Arch Pharm Res Vol 26, No 10, 800-804, 2003

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

Effects of a New Neuroprotective Agent KR-31378 on Liver Cytochrome P450s in Male Sprague Dawley Rats

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(Received August 5, 2003)

The effects of KR-31378, a neuroprotective agent for ischemia-reperfusion damage, on liver microsomal cytochrome P450s (CYPs) were investigated in male Sprague Dawley rats. When rats were treated orally with KR-31378 for 7 consecutive days, CYP3A-selective erythromycin *N*-demethylase (ERDM) activity was significantly induced in a dose-dependent manner. In Western immunoblotting, CYP 3A proteins were clearly induced by treatment with KR-31378. Within 24 h after treatment with 80 mg/kg of KR-31378, ERDM activity was induced in liver microsomes in accompanied by induction of the level of CYP 3A proteins. The present results suggest that KR-31378 might modulate the expression of CYP 3A enzymes in humans.

Key words: Induction, Cytochrome P450, KR-31378, Rat, *In vivo*

INTRODUCTION

KR-31378 [(2*S*,3*S*,4*R*)-*N*-(6-amino-3,4-dihydro-2-dimethoxymethyl-3-hydroxy-2-methyl-2*H*-benzopyranyl)-*N'*-benzyl-*N''*-cyanoguanidine] (Fig. 1) was synthesized for the first time as a new therapeutic agent for neuronal diseases (Yoo *et al.*, 2001). The authors found that KR-31378 possesses both anti-oxidant and potassium channel modulating activities (Yoo *et al.*, 2001). Moreover, KR-31378 not only has been shown to protect cultured rat cortex neurons against iron-induced oxidative injury *in vitro*, but also has been shown to cause a significant reduction of infarct volume at 24 h following occlusion in a rat model of transient cerebral-ischemia (Hong *et al.*, 2002; Kim *et al.*, 2002). Currently, KR-31378 is under preclinical studies as a new neuroprotective agent for ischemia and reperfusion damage. Although the preclinical studies are still under way, KR-31378 appeared to be relatively non-toxic that the oral LD_{50} value of KR-31378 in rats was greater than 2000 mg/kg (unpublished data). A pharmacokinetic study in rats has shown that the absorption of KR-31378 from the gastrointestinal tract was almost complete, and that the first-pass effect would not be considerable following an oral administration (Kim *et al.*, 2000). *N*-acetyl-KR-31378 has been identified in the plasma as a metabolite following an intravenous or an oral administration of KR-31378 to rats (unpublished data). Taken together, KR-31378 would be a good candidate for neuronal diseases with a relatively low toxicity.

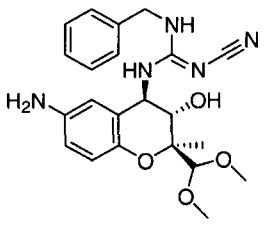

Fig. 1. Structure of KR-31378

In the present studies, *in vivo* effects of KR-31378 on liver microsomal activities of CYP enzymes were investigated in male Sprague Dawley rats. Because the information on the *in vivo* effect of KR-31378 on CYP enzymes was absent, the dose response and time course studies were performed. Liver microsomes isolated from KR-31378-treated rats were used to determine CYP-associated

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monooxygenase activities and individual CYP protein levels.

MATERIALS AND METHODS

Animals

Specific pathogen-free male Sprague Dawley rats were obtained from Biogenomics (Seoul, Korea). The animals at 5 weeks of age were acclimated for at least 1 week prior to the experiments. Upon arrival, the rats were randomized and housed four per cage. All animals were maintained on gamma-irradiated Jeil Lab Chow (Taejon, Korea) and tap water *ad libitum*. The animal quarters were strictly maintained at 23±3°C and 40-60% relative humidity. A 12 h light/dark cycle was used with an intensity of 150-300 Lux.

Materials

KR-31378 (purity, >99.0%) was synthesized and provided by Dongbu Hannong Co. (Taejeon, Korea). Ethoxyresorufin, pentoxyresorufin, erythromycin, chlorzoxazone, resorufin, dicumarol, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADPH and nitrocellulose filters were purchased from Sigma Chemical Company (St. Louis, MO, USA). The primary antibodies to individual CYP proteins were purchased from Easybio System (Seoul, Korea). Alkaline phosphatase-conjugated goat anti-rabbit IgG antibody, alkaline phosphatase substrate kit and a kit for protein determination were purchased from Bio-Rad Laboratory (Richmond, CA, USA). All other chemicals used were of reagent grade commercially available.

