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# *In Vitro* Metabolism of a New Neuroprotective Agent, KR-31543 in the Human Liver Microsomes : Identification of Human Cytochrome P450

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KR-31543, (2*S*,3*R*,4*S*)-6-amino-4-[*N*-(4-chlorophenyl)-*N*-(2-methyl-2*H*-tetrazol-5-ylmethyl) amino]-3,4-dihydro-2-dimethoxymethyl-3-hydroxy-2-methyl-2*H*-1-benzopyran, is a new neuroprotective agent for preventing ischemia-reperfusion damage. This study was performed to identify the metabolic pathway of KR-31543 in human liver microsomes and to characterize cytochrome P450 (CYP) enzymes that are involved in the metabolism of KR-31543. Human liver microsomal incubation of KR-31543 in the presence of NADPH resulted in the formation of two metabolites, M1 and M2. M1 was identified as *N*-(4-chlorophenyl)-*N*-(2-methyl-2*H*-tetrazol-5-ylmethyl)amine on the basis of LC/MS/MS analysis with a synthesized authentic standard, and M2 was suggested to be hydroxy-KR-31543. Correlation analysis between the known CYP enzyme activities and the rates of the formation of M1 and M2 in the 12 human liver microsomes have showed significant correlations with testosterone 6β-hydroxylase activity (a marker of CYP3A4). Ketoconazole, a selective inhibitor of CYP3A4, and anti-CYP3A4 monoclonal antibodies potently inhibited both *N*-hydrolysis and hydroxylation of KR-31543 in human liver microsomes. These results provide evidence that CYP3A4 is the major isozyme responsible for the metabolism of KR-31543 to M1 and M2.

**Key words:** KR-31543, *In vitro* metabolism, CYP3A4, Human liver microsomes

## INTRODUCTION

KR-31543, (2*S*,3*R*,4*S*)-6-amino-4-[*N*-(4-chlorophenyl)-*N*-(2-methyl-2*H*-tetrazol-5-ylmethyl)amino]-3,4-dihydro-2-dimethoxymethyl-3-hydroxy-2-methyl-2*H*-1-benzopyran, is a novel benzopyran analog possessing both antioxidant and potassium channel modulating activities (Yoo *et al.*, 2001). KR-31543 has shown its ability to protect cultured rat cortex neurons against iron-induced oxidative injury *in vitro*, and it has also shown a significant reduction in infarct volume at 24 h following occlusion in the rat model of transient cerebral-ischemia (Hong *et al.*, 2002). The $LD_{50}$ value of KR-31543 was greater than 1200 mg/kg after oral administration to the mice. KR-31543 is currently being evaluated in preclinical studies as a new neuroprotective agent for ischemia and reperfusion damage. The absolute oral bioavailability for KR-31543 was 27.4% at a dose of 20 mg/kg in rats due to hepatic and intestinal first-pass effects (Lee *et al.*, 2003). In rats, KR-31543 is metabolised to *N*-(4-chlorophenyl)-*N*-(2-methyl-2*H*-tetrazol-5-ylmethyl)amine by *N*-hydrolysis, which is primarily catalysed by CYP3A1/2 (Kim *et al.*, 2002).

The present study was conducted to identify the potential metabolites of KR-31543 in human liver microsomes and to characterise the human cytochrome P450 (CYP) enzyme responsible for the oxidative metabolism of KR-31543 by using correlation analysis, cDNA-expressed CYP isozyme, and inhibition studies. Such information can be of considerable clinical impact in regard to potential drug interactions and to the interindividual variation of drug metabolism.

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## MATERIALS AND METHODS

### Materials and reagents

KR-31543 and its putative metabolite, *N*-(4-chlorophenyl)-*N*-(2-methyl-2*H*-tetrazol-5-ylmethyl)amine, were synthesized by the Korea Research Institute of Chemical Technology (Taejeon, Korea) with a purity > 99.0%. $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, furafylline, diethyldithiocarbamate, ketoconazole, sulfaphenazole, and quinidine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methanol, acetonitrile, and methylene chloride (spectroscopy grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA), and the other chemicals were of the highest quality available.

