). Comparison of the goodness-of-fit values generated from these data indicates that a substrate inhibition equation enzyme model provided a better fit than did other models. The corresponding Eadie-Hofstee plot indicated a "hook" in the upper region of this plot (data are not-shown), which is characteristic of substrate inhibition. The K_m, V_{max}, and K_{si} estimated from these data, respectively, were 3.1 μM, 2.56 pmol/min/pmol CYP, and > 200 μM. The respective data obtained are listed in Table II. The K_m values for the formation of *O*-desmethyl-KR-32570 by CYP1A2 were much smaller compared with those obtained in CYP3A4. Accordingly, the in vitro intrinsic clearance for the formation of *O*-desmethyl-KR-32570 in CYP1A2 was 28.7-fold higher than that of CYP3A4.

DISCUSSION

KR-32570 was metabolised to six major metabolites, i.e., hydroxy-KR-32570, *O*-desmethyl-KR-32570, hydroxy-*O*-desmethyl-KR-32570, and three their glucuronide conjugates in human liver microsomes (Kim *et al.*, 2006). Among the six metabolites, hydroxy-KR-32570 and *O*-desmethyl-KR-32570 were formed as the major metabolites.

The oxidation of KR-32570 to hydroxy-KR-32570 in human liver microsomes is characterized by a simple saturation curve (Fig. 2). *O*-Demethylation of KR-32570 in human liver microsomes is also characterized by a Hill equation (Fig. 2). A similar kinetic profile has been observed with another CYP3A4-mediated diazepam hydroxylation in human liver microsomes (Andersson *et al.*, 1994) and in c-DNA expressed CYP3A4 (Kenworthy *et al.*, 2001). This cooperative binding effects associated with substrates of CYP3A4 are well documented, and it has become widely accepted that such events may be due to the binding of multiple molecules to the enzyme (Kenworthy *et al.*, 2001; Shou *et al.*, 1999), either within the active site or at separate, distant locations on the enzyme (Ueng *et al.*, 1997). Previous work also suggest

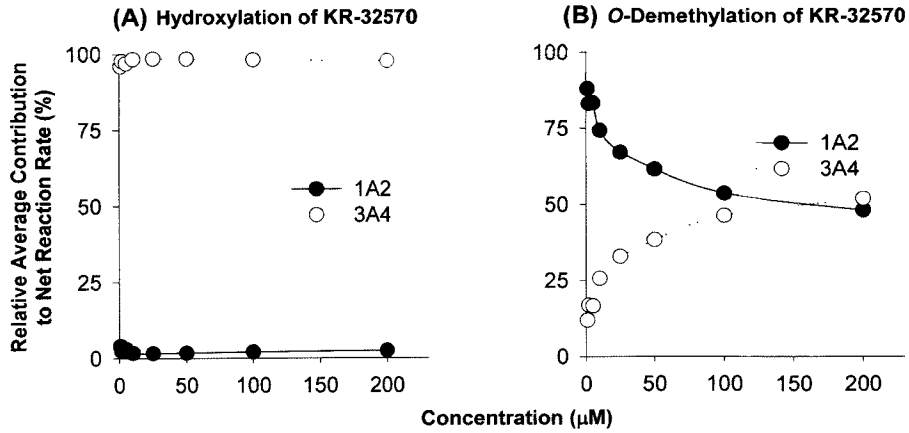

Fig. 4. Abundance adjusted simulations of the relative contributions of CYP1A2 (●), and CYP3A4 (○) to KR-32570 hydroxylation (A) and *O*-demethylation (B) in relation to KR-32570 concentrations. Solid lines, calculated relative contribution based on the normalized rate for each CYP isoform (see Discussion).

many CYPs including CYP1A2 (Ekins *et al.*, 1998) and CYP2C19 (Venkatakrishnan *et al.*, 1998), not only CYP3A4, appear to behave as allosteric enzymes.

The formation of *O*-desmethyl-KR-32570 from KR-32570 by rCYP1A2 exhibited substrate inhibition (Fig. 3B), unlike the kinetic data obtained in human liver microsomes, which were characterized by a sigmoidal saturation curve (Fig. 2). Similar substrate inhibition profiles have been observed previously with CYP1A2-mediated ethoxyresorufin metabolism (Lin *et al.*, 2001), CYP2B6-mediated 8,14-dihydroxyefavirenz formation (Ward *et al.*, 2003), and CYP3A-mediated triazolam hydroxylation (Schrag and Wienkers, 2001), which are suggestive of multiple substrate-binding sites (or multiple regions within a single active site).

Contributions of each cytochrome to KR-32570 metabolism were normalized for mean values of the relative abundance of individual cytochromes in the liver (Rodrigues, 1999). Briefly, the reaction rates measured with individual cDNA-expressed CYP isoforms were normalized with respect to the nominal specific content of the corresponding CYP in native human liver microsomes. In this study, we adapted the data of immunologically determined CYP isoform liver contents reported by other researchers (Shimada *et al.*, 1994), i.e., 28.8% for CYP3A4, and 12.7% for CYP1A2. In turn, the normalized rates for each cDNA-expressed CYP were summed, yielding a 'total normalized rate' (TNR = $\Sigma f_i \cdot V_i$), and the normalized rate for each CYP isoform (= $f_i \cdot V_i$) was expressed as a percentage of the net reaction rate (= $100 \cdot f_i \cdot V_i / \Sigma f_i \cdot V_i$, where f_i indicates the fraction of each CYP isoform content in the human liver, and $V_i = V_{maxi} \cdot [S_i]/K_{mi} + [S_i]$). This simulation shows that CYP3A4 is the major CYP isoform responsible for hydroxylation from KR-32570 over the whole concentration range test (0-200 μM). However, the percentage of the *O*-demethylation rate by CYP1A2 (around 85%) decreased with increasing substrate concentration and reached 50% at 200 μM, whereas CYP3A4 contribution increased with substrate concentration (Fig. 4). Although two CYP isoforms, CYP1A2 and CYP3A4, were involved in the hydroxylation of KR-32570, the K_m values estimated by incubation with c-DNA expressed enzymes indicated that the KR-32570 had a higher affinity to CYP3A4 (mean K_m = 9.5 μM), compared with the affinity to CYP1A2 (mean K_m > 200 μM). In the case of demethylation of KR-32570, CYP1A2 has a higher affinity to KR-32570 than CYP3A4. Taken together, these results suggest that CYP3A4 and CYP1A2 are the major enzymes involved in the hydroxylation and *O*-demethylation of KR-32570 in the lower substrate concentration.

In conclusion, this study demonstrates that the formations of hydroxy- and *O*-desmethyl-KR-32570 from KR-32570 are the major metabolic pathways in human liver microsomes. CYP1A2 and CYP3A4 are responsible for KR-32570 oxidations. The possibility of drug-drug interactions would be predicted when prescribing KR-32570 concomitantly with known inhibitors of CYP3A4 and CYP1A2.

ACKNOWLEDGEMENTS

This work was supported by a grant (CBM2-B412-001-1-0-0) from the Center for Biological Modulators of the 21st Century Frontier R&D Program, Ministry of Science and Technology, Korea, and the Korea Health 21 R&D Project, Ministry of Health and Welfare, R. O. K (03-PJ10-PG13-GD01-0002).

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