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Metabolism of a New Neuroprotective Agent for Ischemia-Reperfusion Damage, KR-31543 in the Rats using Liquid Chromatography/Electrospray Mass Spectrometry

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KR-31543,(2S,3R,4S)-6-amino-4-[N-(4-chlorophenyl)-N-(2-methyl-2H-tetrazol-5-ylmethyl)amino]-3,4-dihydro-2-dimethoxymethyl-3-hydroxy-2-methyl-2H-1-benzopyran is a new neuroprotetive agent for ischemia-reperfusion damage. The *in vitro* and *in vivo* metabolism of KR-31543 in rats has been studied by LC-electrospray mass spectrometry. Rat liver microsomal incubation of KR-31543 in the presence of NADPH resulted in the formation of a metabolite M1. M1 was identified as N-(4-chlorophenyl)-N-(2-methyl-2H-tetrazol-5-ylmethyl)amine on the basis of LC-MS/MS analysis with the synthesized authentic standard. Rat CYP3A1 and 3A2 are the major CYP isozymes involved in the formation of M1.

Key words: KR-31543, LC/MS/MS, Metabolism, CYP, Neuroprotective

INTRODUCTION

In spite of considerable research efforts for the development of neuroprotective agents to save neurons from the biochemical and metabolic outcomes of ischemic brain injury, most of the neuroprotective agents studied so far have shown a lack of clinical efficacy (De Keyser *et al.*, 1999). Both inhibition of lipid peroxidation induced by reactive oxygen species and stabilization of membrane have been proposed as neuroprotective strategies in stroke (Hong *et al.*, 2002).

KR-31543,(2*S*,3R,4S)-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 to protect cultured rat cortex neurons against iron-induced oxidative injury *in vitro*, and also shown a significant reduction in infarct volume at 24 h following occlusion in the rat model of transient cerebral-ischemia. The LD_{50} value of KR-31543 was greater than 1200 mg/kg after oral administration to 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oral bioavailability for KR-31543 was 27.4% at 20 mg/kg in rats due to hepatic and intestinal first-pass effects (unpublished data).

The present study was in support of early drug discovery/development efforts, and experiments were conducted for mass spectral qualitative structural elucidation of the predominant metabolites of KR-31543 from *in vitro* incubation with rat hepatic microsomes and *in vivo* metabolites in the bile samples obtained after oral administration of KR-31543. Cytochrome P-450 (CYP) isozymes responsible for the metabolism of KR-31543 in rats were characterized using rat c-DNA expressed CYP isozymes and immunoinhibition study.

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MATERIALS AND METHODS

Materials and reagents

KR-31543 and its putative metabolites, *N*-(4-chlorophen-

yl)-*N*-(2-methyl-2*H*-tetrazol-5-ylmethyl)amine and (2*S*,3R, 4S)-6-acetylamino-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 (*N*-acetyl-KR-31543) were synthesized by Korea Research Institute of Chemical Technology (Taejeon, Korea) with >99.0% purity. Glucose-6-phosphate (G-6-P), G-6-P dehydrogenase and $NADP^+$ were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pooled male rat liver microsomes, rat CYP3A2 polyclonal antiserum (anti-CYP3A2) and CYP1A1, 1A2, 2A2, 2B1, 2C6, 2C11, 2C12, 2C13, 2D1, 2D2, 2E1, 3A1 and 3A2 Supersomes® were obtained from Gentest (Woburn, MA, USA). Methanol, ethanol, acetonitrile and methylene chloride (HPLC grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and the other chemicals were of the highest quality available.

KR-31543 *in vitro* metabolism in rat liver microsome and rat CYP supersomes®

Reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.4), 3 mM magnesium chloride, rat liver microsomes (100 μg protein) or rat CYP supersomes® (5 pmol), NADPH-generating system (1.3 mM $NADP^+$, 3.3 mM G-6-P and 0.5 unit G-6-P dehydrogenase), and 20 μM KR-31543 in total volume of 250 μL. The control incubations were conducted in the absence of NADPH-generating system. The samples were incubated at 37 for 30 min and the reaction was stopped by adding acetonitrile (1 mL). The reaction mixture was centrifuged and the supernatant was evaporated. The residue was dissolved in 40% acetonitrile (50 μL) and the aliquot (5 μL) was analyzed by LC-MS and MS/MS for the identification of the metabolite.

In vivo metabolism of KR-31543 in rat bile

Two male Sprague-Dawley rats (230 ± 10 g, Biogenomics, Korea) were anaesthetized with ether and the bile duct was catheterized using PE-10 tubing. The bile samples were collected for 12 hr after oral administration of KR-31543 in 50% polyethylene glycol solution at a dose of 10 mg/kg. Bile sample (2 mL) was adjusted to pH 7.0 and extracted with methylene chloride (2 mL) and centrifuged. The organic phase was evaporated to dryness under nitrogen gas, and the residue was dissolved in 40% acetonitrile (100 μL). The aliquot was analyzed by LC-MS method to identify unchanged KR-31543 and its possible metabolites.