Human liver microsomes (coded H3, H23, H42, H43, H56, H66, H70, H89, H93, H112, HK23, H161, and HK34) and microsomes derived from baculovirus infected insect cells, which were transfected with human P450 cDNA (Supersomes™) over-expressing CYP1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, were purchased from Gentest (Woburn, MA, USA). Human selective monoclonal antibodies for CYP3A4, CYP2E1, CYP2D6, and CYP1A2 and antiserum for CYP2C were also obtained from Gentest.

### KR-31543 *in vitro* metabolism in human liver microsome and P450 Supersomes®

KR-31543 (at a final concentration of 10 μM, added in acetonitrile, final solvent concentration not exceeding 0.5%, *v/v*) was incubated with an NADPH generating system (1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate and 0.8 U/mL glucose-6-phosphate dehydrogenase) with pooled human liver microsome H161 (0.4 mg/mL microsomal protein) or microsomes expressing one of the following P450s: CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, or 3A4 (final concentration of 50 pmol CYP/mL) in a 50 mM potassium phosphate buffer (pH 7.4) to give a final incubation volume of 200 μL. Incubations were initiated by the addition of a NADPH generating system after a 3 min preincubation period and were continued at 37°C for 15 min in a shaking water bath. These initial velocity conditions were linear with respect to both protein and time of incubation. The reaction was terminated by the addition of 200 μL of acetonitrile. Following the mixing with a vortex mixer and centrifugation, the aliquot of supernatant was analyzed by the LC method with mass detection for the identification of the metabolite or the LC method with UV detection for the quantification.

### LC/MS/MS analysis of KR-31543 and metabolites

For the identification of KR-31543 and its metabolites, a tandem quadrupole mass spectrometer (Quattro LC, Micromass, Manchester, UK), coupled with a Nanospace SI-2 LC system with UV detector (Shiseido, Tokyo, Japan), was used. The separation was performed on a Luna $C_8$ column (3 μm, 2 mm i.d.×100 mm, Phenomenex, Torrance, CA, USA) using the mobile phase that consisted of acetonitrile and 10 mM ammonium formate (42:58, v/v) at a flow rate of 0.2 mL/min. The column temperature was 30°C. For the identification of the metabolites, mass spectra were recorded by electrospray ionization with a positive mode. The ion source and desolvation temperature were held at 120°C and 350°C, respectively, and the cone voltage was 15 V. The molecular ions of the analytes were extracted and fragmented through collision-induced dissociation, which was achieved with argon collision gas and 10 eV of collision energy. For the quantification of KR-31543 and its metabolites, M1 and M2, in the microsomal incubates, the LC method using a UV detector was set at 260 nm. The values of M2 were calculated using a standard curve of KR-31543 because a pure sample of M2 was not available.

### Correlation analysis

Comparative metabolic rates of KR-31543 of 12 different human liver microsomes were investigated by incubating 10 μM of KR-31543 with 0.4 mg/mL microsomal protein for 15 min. The rates of M1 and M2 formation were then correlated with the rates of specific cytochrome P450 activities in human liver microsomes that were reported by Gentest (data available at www.gentest.com/products/enzym_micro/prod_inserts/H161_1.shtm) using the Pearson product moment correlation. For pairs with *p* values below 0.050, they were considered to have a significant relationship between the two variables.

### Inhibition of KR-31543 metabolism

Immunoinhibition studies were performed by incubating human liver microsomes with various amounts of human selective monoclonal antibodies for CYP1A2, CYP2E1, CYP2D6, or CYP3A4, antiserum for CYP2C, for 15 min on ice. And then, potassium phosphate buffer, KR-31543 (10 μM) and NADPH-generating system were added and incubated at 37°C for 15 min. As a control, comparable incubations were done with microsomes and a 25 mM Tris buffer.

Chemical inhibition of M1 and M2 formation was determined by using a pool of human liver microsomes and several well-characterized specific inhibitors, namely furafylline (CYP1A2) (Sesardic *et al.*, 1990), diethyldithiocarbamate (CYP2E1) (Guengerich *et al.*, 1991), sulfaphenazole (CYP2C9) (Baldwin *et al.*, 1995), quinidine (CYP2D6) (Otton *et al.*, 1988), and ketoconazole (CYP3A4) (Maurice *et al.*, 1992). Inhibited activities were compared with activities of appropriate control incubations without the chemical inhibitors. Microsomes were preincubated with chemical inhibitors and the NADPH-generat-

ing system at 37°C for 10 min before the addition of KR-31543.