Animal treatment and microsome preparation

Animals were treated orally with KR-31378 in 0.5% CMC at 5, 20 and 80 mg/kg/5 mL for 7 consecutive days in the dose response studies. 0.5% CMC at 5 mL/kg was given for vehicle control. Livers were isolated 24 h after the last dosing. For the time course studies, 80 mg/kg/5 mL of KR-31378 in 0.5% CMC was administered orally for 1, 3 or 5 days. The microsomes were prepared from individual livers, as described elsewhere (Kim *et al.*, 1995), and were resuspended in 0.1 M potassium phosphate buffer pH 7.4, containing 20% glycerol and 0.1 mM EDTA. Aliquots of the liver microsomes were stored at -70°C until use. The content of microsomal protein was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Monooxygenase assays

Ethoxyresorufin *O*-deethylase (EROD) activity was determined spectrofluorometrically as described by Blank *et al.* (1987) with a slight modification. The reaction mixture (2 mL) consisted of 0.1 M potassium phosphate buffer, pH 7.4, containing 2 mg/mL of bovine serum albumin, 10 μM dicumarol, 5 mM glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase, 5 μM NADPH and 2.5 μM 7-ethoxyresorufin. The formation of resorufin was monitored fluorometrically at an excitation maximum of 550 nm and an emission maximum of 585 nm. Pentoxyresorufin *O*-depentylase (PROD) activity was determined spectrofluorometrically by measuring the amount of resorufin produced from 7-pentoxyresorufin (Lubet *et al.*, 1985). The assay procedure was exactly the same as the assay of EROD, except that the substrate concentration was 2.0 μM. The activities of chlorzoxazone hydroxylase were determined by measuring the amounts of hydroxychlorzoxazone formed, in an HPLC system. For the chlorzoxazone hydroxylase assay, the method of Guengerich and Kim (1991) was slightly modified. The reaction mixture (1 mL) contained 100 μM chlorzoxazone, 5 mM glucose 6-phosphate, 1.0 mM $NADP^+$ and 1 unit of glucose 6-phosphate dehydrogenase in 50 mM potassium phosphate buffer, pH 7.4. After the reaction termination by phosphoric acid, the product was extracted with dichloromethane and methanol. Erythromycin *N*-demethylase (ERDM) activity was determined by measuring the amount of formaldehyde formed, as described previously (Nash, 1953; Jeong *et al.*, 1998).

Western immunoblotting analyses

Microsomal proteins (10 μg/well) were resolved on 10% SDS-PAGE and were transferred to nitrocellulose filters. The filters were incubated with 2.5% non-fat dry milk for 30 min to block the nonspecific binding, and then were incubated with rabbit polyclonal antibodies against either rat CYPs 1A1/2, 2B1/2, 2E1 or 3A1/2, followed by an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody. For immunoblotting, the nitrocellulose filters were developed with a mixture of 5-bromo-4-chloro-3-indolylphosphate, nitroblue tetrazolium and 0.1 M Tris buffer (1:1:10) under an instruction provided by the manufacturer.

Statistics

The monooxygenase activity was expressed as a mean ± S.E. and a Dunnetts t-test was used to compare the significance of the data obtained. Significant values were taken as $*p<0.05$.

RESULTS

In Table I, effects of KR-31378 on liver microsomal activities of CYP-associated monooxygenases were shown in male Sprague Dawley rats. The rats were treated orally with 5, 20 and 80 mg/kg of KR-31378 for 7 consecutive days. The ERDM activity was significantly induced by the treatment with KR-31378. The activities of PROD, EROD

Table I. Effects of KR-31378 on CYP-associated monooxygenase activities in liver microsomes of male Sprague Dawley rats

Enzyme	Activity (pmole/min per mg protein)			
	Vehicle	5 mg/kg	20 mg/kg	80 mg/kg
Ethoxyresorufin *O*-deethylase (CYP 1A1/2)	1477 ± 167	1545 ± 264	1161 ± 276	1330 ± 155
Pentoxyresorufin *O*-dealkylase (CYP 2B1/2)	399 ± 100	493 ± 72	489 ± 12	538 ± 72
Erythromycin *N*-demethylase (CYP 3A1/2)	160 ± 13	230 ± 14	210 ± 14	440 ± 33*
Chlorzoxazone 4-hydroxylase (CYP 2E1)	471 ± 36	418 ± 22	413 ± 40	611 ± 56

