Stereoselective Pharmacokinetics of RS-8359, A Selective and Reversible Inhibitor of A-Type Monoamine Oxidase, in Rats, Mice, Dogs, and Monkeys

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RS-8359, ($\pm$)-4-(4-cyanoanilino)-5,6-dihydro-7-hydroxy-7H-cyclopenta[d]-pyrimidine, inhibits, selectively and reversibly, A-type monoamine oxidase (MAO-A). In order to clarify the stereoselective metabolism of this drug, plasma concentrations of the [R] and [S]-enantiomers of RS-8359 were determined by chiral column HPLC after oral administration of each enantiomer to rats, mice, dogs, and monkeys. After administration of the [R]-enantiomer, high levels were retained in all animal species studied. On the other hand, when the [S]-enantiomer was administered, plasma concentrations decreased rapidly in rats and mice, and extremely rapidly in dogs, while in monkeys, only a trace amount was detected immediately after dosing. Thus, it was found, as a common phenomenon in rats, mice, dogs, and monkeys, that plasma concentrations of the [S]-enantiomer were markedly lower than those of the [R]-enantiomer. Secondly, the [R]-enantiomer was observed in plasma after administration of the [S]-enantiomer, and the [S] to [R] chiral inversion rate was estimated from $AUC([R])$ after [S]/ $AUC([R])$ after [R]. The percentage was 45.8% in rats, 3.8% in mice, 0.8% in dogs, and 4.2% in monkeys. Further, the [S]-enantiomer was detected in plasma of SD rats dosed with the [R]-enantiomer, suggesting [R] to [S] chiral inversion in rats. These results show marked species differences in the chiral inversion of the cyclopentanol group of RS-8359. A mechanism of chiral inversion is discussed based on experiments using isolated rat hepatocytes.

Key words RS-8359; stereoselective metabolism; chiral inversion; species difference

RS-8359, ($\pm$)-4-(4-cyanoanilino)-5,6-dihydro-7-hydroxy-7H-cyclopenta[d]-pyrimidine, is a novel compound producing selective and reversible inhibition of A-type monoamine oxidase,¹⁻³⁾ and it is under development as an antidepressant. This drug has enantiomers due to the secondary hydroxy group at the 7-position of the cyclopentane ring. It is well known that a marked difference in plasma concentration between enantiomers occurs as a result of stereoselective metabolism, and a wide variety of drug-metabolizing enzymes are responsible for such stereoselective metabolism.⁴⁻⁶⁾ In particular, many papers have described the chiral inversion of pharmacologically inactive *R*(-)-enantiomers of the 2-arylpropionic acid non-steroidal anti-inflammatory drugs (NSAIDs) to their active *S*(+)-enantiomers.⁷⁻⁹⁾ In general, a detailed grasp of the metabolic profiles of each enantiomer is needed to understand the pharmacological and toxicological features of enantiomeric drugs. In this study, plasma concentrations of the enantiomers after oral administration of each enantiomer of RS-8359 to rats, mice, dogs, and monkeys were determined. The chiral inversion of the secondary alcohol of RS-8359 and species differences discovered will be discussed.

MATERIALS AND METHODS

Chemicals The [R]- and [S]-enantiomers of RS-8359, authentic samples of metabolites (*i.e.*, 5,7-*trans*-diol, 5,7-*cis*-diol, 2-keto and 7-keto derivatives), and TUK-8360 (an internal standard) were all provided by Ube Kosan Co., Ltd. (Yamaguchi, Japan). The optical purity of the [R]- and [S]-enantiomers was estimated by chiral column HPLC under conditions (b) described below and was 99.0% for both enan-

tiomers. [S]-RS-8359 labeled with ¹⁴C was optically separated at Sumika Chemical Analysis Service, Ltd. (Osaka, Japan) from [¹⁴C]RS-8359, which was synthesized at Daiichi Pure Chemicals Co., Ltd. (Ibaraki, Japan). The specific radioactivity was 2.04 MBq/mg. The radiochemical purity was over 98.5% determined by TLC using thin-layer, Kieselgel 60 F254 plates (Merck, thickness 0.25 mm) and developing solvents, (A) ethanol-diethylether (4:6), (B) chloroform-methanol-aq. ammonia (4:1:0.1). The optical purity of [¹⁴C][S]-RS-8359 was 99.5%. The chemical structures of these compounds are shown in Fig. 1.