## RESULTS

### Identification of KR-31543 metabolites in human liver microsomes

Following the incubations of KR-31543 with human liver microsomes in the presence of a NADPH-generating system, the unchanged KR-31543 and two metabolites (M1 and M2 in order of elution) were profiled, characterized, and tentatively identified using LC/MS analysis (Fig. 1). LC/MS/MS analysis of the unchanged KR-31543 and its two metabolites produced the informative and prominent product ions for structural elucidation (Fig. 2). MS/MS spectrum of KR-31543, having a protonated molecular ion ($MH^+$) at *m/z* 475, showed major fragment ions at *m/z* 252 (dihydro-2-dimethoxymethyl-3-hydroxy-2-methyl-2*H*-1-benzopyran), *m/z* 220 (additional loss of methoxy group from *m/z* 252), *m/z* 188 (additional loss of methoxy group from *m/z* 220), and *m/z* 443 (the loss of methoxy group).

Metabolite M1, the major metabolite peak with the $MH^+$ ion of *m/z* 224 (251 amu less than parent KR-31543), was identified as *N*-(4-chlorophenyl)-*N*-(2-methyl-2*H*-tetrazol-5-ylmethyl)amine by co-chromatography and the MS/MS spectral data of the authentic standard. M1, which resulted from the hydrolysis of dihydro-2-dimethoxymethyl-3-hydroxy-2-methyl-2*H*-1-benzopyran moiety at nitrogen of methylamino moiety, showed fragment ions at *m/z* 140 (the loss of 2-methyl-2*H*-tetrazole group) and *m/z* 127 (4-chlorophenylamino group). Although M1 was identified as a major metabolite, the remaining benzopyran moiety of KR-31543 was not detected in this *in vitro* system.

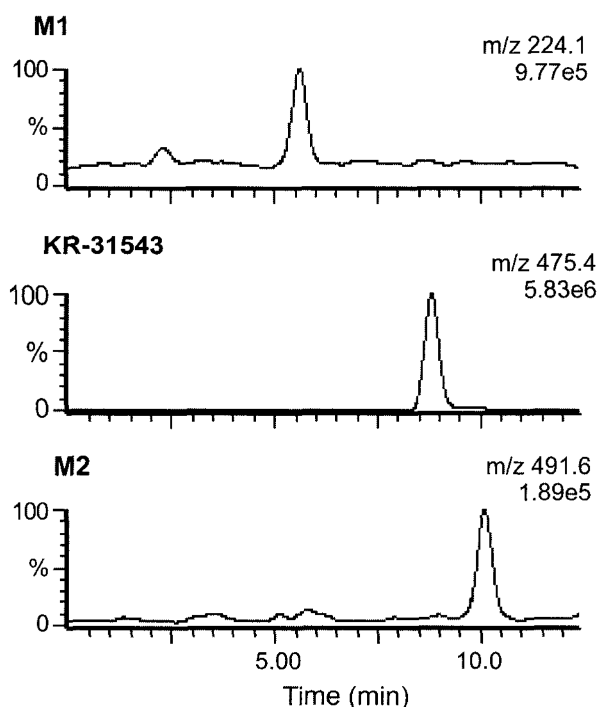


**Fig. 1.** Extracted ion chromatograms of KR-31543 and two metabolites, M1 and M2 of the human liver microsomal incubates of KR-31543 in the presence of NADPH-generating system.