To characterize the nature of glucuronidation, the bile (0.5 mL) was incubated with β-glucuronidase (100 unit) at 37°C for 2 hr, KR-31543 and its metabolites were extracted and analyzed as described above.

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

For the analysis 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 2 C_8 column (3 μm, 2 mm i.d. × 100 mm, Phenomenex, Torrance, CA, USA) using the mobile phase consisted of acetonitrile and 5 mM ammonium formate (45 : 55, v/v) at a flow rate of 0.2 mL/min. The column temperature was 30°C. The analytes were analyzed using a UV detector set at 260 nm for quantitation in the kinetic study.

For the identification of the metabolites, mass spectra were recorded with positive ionization electrospray mode. The ion source and desolvation temperature were held at 120°C and 230°C, respectively, and the cone voltage was 15 eV. The molecular ions of the analytes were extracted and fragmented by collision-induced dissociation, which was achieved with argon collision gas and 10 eV collision energy.

Kinetics of KR-31543 metabolism

Enzyme kinetics of KR-31543 metabolism was investigated by incubating KR-31543 in pooled rat liver microsomes in duplicates at concentrations from 0.5 to 200 μM. KR-31543 was incubated with approximately 0.4 mg/mL microsomal protein and NADPH-generating system at 37°C for 30 min. Estimates for the apparent V_{max} and K_m values for the formation of M1 were determined by nonlinear regression.

Immunoinhibition of KR-31543 metabolism

Immunoinhibition study was conducted by incubating rat liver microsomes with anti-CYP3A2 (0, 10, 20 and 50 μL) at 23°C for 20 min prior to the incubation of KR-31543 by adding other components required for catalytic activity. In the control experiments, the pre-immune IgG was used instead of anti-CYP3A2.

RESULTS

Identification and characterization of metabolite of KR-31543

For metabolites identification, LC/ESI-MS analysis was conducted after the incubation of KR-31543 with rat liver microsomes in the presence and absence of NADPH-generating system. Following the incubations of KR-31543 with rat liver microsomes in the presence of NADPH-generating system, unchanged KR-31543 and a major putative metabolite (M1) were observed (Fig. 1). In contrast, no metabolite including M1 was produced in the absence of NADPH, thus, suggesting the involvement of CYP enzymes for the formation of M1.

LC/MS/MS spectra of unchanged KR-31543 and metabolite M1 shows the informative and prominent product ions for the structural elucidation (Fig. 2). MS/MS spectrum of KR-31543, having a protonated molecular ion

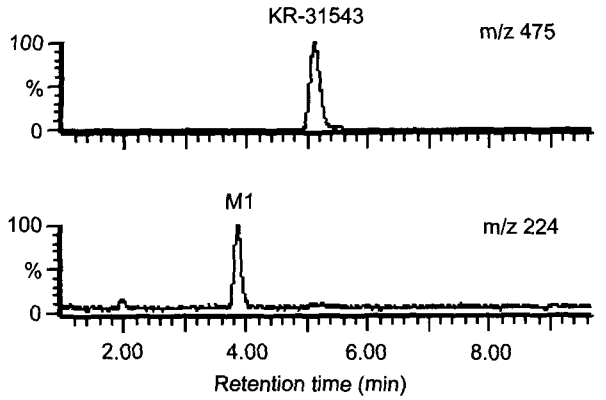

Fig. 1. LC-ESI-MS ion chromatograms of the rat liver microsomal incubation of KR-31543 in the presence of NADPH-generating system.

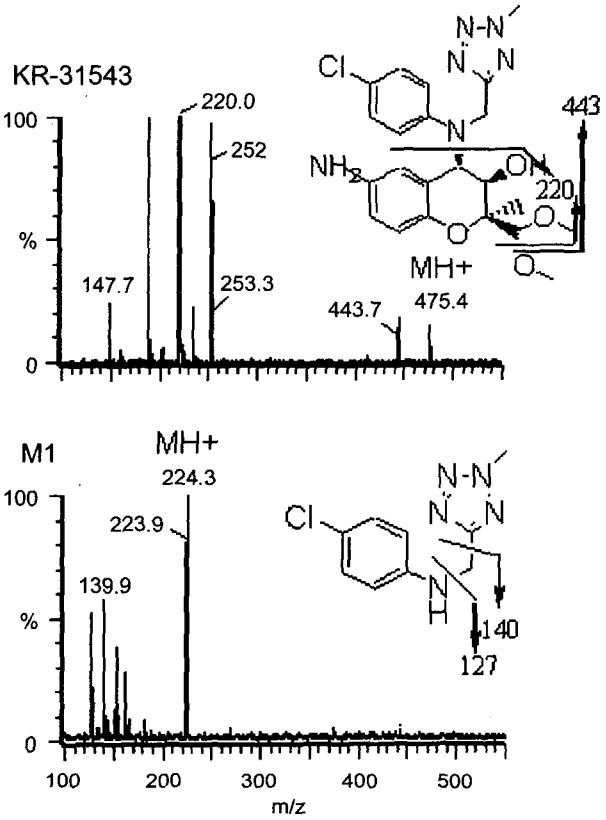

Fig. 2. ESI-MS/MS spectra of KR-31543 and its metabolite M1.