Rats were treated orally with 5, 20 and 80 mg/kg of KR-31378 for 7 consecutive days. Twenty four hr after the last treatment, th e livers were removed to prepare the microsomes. Each value represents the mean activity ±S.E. of four animals. *Significantly different from the vehicle control at $p<0.05$.

and chlorzoxazone 4-hydroxylase were not changed significantly by KR-31378. In Fig. 2, the results of Western immunoblotting analyses are shown. Liver microsomes isolated from KR-31378-treated rats were subjected to the SDS-PAGE, followed by the incubation of blotted filters with polyclonal antibodies raised against rat CYP 1A1/2, 2B1/2, 2E1 and 3A1/2, respectively. CYP 3A1/2 proteins were substantially induced in rat liver microsomes prepared from rats treated with 80 mg/kg of KR-31378 for 7 consecutive days. The results were consistent with the induction of CYP 3A-selective ERDM activities. Meanwhile, CYP 2B1/2 proteins were also induced in the present studies without an induction of PROD activities. The levels of CYP 1A1/2 and 2E1 were not changed by KR-31378 (data not shown).

In Table II, the time course effects of KR-31378 on liver microsomal activities of CYP-associated monooxygenases were investigated within 5 days. ERDM activities were significantly induced from 1 day after the treatment with 80 mg/kg of KR-31378. The PROD activities were slightly induced without significance. The time course effects of KR-31378 on CYP 3A and 2B expression are shown in Fig. 3A and 3B, respectively. CYP 3A proteins were induced in the rat liver microsomes from 1 day after the KR-31378 treatment and reached a maximum induction at 3 days after the treatment. CYP 2B proteins were also induced from 1 day after the KR-31378 treatment and reached a maximum induction at 3 days after the treatment. The levels of CYP 1A1/2 and 2E1 were not changed by KR-31378 (data not shown).

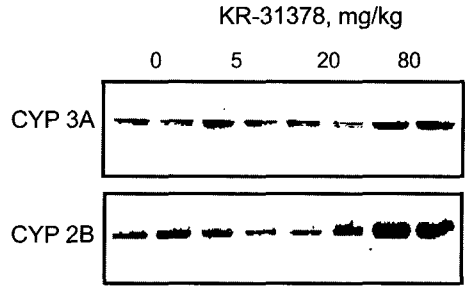

Fig. 2. Induction of CYP 3A and 2B by KR-31378 in liver microsomes of male Sprague Dawley rats. The rats were administered orally with KR-31378 in 0.5% CMC for 7 consecutive days. The microsomal proteins (10 μg/well) were resolved on a 10% SDS-PAGE and transferred onto a nitrocellulose filter for the Western immunoblotting. The filter was incubated with the primary antibody against either CYP 3A or CYP 2B proteins, followed by an incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies. Then the filter was developed using the 1:1:10 mixture of 5-bromo-4-chloro-3-indolyl-phosphate, nitroblue tetrazolium and 0.1 M Tris buffer. The results shown were from the pooled microsomes in duplicates.

DISCUSSION

Interaction of CYP enzymes with certain chemicals may cause either toxicant activation or detoxication, drug tolerance and severe drug-drug interactions in body (Wrighton

Table II. Effects of KR-31378 on CYP-associated monooxygenase activities in liver microsomes of male Sprague Dawley rats. A time-course study

Enzyme	Activity (pmole/min per mg protein)			
	Vehicle	1 day	3 days	5 days
Ethoxyresorufin *O*-deethylase	1037 ± 160	1100 ± 47	1283 ± 80	1345 ± 165
Pentoxyresorufin *O*-dealkylase	863 ± 80	910 ± 70	1040 ± 70	935 ± 102
Erythromycin *N*-demethylase	240 ± 19	469 ± 11*	444 ± 47*	456 ± 45*
Chlorzoxazone 4-hydroxylase	129 ± 11	174 ± 29	192 ± 9	192 ± 17

Rats were treated orally with 80 mg/kg of KR-31378 for 1, 3 or 5 days. At the designated time, the livers were removed to prepar e the microsomes. Each value represents the mean activity ±S.E. of four animals. *Significantly different from the vehicle control at $p<0.05$.