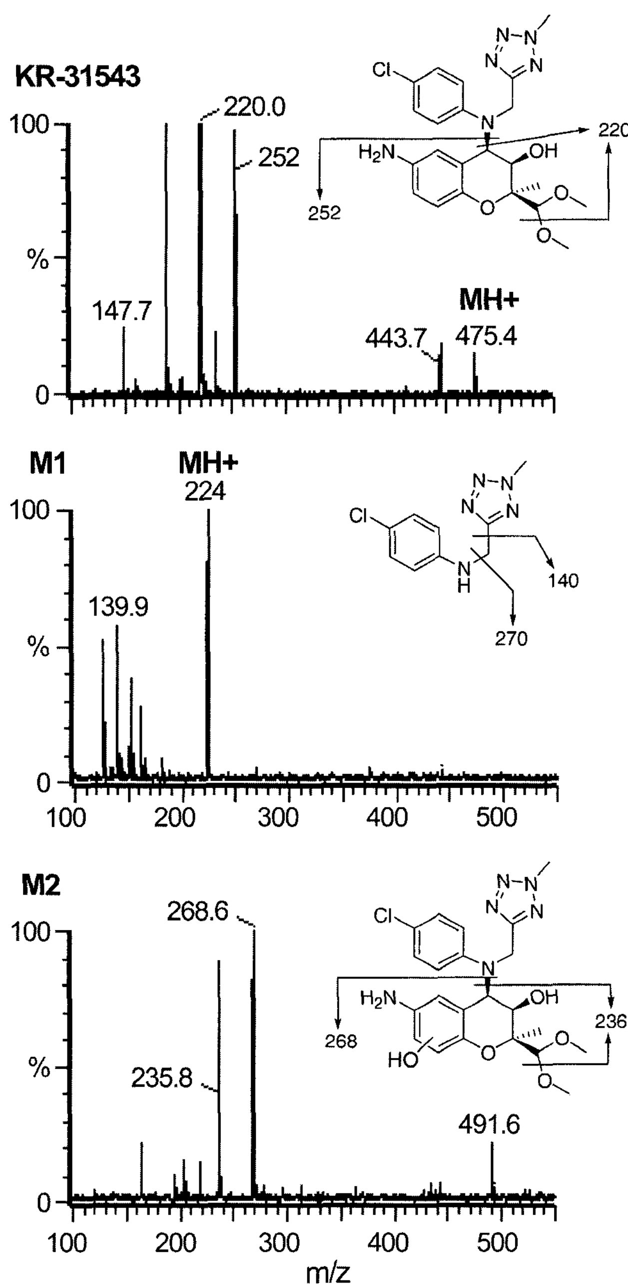


**Fig. 2.** MS/MS spectra of KR-31543 and its two metabolites M1 and M2 obtained by LC/MS/MS analysis of the human liver microsomal incubates of KR-31543 in the presence of NADPH-generating system.

Metabolite M2, the minor metabolite peak, possessed 16 amu more than KR-31543 [$MH^+$ ion at *m/z* 491] (Fig. 1). The fragment ions at *m/z* 268 [the loss of *N*-(4-

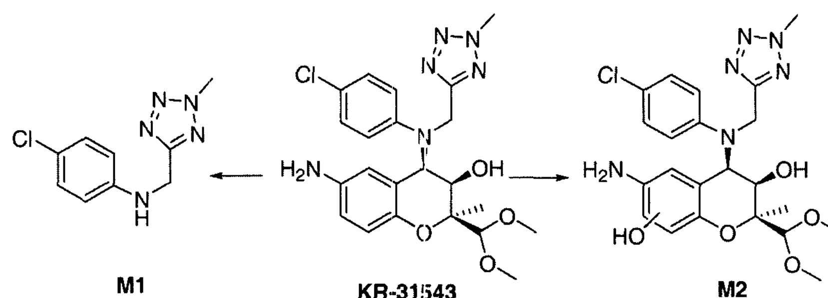


**Fig. 3.** Proposed metabolic pathway of KR-31543 in human liver micrsomes

chlorophenyl)-*N*-(2-methyl-2*H*-tetrazol-5-ylmethyl)amine] and *m/z* 236 (a loss of methoxy group of *m/z* 268) suggested that M2 might result from the hydroxylation at the benzopyran moiety. M2 was tentatively identified as hydroxy-KR-31543. The exact site for hydroxylation could not be determined.

Based on these results, the possible metabolic pathway of KR-31543 in human liver microsomes is proposed in Fig. 3.