(MH^+) at *m/z* 475, gave major fragment ions at *m/z* 252 (dihydro-2-dimethoxymethyl-3-hydroxy-2-methyl-2H-1-benzopyran), *m/z* 220 (additional loss of methoxy group from *m/z* 252), *m/z* 188 (additional loss of two methoxy groups from *m/z* 220) and *m/z* 443 (the loss of methoxy group).

Metabolite M1, a 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 based on the comparison of chromatographic retention and MS/MS spectra of the authentic

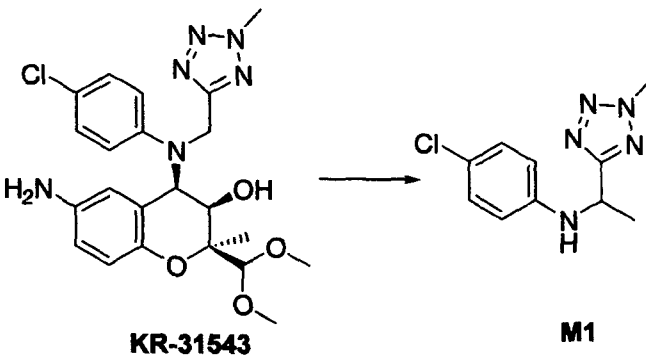

Fig. 3. Proposed metabolic pathway of KR-31543 in the rats.

standard. M1, resulting 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-chlorophenyl-amino group). No metabolite was produced from the incubation of M1 with rat liver microsomes. Although M1 was identified as a major metabolite, the remaining benzopyran moiety of KR-31543 was not detected in this *in vitro* system. A potential metabolite, (2*S*,3R,4S)-6-amino-4-[*N*-(4-chlorophenyl)-*N*-(2-methyl-2*H*-tetrazol-5-ylmethyl)amino]-3,4-dihydro-2-hydroxymethyl-3-hydroxy-2-methyl-2*H*-1-benzopyran (KR-31952), which could be produced by the oxidation of acetal group to alcohol group, was not detected.

The profile of the neutral extract of KR-31543-treated rat bile samples was similar to that from rat liver microsomal incubation, giving the unchanged KR-31543 and M1. After the enzymatic hydrolysis of the bile with β-glucuronidase, there was no change in the amount of M1 or KR-31543. Potential metabolites, *N*-acetyl-KR-31543, (2*S*,3R,4S)-6-acetylamino-4-[*N*-(4-chlorophenyl)-*N*-(2-methyl2*H*-tetrazol-5-ylmethyl)amino]-2-dimethoxy-methyl-2-methyl-2*H*-1-benzopyran (dehydrated-*N*-acetyl-KR-31543) and KR-31952, were not detected in the bile.

Based on these results, the possible metabolic pathway of KR-31543 in rat liver is proposed in Fig. 3. KR-31543 was metabolized in the rats by *N*-hydrolysis to *N*-(4-chlorophenyl)-*N*-(2-methyl-2*H*-tetrazol-5-ylmethyl)amine (M1).

Kinetics of KR-31543 metabolism

In male rat liver microsomes, the formation of M1 followed Michelis-Menten kinetics, and the apparent kinetic constants for the formation of M1 in rat liver microsomes were K_m values of 69.9 iM and V_{max} values of 188 pmol/min/mg protein, respectively. Intrinsic clearance (CL_{int}, V_{max}/K_m) for M1 was 2.7 μL/min/mg protein.

Identification of CYP isozymes involved in KR-31543 metabolism

Metabolism of KR-31543 varied significantly in the thir-

Table 1. Formation rate of N-(4-chlorophenyl)-N-(2-methyl-2H-tetrazol-5-ylmethyl) amine (M1) from KR-31543 by rat CYP isozymes.

