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In Vitro Metabolism of a New Cardioprotective Agent, KR-33028 in the Human Liver Microsomes and Cryopreserved Human Hepatocytes

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KR-33028 (*N*-[4-cyano-benzo[b]thiophene-2-carbonyl]guanidine) is a new cardioprotective agent for preventing ischemia-reperfusion injury. This study was performed to identify the metabolic pathway of KR-33028 in human liver microsomes and to compare its metabolism with that of cryopreserved human hepatocytes. Human liver microsomal incubation of KR-33028 in the presence of NADPH and UDPGA resulted in the formation of four metabolites, M1, M2, M3, and M4. M1 and M2 were identified as 5-hydroxy-KR-33028 and 7-hydroxy-KR-33028, respectively, on the basis of LC/MS/MS analysis with the synthesized authentic standard. M3 and M4 were suggested to be dihydroxy-KR-33028 and hydroxy-KR-33028-glucuronide, respectively. Metabolism of KR-33028 in cryopreserved human hepatocytes resulted in the formation of M1, M2, and M4. These data show a good correlation between major metabolites formed in human liver microsomes and cryopreserved human hepatocytes. In addition, KR-33028 was found to inhibit moderately the metabolism of CYP1A2 substrates. Based on the results obtained metabolic pathway of KR-33028 is proposed.

Key words: KR-33028, Metabolism, Microsomes, Hepatocytes, LC/MS/MS

INTRODUCTION

In general, xenobiotics undergo extensive metabolic transformation in living organisms through various metabolic reactions. Microsomal mixed function oxidase is the primary enzyme for phase one reactions which convert xenobiotics into more soluble products. Although these products are generally less toxic than the parent compound, more toxic metabolites may also result. Such *in vitro* studies with microsomal preparations provide specific details of the chemical identity of metabolites and intermediates, the pattern of their formation, and the metabolic pathways of xenobiotics (Kim *et al.*, 2002; Park *et al.*, 2002).

KR-33028 (*N*-[4-cyano-benzo[b]thiophene-2-carbonyl] guanidine) is a new cardioprotective agent for preventing ischemia-reperfusion injury (Lee *et al.*, 2005). It was known to inhibit Na^+/H^+ exchanger isoform-1 (NHE-1, IC_{50} = 2.0 μM), the enzyme responsible for the excessive Ca^{2+} influx during ischemia and reperfusion. KR-33028 is currently being evaluated in preclinical studies as a new cardioprotective agent for ischemia and reperfusion injury. KR-33028 not only has been shown to recover the cardiac contractability above the 60% of basal RPP (% recovery of rate pressure product) value, but also has been shown to attenuate the cardiac contracture and protect myocyte damage in the isolated rat heart ischemia model (Lee *et al.*, 2005). Furthermore, KR-33028 greatly limited the infarct size in the *in vivo* rat myocardial

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infarction model. Although the preclinical studies are still under way, KR-33028 appeared to be relatively non-toxic that the oral LD_{50} value of KR-33028 in rats was greater than 1500 mg/kg (unpublished data). Taken together, KR-33028 would be a good candidate for cardiovascular diseases with a relatively low toxicity.

The present study was in support of early drug discovery and developments efforts, and experiments were conducted for mass spectral qualitative structural elucidation of the predominant metabolites of KR-33028 from *in vitro* incubation with human liver microsomes and cryopreserved human hepatocytes. This study further examined the effects of KR-33028 on the metabolism of nine major CYP isoform-specific substrates to assess the probability of drug interaction. Such information may be of considerable clinical impact in regard to safety.

MATERIALS AND METHODS

Chemicals and reagents

KR-33028 and its putative metabolites, 5-hydroxy-KR-33028 (*N*-[4-cyano-5-hydroxy-benzo[b]thiophene-2-carbonyl] guanidine) and 7-hydroxy-KR-33028 (*N*-[4-cyano-7-hydroxy-benzo[b]thiophene-2-carbonyl]guanidine), were synthesized by the Korea Research Institute of Chemical Technology (Taejeon, Korea) with a purity > 99.0%. Uridine diphosphoglucuronic acid (UDPGA), saccharic acid-1,4-lactone, alamethicin, 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), cryopreserved human hepatocytes (coded BD454503), cryopreserved hepatocytes one step purification kit, ISOM's media, and HepatoSTIM hepatocyte culture medium were purchased from BD Gentest (Woburn, MA, U.S.A.).