Collagenase was obtained from Boehringer Mannheim GmbH (Germany). *N*-[2-Hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) was from Sigma Chemicals Co. (St. Louis, MO). All other reagents and solvents were of reagent grade.

Administration to Animals and Blood Sampling Wistar-Imamichi (WI) rats (body weight, 230–250 g; Imamichi Institute for Animal Reproduction, Saitama, Japan), Sprague-Dawley (SD) rats (body weight, 230–250 g; Japan Charles River, Kanagawa, Japan), ddY mice (body weight, 30–35 g; Japan SLC, Shizuoka, Japan), beagle dogs (body weight, 9.5–10.7 kg) and cynomolgus monkeys (body weight, 3.0–3.7 kg) were all male and were used after overnight fasting. The enantiomers of RS-8359 were dissolved in acid solution (4.0 g NaCl and 4.8 ml 10% HCl were dissolved in distilled water, to make 1000 ml) or suspended in 0.5% carboxymethyl cellulose (CMC) solution, and administered orally to rats (5 mg/kg), mice (10 mg/kg), dogs (30 mg/kg), and monkeys (30 mg/kg). The enantiomers were administered in crossover fashion to the same dogs and monkeys with an interval of one week. At fixed times, blood was collected in heparinized

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Fig. 1. Chemical Structures of RS-8359 and Related Compounds

syringes from the hearts of mice under chloroform anesthesia, and from the veins of the other animals. The blood was immediately centrifuged at 10000 rpm for 3 min at 4°C to obtain plasma which was stored at -20°C until analysis.

An equimolar mixture of [¹⁴C][S]-RS-8359 and [R]-RS-8359, designated a radioisotopic pseudo-racemate in this study, was administered at a dose of 10 mg/kg to WI rats. Blood sampling was carried out as described above.

Preparation of Hepatocytes Hepatocytes were prepared from livers of male WI and SD rats according to the methods described by Moldeus *et al.*¹⁰ Incubations (2×10⁶ cells/ml) were carried out in Krebs-Henseleit buffer containing HEPES (12.6 mM) in rotating 50-ml round-bottomed flasks at 37°C for 120 min, during continuous gassing with 95% O₂-5% CO₂. The concentration of substrates was 5 μg/ml. At fixed times, aliquots (1.0 ml) of the reaction mixtures were taken and centrifuged at 10000 rpm for 2 min. The supernatant obtained was analyzed by HPLC as described below.

HPLC Determination of Metabolites To plasma (0.2 ml) were added TUK-8360 (1.0 μg/0.1 ml methanol), as an internal standard, and two volumes of methanol. After separation of the precipitates by centrifugation, the supernatant was evaporated to dryness under a stream of nitrogen. The concentration of RS-8359 as a racemate was determined by HPLC (a), and simultaneously the effluent corresponding to the peak of RS-8359 was collected using a fraction collector. To the effluent was added 0.1 M carbonate buffer (pH 9, 1.0 ml), followed by extraction with ethyl acetate (2.0 ml). After concentration of the organic layer to dryness, the enantiomeric ratios of RS-8359 were determined by chiral column HPLC (b). The plasma concentration of each enantiomer was calculated by multiplying the concentrations of racemic RS-8359 by the ratio of each enantiomer. For the experiments using the radioisotopic pseudo-racemate, the [R]-enantiomer was fractionated on the chiral column HPLC, and the radioactivity was measured. The radioactivity, derived

from the [S]-enantiomer *via* chiral inversion, was converted to a weight by the specific radioactivity. Measurement of radioactivity was carried out by liquid scintillation counting, using a Packard 460C.