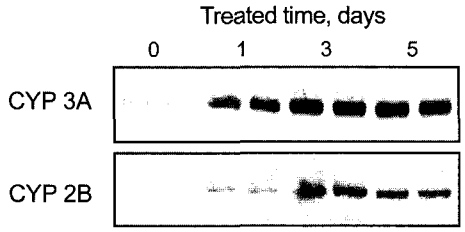

Fig. 3 Induction of CYP 3A and 2B by 80 mg/kg of KR-31378 in liver microsomes of male Sprague Dawley rats. A time-course study. Rats were administered orally with 80 mg/kg of KR-31378 in 0.5% CMC for 1, 3 or 5 days. At the designated time, the livers were removed to prepare the microsomes. The microsomal proteins (10 μg/well) were resolved on a 10% SDS-PAGE and transferred onto a nitrocellulose filter for the Western immunoblotting. The filter was incubated with the primary antibody against either CYP 3A or 2B proteins, followed by an incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG. The results shown were from the pooled microsomes in duplicates.

and Stevens, 1992). For these reasons, the studies on the expression of CYP enzymes by new chemical entities are essentially required in development processes not just for new drugs, but also for new pesticides. Particularly, drugs for long-term administration should be evaluated whether or not the drugs have potential to modulate CYP expression. In the present studies, the *in vivo* effects of KR-31378 on CYP expression were investigated in male Sprague Dawley rats.

We found that CYP 3A1/2-selective ERDM activity was significantly induced by the treatment of male Sprague-Dawley rats with 80 mg/kg of KR-31378 for 7 consecutive days (Table I). In the Western immunoblotting analyses, the levels of CYP 3A1/2 protein were increased from 1 day after the treatment with KR-31378 and reached a maximum induction at 3 days after the treatment (Fig. 2). However, the ERDM activity was fully induced from 1 day after the treatment. Except this discrepancy, our present results indicated that the induction of ERDM activity by KR-31378 might result from the induction of CYP 3A1/2 proteins.

Interestingly, the treatment with KR-31378 at the dose of 80 mg/kg elevated the CYP 2B1/2 protein levels from 1 day after the treatment, but not the enzyme activity of PROD, a CYP 2B1/2-selective enzyme (Table I and Fig. 2). Although the exact mechanism should be investigated further, the possible mechanism for the discrepancy between protein levels and enzyme activity would be that KR-31378 ligands to active sites of CYP proteins, preventing the enzymes to be fully functioned. For example, we have recently found that KR-31378 inhibited baculovirus/insect cell-expressed human CYP 3A4, CYP 2C9 and CYP 2D6 activities *in vitro* with IC_{50} values of 2.9 μM, 73.7 μM and 17.4 μM, respectively (our unpublished data). Although the inhibitory effects of KR-31378 on CYP 2B1/2 activities were not determined, these results indicated that KR-31378 might be able to interact with certain CYP proteins with different affinities. In this regard, CYP 3A-selective ERDM activity would also be affected by KR-31378 in the present studies. If our hypothesis is correct, the binding affinity of KR-31378 with CYP 2B1/2 should be stronger than that with CYP 3A4. The other possible mechanism would be the inhibition patterns of KR-31378. If KR-31378 specifically inhibits CYP 2B1/2 as a mechanism-based inactivator, the expressed enzymes may not be able to function actively. Currently, an attempt to understand the mechanism of discrepancy between enzyme activities and protein levels of CYP 2B1/2 caused by KR-31378 are being made.

The induction of CYP enzymes may cause drug tolerance and/or drug-drug interactions. Particularly, CYP 3A enzymes are very important in these matters, because CYP 3A is a subfamily of the most abundant drug-metabolizing CYP enzymes in human, and because large portions of drugs currently available are predominantly metabolized by CYP 3A enzymes. Fortunately, however, the induction of CYP 3A proteins by KR-31378 was only clear at the highest dose that is much higher than the expected clinical dose. Likewise, the induction of CYP 2B1/2 by KR-31378 was clear only at the highest dose. In addition, CYP 2B enzymes are neither expressed much in human nor involved in drug metabolism significantly. Although the long-term effects of KR-31378 on CYP enzyme expression need to be investigated in the future, it was concluded that KR-31378 might be a modest CYP inducer in rats.

ACKNOWLEDGEMENT

This work was partially supported by the Project from the Center for Biological Modulators (CBM-02-B-8), Ministry of Science and Technology, Republic of Korea.

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