In vitro metabolism of KR-33028 by human liver microsomes

For microsomal metabolism of KR33028, 0.3 mg of human liver microsomes, 0.1 M potassium phosphate buffer (pH 7.4), and 50 μg of alamethicin/mg of microsomal protein were mixed and placed on ice for 15 min. 100 μM KR-33028, 5 mM sacchrolacton was added, and the mixture was preincubated at 37°C for 5 min. To initiate the reaction, NADPH-generating system (including 3.3 mM G6P, 1.3 mM β-$NADP^+$, 3.3 mM $MgCl_2$, 1.0 unit/mL G6PDH) and 5 mM UDPGA was added, and the reaction mixtures (final volume 250 μL) were incubated at 37°C for 30 min in a shaking water bath. The control incubations were conducted with heat-denatured microsomal preparations (80°C for 10 min). In all experiments, KR-33028 was dissolved in methanol, and the final concentration of organic solvent did not exceed 1%. The reaction was terminated by the addition of 100 μL of acetonitrile on ice. The incubation mixtures were then centrifuged at 20,000 g at 4°C for 5 min. Aliquots of the supernatant were analysed by LC/MS and MS/MS for the identification of the metabolites.

In vitro metabolism of KR-33028 by cryopreserved human hepatocytes

Cryopreserved human hepatocytes were thawed using the BD Gentest one step purification kit according to the procedures provided by the manufacturer. Briefly, the cells were rapidly thawed in a 37°C water bath and then added to the pre-warmed ISOM's medium. Cells were pelleted at 50 g at room temperature for 5 min. The cell pellet was reconstituted in fresh incubation medium (HepatoSTIM hepatocyte culture medium), and cell viability was determined by the trypan blue method. Cell suspension was placed in a 37°C incubator supplemented with 5% CO_2 before use.

Hepatocytes were prepared in a 96-well plate for in a 37°C incubator. The final culture volume and cell concentration were 125 μL and 0.5×10^6 viable cells/mL, respectively. The incubation mixtures containing KR-33028 were incubated at 37°C for 2 h under 5% CO_2. The reaction was terminated by the addition of 125 μL of acetonitrile on ice. The mixtures were centrifuged at 20,000 g at 4°C for 5 min. Aliquots of the supernatant were analysed by LC/MS and MS/MS for the identification of the metabolites.

LC/MS/MS analysis of KR-33028 and its metabolites

For the identification of KR-33028 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 (15:85, 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 optimized 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. The analytical data were processed by Analyst software (version 1.2).

Inhibitory potency of KR-33028 on CYP activities in human liver microsomes

The inhibitory potency of KR-33028 was determined with cytochrome P450 assays in the absence and presence of KR-33028 (final concentrations of 0.5~100 μM with methanol concentration less than 0.5%) using pooled human liver microsomes. All experiments were performed in duplicate. Phenacetin *O*-deethylase, coumarin 7-hydroxylase, bupropion 4-hydroxylase, paclitaxel 6α-hydroxylase, tolbutamide 4-hydroxylase, *S*-mephenytoin 4-hydroxylase, dextromethorphan *O*-demethylase, chlorzoxazone 6-hydroxylase, and midazolam 1-hydroxylase activities were determined as probe activities for CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4, respectively, using cocktail incubation and tandem mass spectrometry, as described previously (Kim *et al.*, 2005).