## Correlation of rates of KR-31543 metabolism with enzyme activities on human liver microsomes

The rates of M1 and M2 formation from KR-31543 in 12 different human liver microsomes ranged from 80.4 to 519.9 and 16.6 to 62.7 pmol/min/mg protein, respectively. As shown in Table I, a good correlation was observed between the rates of testosterone 6β-hydroxylation, a marker of CYP3A4, and the formation of M1 and M2 ($r$ = 0.944 and 0.878, respectively). No significant relationships of KR-31543 oxidation were observed with coumarin 7-hydroxylase (CYP2A6), paclitaxel 6α-hydroxylase (CYP2C8), diclofenac 4'-hydroxylase (CYP2C9), *S*-mephenytoin 4'-hydroxylase (CYP2C19), bufuralol 1'-hydroxylase (CYP2D6), phenacetin O-deethylase (CYP1A2), and chlorozoxazone 6-hydroxylase (CYP2E1).

**Table I.** Correlation of CYP activity and the formation rates of M1 and M2 from KR-31543 in 12 different human liver microsomes

| Enzymatic activity | CYP isozyme | Correlation coefficient (*r*) | |
|---|---|---|---|
| | | M1 | M2 |
| phenacetin O-deethylase | 1A2 | -0.0241 | 0.0730 |
| coumarin 7-hydroxylase | 2A6 | 0.187 | -0.0215 |
| paclitaxel 6α-hydroxylase | 2C8 | 0.560 | 0.325 |
| diclofenac 4'-hydroxylase | 2C9 | 0.330 | 0.434 |
| [S]-mephenytoin 4'-hydroxylase | 2C19 | 0.0775 | 0.283 |
| bufuralol 1'-hydroxylase | 2D6 | -0.317 | -0.479 |
| chlorozoxazone 6-hydroxylase | 2E1 | 0.0227 | 0.0229 |
| testosterone 6β-hydroxylase | 3A4 | 0.944* | 0.878* |

*: significant relationship ($p<0.05$)

## Metabolism of KR-31543 in expressed human CYP isozymes

KR-31543 oxidation activities were investigated in nine human CYP expression isozymes (1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) derived from baculovirus infected insect cells (Table II). Of all the CYP isozymes tested, CYP3A4 showed the greatest M1 formation activity. Also, only CYP3A4 had the M2 formation activity. CYP3A4 appears to play a major role in KR-31543 metabolism.

## Chemical Inhibition of KR-31543 metabolism in human liver microsomes

The effects of selective inhibitors of CYP450s on KR-31543 metabolism were investigated in pooled human liver microsomes (Fig. 4). Ketoconazole, a selective inhibitor of CYP3A4, effectively inhibited the formation of M1 and M2. Furafylline (CYP1A2), diethyldithiocarbamate (CYP2E1), quinidine (CYP2D6), and sulfaphenazole (CYP2C9) showed weak or no inhibitory effects on the formation of M1 and M2.

**Table II.** KR-31543 metabolism in human CYP expression isozymes derived from baculovirus infected insect cells

| Human CYPs | Metabolite formation (pmol/pmol protein/min) | |
|---|---|---|
| | M1 | M2 |
| CYP1A1 | 0.29 | N.D. |
| CYP1A2 | 0.25 | N.D. |
| CYP2B6 | 0.18 | N.D. |
| CYP2C8 | 0.19 | N.D. |
| CYP2C9 | 0.23 | N.D. |
| CYP2C19 | 0.21 | N.D. |
| CYP2D6 | 0.25 | N.D. |
| CYP2E1 | 0.13 | N.D. |
| CYP3A4 | 1.63 | 0.16 |

N.D.: not detected (<0.1 pmol/pmol protein/min)
KR-31543 (10 μM) and human CYP isozymes were incubated with the presence of a NADPH-generating system for 15 min at 37°C. Results are expressed as means of duplicate assays, which differed by <10%.