(a) Octadecyl silica (ODS) column HPLC: column; YMC Pack ODS A-312 (6.0 mm i.d.×150 mm, YMC Co., Ltd., Kyoto, Japan), effluent; acetonitrile-0.5% ammonium acetate (25:75), flow rate; 1.0 ml/min, detection: UV 315 nm.

(b) Chiral column HPLC: column; Chiralpak WE (4.6 mm i.d.×250 mm, Daicel Chemical Industries Ltd., Tokyo, Japan), effluent; acetonitrile-0.25 mM copper sulfate (80:10), flow rate; 0.8 ml/min, detection; UV 315 nm.

Pharmacokinetic Parameters After oral administration of RS-8359 enantiomers to rats, mice, dogs, and monkeys, the *C*_{max} (maximum plasma concentration), and *T*_{max} (time to reach *C*_{max}) were obtained directly from the observed data. The *AUC* (area under the plasma concentration-time curve) was calculated from the trapezoidal rule.

Calculation of Chiral Inversion Rate The extent of gastrointestinal absorption of both RS-8359 enantiomers was found to be approximately quantitative from the experiments using [¹⁴C]-labeled compounds (data not shown). The rate of chiral inversion was calculated from the following equations according to Lee *et al.*¹¹⁾

$$\begin{aligned}
 &[S] \text{ to } [R] \text{ chiral inversion (\%)} \\
 &= AUC([R] \text{ after } [S]) / AUC([R] \text{ after } [R]) \times 100 \\
 &[R] \text{ to } [S] \text{ chiral inversion (\%)} \\
 &= AUC([S] \text{ after } [R]) / AUC([S] \text{ after } [S]) \times 100
 \end{aligned}$$

where, for example, *AUC*([R] after [S]) is the *AUC* of the [R]-enantiomer after administration of the [S]-enantiomer.

RESULTS

HPLC Separation The plasma concentration of each enantiomer of RS-8359 was determined by a combination of ODS and chiral column HPLC. Typical chromatograms for the respective HPLC of rat plasma, as an example, are shown in Fig. 2. There was no interference from biological components in the determination of RS-8359 by both HPLC methods.

Plasma Concentrations in Rats The plasma concentrations after oral administration of RS-8359 enantiomers to SD rats, at a dose of 5 mg/kg, are given in Fig. 3. After administration of the [R]-enantiomer, the *T*_{max}, *C*_{max}, and *AUC* for the [R]-enantiomer were 2 h, 3.05 μg/ml, and 16.9 μg·h/ml, respectively (Table 1). Unlike WI rats,¹²⁾ after administration of the [R]-enantiomer to SD rats, a small amount of the [S]-enantiomer was detected together with a high concentration of the [R]-enantiomer (*AUC*[S], 0.92 μg·h/ml). When the [S]-enantiomer was administered, almost equal amounts of the [S]- and [R]-enantiomers were found in plasma (7.86 μg·h/ml for the [S]-enantiomer; 7.74 μg·h/ml for the [R]-enantiomer). Accordingly, the [S] to [R] chiral inversion in SD rats was 45.8%, which was much higher than the 15.8% in WI rats.¹²⁾

The results of the isotopic pseudo-racemate experiments in WI rats are given in Fig. 4. The *AUC* of the [R]-enantiomer was 2.8 times greater than that of the [S]-enantiomer. The [R]-enantiomer consisted of non-labeled and ¹⁴C-labeled components, the latter being produced from the [¹⁴C][S]-

enantiomer by chiral inversion. The AUC s of the cold and radioactive components were 26.07 and 3.82 $\mu\text{g}\cdot\text{h}/\text{ml}$, respectively. As a result, 12.8% of the $[R]$ -enantiomer present in plasma after administration of the racemate was shown to be derived from the $[S]$ -enantiomer.