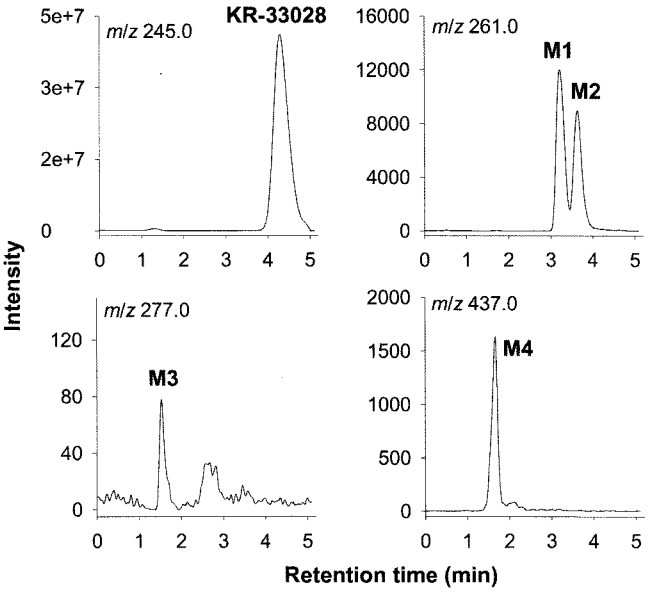

Fig. 1. Extracted ion chromatograms of KR-33028 and four metabolites of the human liver microsomal incubates of KR-33028 in the presence of NADPH-generating system and UDPGA

RESULTS

Identification of KR-33028 metabolites in human liver microsomes

Following the incubations of KR-33028 with human liver microsomes in the presence of a NADPH-generating system and UDPGA, the unchanged KR-33028 and four metabolites (M1~M4) were profiled, characterized, and tentatively identified using LC/MS analysis (Fig. 1). LC/MS/MS analysis of the unchanged KR-33028 and its four metabolites produced the informative and prominent product ions for structural elucidation. MS/MS spectrum of KR-33028, having a protonated molecular ion MH^+ at *m/z*

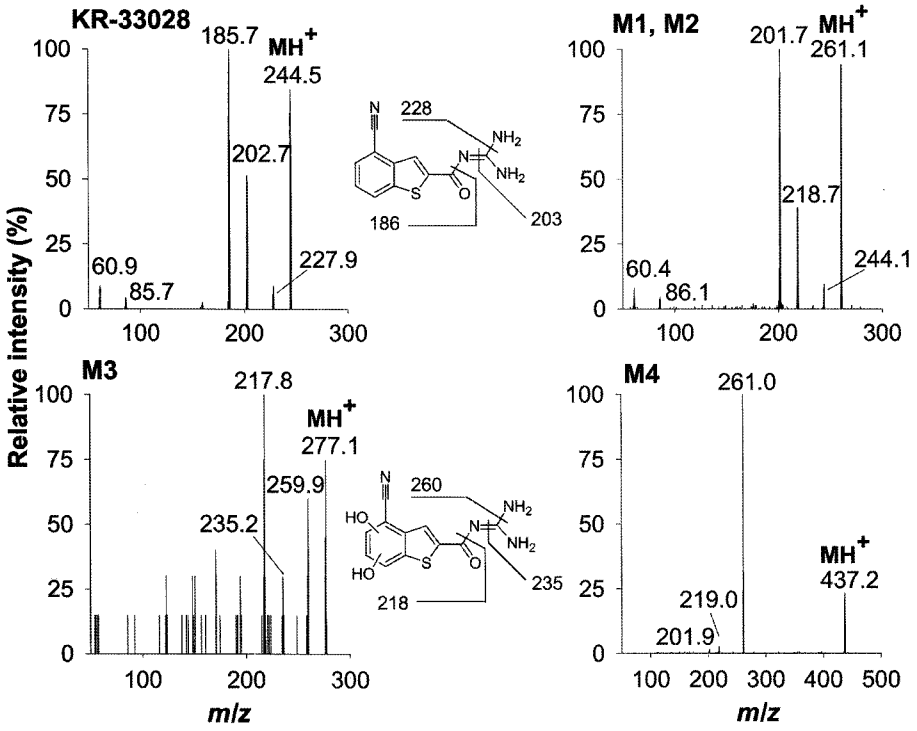

Fig. 2. MS/MS spectra of KR-33028 and its four metabolites obtained by LC/MS/MS analysis of the human liver microsomal incubates of KR-33028 in the presence of NADPH-generating system and UDPGA

245, showed major fragment ions at *m/z* 228 (the loss of amine group), *m/z* 203 (the loss of methanediamine group), and *m/z* 186 (the loss of guanidine group) (Fig. 2).