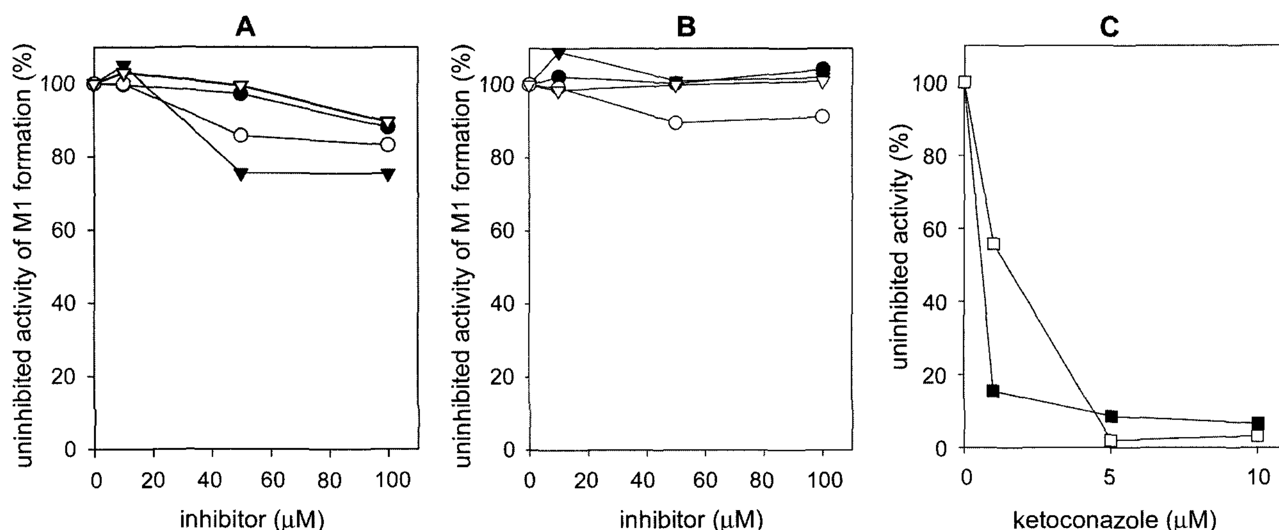


**Fig. 4.** Inhibition of KR-31543 metabolism to (A) M1 and (B) M2 in human liver microsomes by several chemical inhibitors. Pooled human liver microsome (H161) was used, with preincubation in the presence of a NADPH-generating system. (A) and (B) diethyldithiocarbamate (▽), furafylline (○), quinidine (●) and sulfaphenazole (▼); (C) ketoconazole for the formation of M1 (■) and M2 (□).

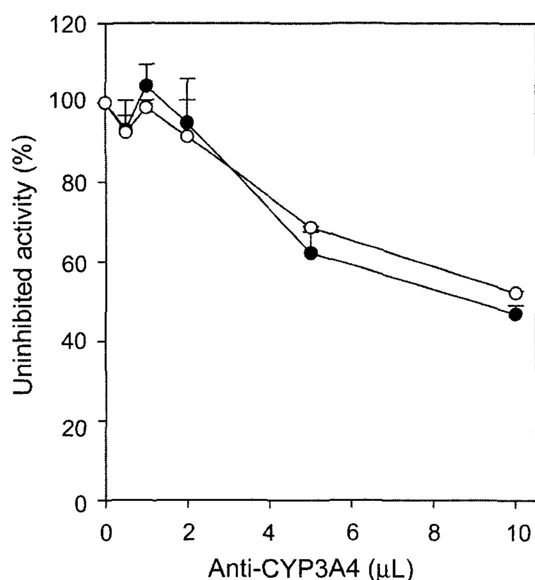


**Fig. 5.** Effect of anti-CYP3A4 on the metabolism of KR-31543 to M1 (○) and M2 (●). Pooled human liver microsome (H161) was preincubated with anti-CYP3A4.

### Immunoinhibition of KR-31543 oxidation in human liver microsomes

To determine which CYP is primarily responsible for KR-31543 metabolism, immunoinhibition studies with anti-CYP1A2, anti-CYP2C, anti-CYP2D6, anti-CYP2E1, or anti-CYP3A4 monoclonal antibodies were conducted in pooled human liver microsomes. Only anti-CYP3A4 significantly inhibited M1 and M2 formation in pooled human liver microsome H161 (Fig. 5). The inhibition of KR-31543 oxidation activity by other antibodies was <10% (data not shown). These results strongly suggest that CYP3A4 is responsible for almost all of the KR-31543 metabolism activity in human liver.

## DISCUSSION

The *in vitro* metabolism study, using human liver microsomes, found KR-31543 to be a substrate for CYP-mediated oxidative metabolism. As shown in Fig. 3, KR-31543 was metabolised by two principal metabolic pathways, ie, *N*-hydrolysis to *N*-(4-chlorophenyl)-*N*-(2-methyl-2*H*-tetrazol-5-ylmethyl)amine (M1) and hydroxylation at the benzopyran moiety to hydroxy-KR-31543 (M2). M1 was also identified as the major metabolite in the *in vitro* and *in vivo* metabolism of KR-31543 in rats (Kim *et al.*, 2002). However, hydroxy-KR-31543 (M2) was produced only by human liver microsomes and CYP3A4. The rate of hydroxylation to M2 was lower than that of M1 formation, suggesting that *N*-hydrolysis to M1 may be a major metabolic pathway for the metabolism of KR-31543 in human liver.