Rat CYPs	M1 formation (pmol/pmol protein/min)
CYP1A1	0.21
CYP1A2	0.14
CYP2A2	0.18
CYP2B1	0.12
CYP2C6	0.16
CYP2C11	0.20
CYP2C12	N.D.
CYP2C13	N.D.
CYP2D1	0.29
CYP2D2	0.13
CYP2E1	0.11
CYP3A1	0.80
CYP3A2	0.53

N.D.: not detected (<0.1 pmol/mg protein/min)
KR-31543 (20 μM) and rat CYP isozymes were incubated with the presence of NADPH-generating system at 37°C for 30 min. Results are expressed as means of duplicate assays, which differed by <10%.

teen cDNA expressed CYP isozyme systems investigated (Table I). The rate of M1 formation was higher in CYP3A1 (0.80 pmol/mg protein/min) and CYP3A2 Supersomes® (0.53 pmol/mg protein/min) compared to others. The rate of formation was relatively lower in CYP1A1, CYP1A2, CYP2A2, CYP2B1, CYP2C6, CYP2C11, CYP2D1, CYP2D2 and CYP2E1 (<0.30 pmol/mg protein/min). No detectable concentration of M1 was observed from CYP2C12 and CYP2C13. Therefore, CYP3A1/2 appears to play the major roles in the formation of M1 with lesser contributions from other CYP isozymes.

In order to confirm the involvement of CYP3A2 in the formation of M1, immunoinhibition study using anti-CYP3A2, rat CYP3A2 selective inhibitor serum, was conducted. The effect of varying amount of anti-CYP3A2 on KR-31543 metabolism in rat liver microsomes is illustrated in Fig. 4. More than 70% inhibition of M1 formation was observed as comparable to the control level using 10 μL of anti-CYP3A2 serum. Immunoinhibition study for CYP3A1 could not be conducted due to unavailability of rat CYP3A1 antiserum.

DISCUSSION

This study has characterized the *in vitro* and *in vivo* metabolism of KR-31543 in rats. *In vitro* metabolism study using rat liver microsomes found KR-31543 to be a substrate for CYP-mediated oxidative metabolism, and *N*-(4-chlorophenyl)-*N*-(2-methyl-2*H*-tetrazol-5-ylmethyl)amine (M1) was identified a major metabolite. After oral administration of KR-31543 to rats, M1 was found as a major metabolite in the bile.

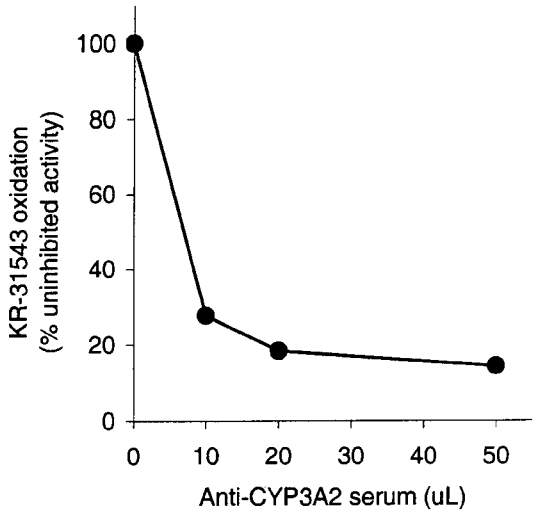

Fig. 4. Effect of anti-CYP3A2 on the formation of M1 from KR-31543. Rat liver microsomes were pre-incubated with anti-CYP3A2.

This study demonstrated that *N*-hydrolysis to M1 is the major pathway of *in vitro* and *in vivo* metabolism for KR-31543. These results support the previous findings in the pharmacokinetic study of KR-31543 in rats. In this pharmacokinetic study, the low bioavailability (~27%) of KR-31543 is likely mainly due to hepatic and intestinal first-pass effects rather than low absorption of KR-31543, which is indicated by very low (4.2%) of unchanged KR-31543 recovered from gastrointestinal tract at 24 hours (unpublished data).

To characterize the major CYP isozymes(s) responsible for M1 formation, *in vitro* metabolism of KR-31543 in microsomes produced from insect cell line transfected with Baculovirus containing a single rat CYP isozyme with supplemental reductase was investigated. Incubation of KR-31543 with CYP3A1 and CYP3A2 Supersomes® produced M1 at a comparable rate to that seen in rat liver microsomes. Furthermore, consistent with the expressed CYP enzymes, the metabolism of KR-31543 to M1 was inhibited by anti-CYP3A2. These studies with CYP expression system and immunoinhibition indicate that CYP3A1/2 have the dominant role in the metabolism of KR-31543 in rats.

Based on the finding from this study, it is postulated that new neuroprotective agent, KR-31543, is metabolized to M1 by *N*-hydrolysis which was primarily catalyzed by CYP3A1 and CYP3A2 in rats.

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REFERENCES

De Keyser J, Sulter G, Luiten PG. Clinical trials with neuroprotective drugs in acute ischemia stroke: are we doing the right thing? *Trends Neurosci.*, 22, 535-540 (1999).

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).

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).