Major metabolites M1 and M2 were identified as 5-hydroxy-KR-33028 and 7-hydroxy-KR-33028, respectively, by co-chromatography and the MS/MS spectral data of the authentic compounds (Fig. 2). The mass spectra of two hydroxy-KR-33028 have a protonated molecular ion peaks MH^+ at *m/z* 261, suggesting one oxygen atom were inserted in the molecules of KR-33028 (MH^+, *m/z* 245). The MS/MS spectrum of hydroxy-KR-33028 also showed fragmentation pattern similar to parent compound (Fig. 3).

Metabolite M3, the minor metabolite peak, gave an MH^+ molecular ion at *m/z* 277, suggesting two oxygen atoms were inserted in the parent compound. The MS/MS spectrum of M3 by fragmenting *m/z* 277 through collision gave the characteristic daughter ions at *m/z* 260, 235, and 218, suggesting the possible dihydroxylation of benzothiophene ring (Fig. 2). Therefore M3 was tentatively identified as dihydroxy-KR-33028. The exact site for hydroxylation could not be determined.

The other minor metabolite M4 has protonated molecular ion peaks MH^+ at *m/z* 437 (Fig. 2). M4 was confirmed as hydroxy-KR-33028-glucuronide by the diagnostic loss of the glucuronyl moiety (176 amu, Liu *et al.*, 2005) to form the aglycon product ion at *m/z* 261 (Fig. 3). The exact site for glucuronidation could not be determined. Based on these results, the possible metabolic pathway of KR-33028 in human liver microsomes is proposed in Fig. 4.

Metabolism of KR-33028 in cryopreserved human hepatocytes

Following the incubations of KR-33028 with cryopreserved human hepatocytes, three metabolites (M1, M2, and M4) were observed (data are not shown). Dihydroxy-KR-33028 (M3) was not observed in human hepatocyte incubation sample. The pattern of metabolism was the same in human liver microsomes and cryopreserved human hepatocytes (Fig. 4).

In vitro inhibition of CYP enzymes by KR-33028

Inhibition of CYP activity was evaluated at concentrations up to 100 μM of KR-33028 to investigate the effect of KR-33028 on CYP-mediated drug interactions (Table I). KR-33028 had a moderate inhibition on phenacetin *O*-deethylase (CYP1A2) with IC_{50} of 19.0 μM. KR-33028 weakly inhibited paclitaxel 6a-hydroxylase (CYP2C8) and midazolam 1-hydroxylase (CYP3A4) with IC_{50} of 38.8 and 34.7 μM, respectively. KR-33028 at a concentration of 100 μM did not affect the other six CYP isoforms.