In order to identify the specific enzymes responsible for KR-31543 metabolism, a combination of chemical inhibition, immunoinhibition, correlation analysis in human liver microsomes, and metabolism by recombinant cDNA-expressed CYP enzymes was employed. Correlation analysis between the known CYP enzyme activities and the rates of the formation of M1 and M2 has showed significant correlations with the testosterone 6β-hydroxylase activity in the 12 human liver microsomes that were investigated. Ketoconazole, which is a selective inhibitor of CYP3A4, and anti-CYP3A4 monoclonal antibodies

potently inhibited both *N*-hydrolysis and hydroxylation of KR-31543. The results collectively suggest that CYP3A4 plays a major role in the metabolism of KR-31543 to *N*-(4-chlorophenyl)-*N*-(2-methyl-2*H*-tetrazol-5-ylmethyl)amine (M1) and hydroxy-KR-31543 (M2).

In the previous reports (Kim *et al.*, 2002; Lee *et al.*, 2003), absolute bioavailability of KR-31543 in rats was low due to the first-pass metabolism. These results suggest that the pharmacokinetics of KR-31543 in humans may be significantly affected by hepatic metabolism. The expression of CYP3A4, the major CYP isozyme in human liver, has been highly variable among human liver samples (Bork *et al.*, 1989; Shimada *et al.*, 1994; Watkins *et al.*, 1985; Wrighton *et al.*, 1990). CYP3A4 has been shown to be inducible by barbiturates, rifampin, and dexamethasone or has been shown to be inhibited by ketoconazole, itraconazole, erythromycin, and protease inhibitors such as ritonavir and indinavir (Baldwin *et al.*, 1995; Lillibridge *et al.*, 1998; Merry *et al.*, 1999; von Moltke *et al.*, 1998; Wang *et al.*, 1999; Wrighton and Ring, 1994). Therefore, any concomitant drugs that can inhibit or induce CYP3A4 may alter the metabolism of KR-31543 and potentially lead to changes in the pharmacokinetics of KR-31543 in humans.

In conclusion, this study demonstrates that the two metabolic pathways (*N*-hydrolysis to M1 and hydroxylation to M2) contribute to the metabolism of KR-31543 in human liver microsomes, and also that CYP3A4 is responsible for the two pathways.

## ACKNOWLEDGEMENT

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## REFERENCES

Baldwin, S. J., Bloomer, J. C., Smith, G. J., Ayrton, A. D., Clarke, S. E., and Chenery, R. J., Ketoconazole and sulphaphenazole as the respective selective inhibitors of P4503A and 2C9. *Xenobiotica*, 25, 261-270 (1995).

Bork, R. W., Muto, T., Beaune, P. H., Srivastava, P. K., Lloyd, R. S., and Guengerich, F. P., Characterization of mRNA species related to human liver cytochrome P-450 nifedipine oxidase and the regulation of catalytic activity. *J. Biol. Chem.*, 264, 910-919 (1989).

Hong, K. W., Kim, K. Y., Lee, J. H., Shin, H. K., Kwak, Y. G., Kim, S. O., Lim, H., and Yoo, S. E., Neuroprotective effect of (2*S*,3*S*,4*R*)-*N*''-cyano-*N*-(6-amino-3,4-dihydro-3-hydroxy-2-methyl-2-dimethoxymethyl-2*H*-benzopyran-4-yl)-*N*'-benzylguanidine (KR-31378), a benzopyran analog, against focal ischemic brain damage in rats. *J. Pharmacol. Exp. Ther.*, 301, 210-216 (2002).

Kim, J., Ji, H. Y., Lee, S. S., Yoo, S. E., Kim. S. O., Lee, D. H., Lim, H., and Lee, H. S., Metabolism of a new neuroprotective agent for ischemia-reperfusion damage, KR-31543 in the rats using liquid chromatography/electrospray mass spectrometry. *Arch. Pharm. Res.*, 25, 664-668 (2002).