Plasma Concentrations in Mice

Fig. 2. Typical ODS (a) and Chiral (b) Column HPLC Chromatograms of Rat Plasma after Oral Administration of RS-8359 Enantiomers

plasma concentration–time curves of each enantiomer after administration of the RS-8359 enantiomers to ddY mice. After administration of the $[R]$ -enantiomer, the $T_{\max}$, $C_{\max}$ and AUC of the $[R]$ -enantiomer were 1 h, 6.82 $\mu\text{g}/\text{ml}$, and 34.3 $\mu\text{g}\cdot\text{h}/\text{ml}$, respectively (Table 1), and a trace amount of the $[S]$ -enantiomer was detected. When the $[S]$ -enantiomer was given, a rapid increase followed by a rapid decrease in the plasma concentration of the $[S]$ -enantiomer was observed ($T_{\max}$, 0.5 h; $C_{\max}$, 4.67 $\mu\text{g}/\text{ml}$; AUC , 5.83 $\mu\text{g}\cdot\text{h}/\text{ml}$), and a substantial amount of $[R]$ -enantiomer was measured (AUC , 1.31 $\mu\text{g}\cdot\text{h}/\text{ml}$). The chiral inversion from $[S]$ to $[R]$ occurred at a rate of 3.8% in ddY mice.

Fig. 3. Plasma Concentrations of Each Enantiomer after Oral Administration of the $[R]$ -Enantiomer (a) and $[S]$ -Enantiomer (b) of RS-8359 to SD Rats (5 mg/kg, $n=4$)

○, $[S]$ -enantiomer; ●, $[R]$ -enantiomer.

Table 1. Pharmacokinetic Parameters of RS-8359 Enantiomers after Oral Administration of Each Enantiomer to Several Animal Species

Species	Dosed isomer	Dose (mg/kg)	Analysed isomer	$T_{\max}$ (h)	$C_{\max}$ ($\mu\text{g}/\text{ml}$)	$AUC(0-24)$ ($\mu\text{g}\cdot\text{h}/\text{ml}$)	S to R inversion (%) ^{a)}
Rat	R	5	$[R]$	2	3.05	16.9 ^{b)}	45.8
			$[S]$	4	0.17	0.92	
	S	5	$[R]$	2	1.72	7.74	
			$[S]$	0.5	3.97	7.86	
Mouse	R	10	$[R]$	1	6.82	34.3	3.8
			$[S]$	2	0.06	0.23	
	S	10	$[R]$	0.5	0.37	1.31	
			$[S]$	0.5	4.67	5.83	
Dog	R	30	$[R]$	1–4	13.88	170.1	0.8
			$[S]$	0.25	0.52	0.24	
	S	30	$[R]$	1	0.16	1.40	
			$[S]$	0.25	6.25	8.74	
Monkey	R	30	$[R]$	3	3.72	51.9	4.2
			$[S]$	—	—	0.15	
	S	30	$[R]$	0.25	0.25	2.17	
			$[S]$	0.25	0.02	0.01	

a) $AUC(R \text{ after } S)/AUC(R \text{ after } R) \times 100$ (%). b) $AUC(0-10)$.

Fig. 4. Plasma Concentrations of Each Enantiomer after Oral Administration of Radiolabeled Pseudoracemic RS-8359 to WI Rats (10 mg/kg, $n=3$)

○, [S]-enantiomer; ●, [R]-enantiomer; ■, [R]-enantiomer derived from [S]-enantiomer.

Fig. 5. Plasma Concentrations of Each Enantiomer after Oral Administration of the [R]-Enantiomer (a) and [S]-Enantiomer (b) of RS-8359 to Mice (10 mg/kg, $n=4$)

○, [S]-enantiomer; ●, [R]-enantiomer.