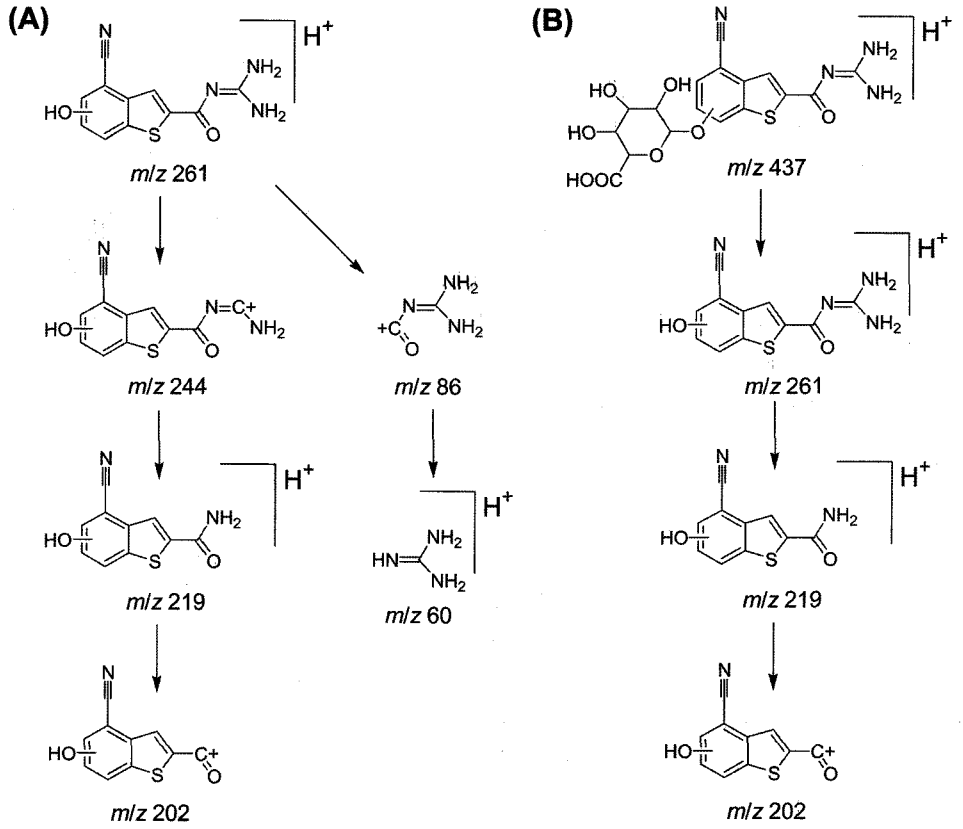

Fig. 3. MS/MS fragmentation scheme for hydroxy-KR-33028 (A) and hydroxy-KR-33028-glucuronide (B)

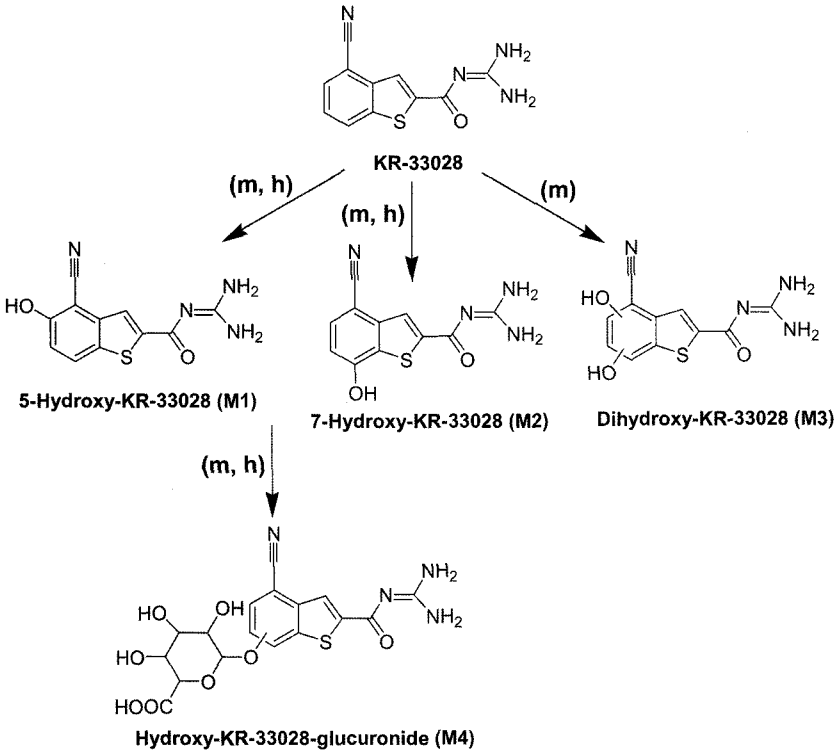

Fig. 4. Proposed metabolic pathway of KR-33028 in human liver microsomes (m) and cryopreserved human hepatocytes (h)

Table I. Inhibitory potency of KR-33028 on specific CYP activities in human liver microsomes