Lee, M. H., Bae, S. K., Kim, E. J., Kim, Y. G., Kim, S. O., Lee, D. H., Lim, H., Yoo, S. E., and Lee, M. G., Dose-independent pharmacokinetics of a new neuroprotective agent for ischemia-reperfusion damage, KR-31543, after intravenous and oral administration to rats: hepatic and intestinal first-pass effects. *J. Pharm. Sci.*, 92, 190-201 (2003).

Lillibridge, J. H., Liang, B. H., Kerr, B. M., Webber, S., Quart, B., Shetty, B., and Lee, C. A., Characterization of the selectivity and mechanism of human cytochrome P450 inhibition by the human immunodeficiency virus-protease inhibitor nelfinavir mesylate. *Drug Metab. Disp.*, 26, 609-616 (1998).

Maurice, M., Pichard, L., and Daujat, M., Effects of imidazole derivatives on cytochromes P450 from human hepatocytes in primary culture. *FASEB J.*, 6, 752-758 (1992).

Merry, C., Barry, M. G., Ryan, M., Tjia, J. F., Hennesy, M., Eagling, V.A., Mulcahy, F., and Back, D. J., Interaction of sildenafil and indinavir when co-administered to HIV-positive patients. *AIDS*, 13, F101-F107 (1999).

Otton, S. V., Crewe, H. K., Lennard, M. S., Tucker, G. T., and Woods, H. F., Use of quinidine inhibition to define the role of the sparteine/debrisoquine cytochrome P450 in metoprolol oxidation by human liver microsomes. *J. Pharmacol. Exp. Therap.*, 247, 242-247 (1988).

Sesardic, D., Boobis, A. R., and Murray, B. P., Furafylline is a potent and selective inhibitor of cytochrome P450 1A2 in man. *Br. J. Clin. Pharmacol.*, 29, 651-663 (1990).

Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., and Guengerich, F. P., Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J. Pharmacol. Exp. Ther.*, 270, 414-423 (1994).

von Moltke, L. L., Greenblatt, D. J., Grassi, J. M., Granda, B. W., Duan, S. X., Fogelman, S. M., Daily, J. P., Harmatz, J. S., and Shader, R. I., Protease inhibitors as inhibitors of human cytochromes P450: High risk associated with ritonavir. *J. Clin. Pharmacol.*, 38, 106-111 (1998).

Wang, J. S., Wen, X., Backman, J. T., Taavitsainen, P., Neubonnen, P. J., and Kivisto, K. T., Midazolam $\alpha$-hydroxylation by human liver microsomes *in vitro*: inhibition by calcium channel blockers, itraconazole and ketoconazole. *Pharmacol. Toxicol.*, 85, 157-161 (1999).

Watkins, P. B., Wrighton, S. A., Mauel, P., Schuets, E. G., Mendezpicon, G., Parker, G. A., and Guzelian, P. S., Identification of an inducible form of cytochrome P-450 in human liver. *Proc. Natl. Acad. Sci. USA*, 82, 6310-6314 (1985).

Wrighton, S. A., Brian, W. R., Sari, M. A., Iwasaki, M., Guengerich, F. P., Raucy, J. L., Molowa, D. T., and Vandenbranden, M., Studies on the expression and metabolic capabilities of human liver cytochrome P-450 IIIA4 (HLp 3). *Mol. Pharmacol.,* 38, 207-213 (1990).

Wrighton, S. A. and Ring B. J., Inhibition of human CYP3A catalyzed 1-hydroxy midazolam formation by ketoconazole, nifedipine, erythromycin, cimetidine and nizatidine. *Pharm. Res.*, 11, 921-924 (1994).

Yoo, S. E., Yi, K. Y., Lee, S., Suh, J., Kim, N., Lee, B. H., Seo, H. W., Kim, S. O., Lee, D. H., Lim, H. and Shin, H. S., A novel anti-ischemic ATP-sensitive potassium channel opener without vasorelaxation: *N*-(6-aminobenzopyranyl)-*N'*-benzyl-*N''*-cyanoguanidine analogue. *J. Med. Chem.,* 44, 4207-4215 (2001).