Plasma Concentrations in Dogs The plasma concentrations of each enantiomer of RS-8359 in dogs are shown in Fig. 6. After administration of the [R]-enantiomer, this enantiomer had already reached a submaximal concentration of 12.13 $\mu\text{g/ml}$ at 15 min, suggesting rapid gastrointestinal absorption. Then, the concentration reached the C_{max} (13.88 $\mu\text{g/ml}$) at 1 h; near- C_{max} levels were maintained until 4 h, and then the concentration decreased slowly. The AUC was 170.1 $\mu\text{g} \cdot \text{h/ml}$. On the other hand, after administration of the [S]-enantiomer, the peak plasma concentration (6.25 $\mu\text{g/ml}$) was reached at 15 min and then decreased quickly. The concentration at 2 h was 0.35 $\mu\text{g/ml}$, and the AUC was only 8.74 $\mu\text{g} \cdot \text{h/ml}$. Following administration of the [S]-enantiomer, a small but significant amount of [R]-en-

Fig. 6. Plasma Concentrations of Each Enantiomer after Oral Administration of the [R]-Enantiomer (a) and [S]-Enantiomer (b) of RS-8359 to Dogs (30 mg/kg, $n=5$)

○, [S]-enantiomer; ●, [R]-enantiomer.

Fig. 7. Plasma Concentrations of Each Enantiomer after Oral Administration of the [R]-Enantiomer (a) and [S]-Enantiomer (b) of RS-8359 to Monkeys (30 mg/kg, $n=3$)

○, [S]-enantiomer; ●, [R]-enantiomer.

tiomer was detected in plasma. The AUC was 1.40 $\mu\text{g} \cdot \text{h/ml}$. Thus, the rate of [S] to [R] chiral inversion was found to be very low (0.8%) in dogs.

Fig. 8. Metabolism of RS-8359 Enantiomers and Intermediate 7-Keto Form of RS-8359 by Rat Hepatocytes (5 $\mu\text{g}/\text{ml}$, $n=3$)

◇, [S]-enantiomer; △, chiral inverted RS-8359; ●, [R]-enantiomer; ▲, *trans*-diol; ■, 2-keto; ○, *cis*-diol; □, 7-keto.

Plasma Concentrations in Monkeys The plasma concentration–time curves of RS-8359 enantiomers in monkeys are given in Fig. 7. After administration of the [R]-enantiomer, the T_{max} , C_{max} , and AUC of the [R]-enantiomer were 3 h, 3.72 $\mu\text{g}/\text{ml}$, and 51.9 $\mu\text{g} \cdot \text{h}/\text{ml}$, respectively. However, despite administration of the [S]-enantiomer, the concentration of this enantiomer only reached trace concentrations, and a small amount of the [R]-enantiomer, produced by chiral inversion, was observed for a long period. The T_{max} and C_{max} of the [R]-enantiomer could not be clearly seen. The AUC was 2.17 $\mu\text{g} \cdot \text{h}/\text{ml}$, which shows the [S] to [R] chiral inversion to be 4.2%.

Metabolism by Isolated Rat Hepatocytes Figure 8 shows the results of metabolic studies of RS-8359 enantiomers and the 7-keto derivative using hepatocytes of SD- and WI-strain rats. The [S]-enantiomer was mainly metabolized to the 7-keto form by SD rat hepatocytes and its rate of production was 26.4% after an incubation period of 120 min. Next, the [R]-enantiomer, with a reverse configuration, was detected in considerably greater amounts (production rate: 16.3%), but the other metabolites were very minor. When the [R]-enantiomer was used as substrate, the production of the

7-keto form increased to 43.4%, although the production of the inverse [S]-enantiomer decreased to 4.2%. On the other hand, the extent of metabolism of RS-8359 enantiomers by WI rat hepatocytes was lower compared with that by the SD strain. The main metabolite of the [S]-enantiomer was the 2-keto derivative (6.5%), and formation of the 7-keto form (2.6%) and the reverse [R]-enantiomer (1.4%) was very low. On incubation of the [R]-enantiomer with WI rat hepatocytes, much more of the 7-keto form was produced (15.5%), but the reverse [S]-enantiomer and other metabolites were not observed at all. These experimental results using hepatocytes indicated that [S] to [R] chiral inversion is more pronounced in SD rats than in WI rats, and also that [R] to [S] chiral inversion also occurred in the SD strain. The *in vitro* findings were in good agreement with the *in vivo* results described above.