Enzyme activity	CYP	IC_{50} (μM)
Phenacetin O-deethylation	1A2	19.0
Coumarin 7-hydroxylation	2A6	>100
Bupropion hydroxylation	2B6	>100
Paclitaxel 6α-hydroxylation	2C8	38.8
Tolbutamide 4-methylhydroxylation	2C9	>100
S-mephenytoin 4-hydroxylation	2C19	>100
Dextromethorphan *O*-demethylation	2D6	>100
Chlorzoxazone 6-hydroxylation	2E1	>100
Midazolam 1-hydroxylation	3A4	34.7

DISCUSSION

Hepatocytes and liver microsomes are routinely used for the prediction of metabolism of drug candidates with the obvious advantages that they are easier to work with than whole animals (Ji *et al.*, 2004; Wrighton *et al.*, 1995). There are good *in vitro* and *in vivo* correlations using hepatocytes and/or microsomes for prediction of metabolism, clearance, and drug-drug interactions (Hewitt *et al.*, 2001; von Moltke *et al.*, 1994). This study was performed to identify the metabolic pathway of KR-33028 in human liver microsomes and to compare its metabolism with that of cryopreserved human hepatocytes.

In vitro metabolism study found KR-33028 to be a substrate for CYP-mediated oxidative metabolism. As shown in Fig. 3, KR-33028 was metabolised by two principal metabolic pathways, ie, oxidation of benzothiophene ring (M1, M2, and M3) and glucuronidation of oxidative metabolite (M4). The rate of hydroxylation to M1 and M2 was higher than that of M3 and M4 formation, suggesting that hydroxylation to M1 and M2 may be a major metabolic pathway for the metabolism of KR-33028 in human liver microsomes. Our results suggest that the pharmacokinetics of KR-33028 in humans may be affected by hepatic metabolism.

In addition, we evaluated *in vitro* metabolism of KR-33028 using cryopreserved human hepatocytes, which is very useful model for *in vitro* metabolism study of xenobiotics (Floby *et al.*, 2004; Hewitt *et al.*, 2001; Li *et al.*, 1999). The pattern of metabolism was similar to human liver microsomes, however, minor metabolite M3 was not observed in cryopreserved human hepatocytes.

Although hepatocytes are very effective in producing phase I and II metabolites, it has not been extensively applied owing to its limitations, including the cost of cryopreserved hepatocytes and low cell viability during cell preparations. In this study, we added UDPGA as well as NADPH generating system in human liver microsomes to produce phase I and II metabolites simultaneously. For *in vitro* metabolism study, this system may be very useful since it is readily available and more economical than hepatocytes.

The effect of KR-33028 itself on the catalytic activities of clinically significant human CYPs (1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) was also investigated in human liver microsomes. KR-33028 was found to be a moderate inhibitor of CYP1A2 activity (IC_{50} = 19.0 μM), supporting the finding of CYP1A2 inhibition by drugs containing guanidine group such as cimetidine and mifentidine (Martinez *et al.*, 1999; Reilly *et al.*, 1988). KR-33028 was less potent to inhibit the metabolism of the substrates for CYP2C8 and CYP3A4 than that of CYP1A2. Coadministration of KR-33028 with CYP1A2 substrates may have moderate effects on the pharmacokinetics of CYP1A2 substrates. These *in vitro* data suggest that *in vivo* interaction studies of KR-33028 remain to be further evaluated to rule out the possible inhibitory potential of KR-33028 with CYP1A2 substrates such as caffeine and theophylline. KR-33028 had no effect on the activities of CYP2A6, 2B6, 2C9, 2C19, 2D6, and 2E1; all of the IC_{50} values for these enzymes were > 100 μM, which is much higher than the values reported for the inhibition of the metabolism of each CYP-specific substrate (Liu *et al.*, 2004; Kim *et al.*, 2001). These findings suggest that clinical interactions between KR-33028 and these CYPs would not be expected.

In conclusion, this study demonstrates that *in vitro* incubations with cryopreserved human hepatocytes and human liver microsomes can be used to study the phase I and II metabolism of xenobiotics. New cardioprotective agent, KR-33028, is metabolised to M1, M2, and M3 by hydroxylation, and hydroxy-KR-33028 was further metabolised to M4 by glucuronidation in human liver.

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