The metabolism of the 7-keto derivative was investigated using rat hepatocytes. The compound was rapidly and stereospecifically reduced to the alcohol corresponding having the [R] configuration, namely [R]-RS-8359. The production rate was 83.9% and 64.5% in SD- and WI-rat hepatocytes, respectively.

Fig. 9. Proposed Mechanism for Chiral Inversion from the [S]- to [R]-Enantiomer of RS-8359

DISCUSSION

RS-8359, which has one asymmetric carbon, is a selective and reversible inhibitor of A-type monoamine oxidase.¹⁻³⁾ In order to clarify the stereoselective metabolism of this compound, the plasma concentrations were determined after oral administration of each enantiomer to rats, mice, dogs, and monkeys. After administration of the [R]-enantiomer, high plasma concentrations were seen in every animal species studied. When the [S]-enantiomer was administered to rats (Fig. 3) and mice (Fig. 5), the plasma concentrations increased at a similar rate to that after administration of the [R]-enantiomer, suggesting almost identical gastrointestinal absorption of the two enantiomers. However, the concentration of the [S]-enantiomer reached the $C_{\max}$ more rapidly and decreased more quickly than that of the [R]-enantiomer. In dogs, the $C_{\max}$ of the [S]-enantiomer had already been reached at 15 min, and then the concentration fell quickly (Fig. 6). Following administration of the [S]-enantiomer to monkeys, the [S]-enantiomer was only detected in trace amounts (Fig. 7). Thus, significant stereoselectivity was found as a common phenomenon in rats, mice, dogs, and monkeys, in that the plasma concentration of the [R]-enantiomer was markedly higher than that of the [S]-enantiomer. The ratios of $AUC[R]/AUC[S]$ were 2.5 and 2.1 in WI¹²⁾ and SD rats, respectively, 5.9 in mice, and 19.6 in dogs. It was impossible to calculate the ratio in monkeys because of the trace amounts of the [S]-enantiomer. The size of these ratios was approximately consistent with the results obtained after administration of racemic RS-8359: 2.6 in WI rats, 3.8 in mice, 31 in dogs, and 238 in monkeys.¹³⁾ From the detailed *in vivo* and *in vitro* experiments relating to this phenomenon, the mechanism proposed is that the [S]-enantiomer is much more rapidly metabolized and excreted than the [R]-enantiomer.¹⁴⁾

The rate of [S] to [R] chiral inversion was estimated from the ratio of the $AUC([R]$ after [S]) and $AUC([R]$ after [R]), and also the rate of [R] to [S] chiral inversion was estimated from the ratio of the $AUC([S]$ after [R]) and the $AUC([S]$ after [S]). The [S] to [R] chiral inversion rate in rats was 15.8% for the WI strain,¹²⁾ whereas a higher rate (45.8%) was found for the SD strain. Further, [R] to [S] reverse chiral inversion was also observed at a rate of 11.7% in SD rats. Similar results were obtained in the *in vitro* experiments using rat hepatocytes. In addition to oxidation/reduction by dehydrogenase as a mechanism for chiral inversion of secondary alcohols,^{15,16)} a conjugation reaction has recently been reported for stiripentol.¹⁷⁾ In the case of RS-8359, an oxidation/reduction mechanism, as shown in Fig. 9, was suggested from the finding that the main metabolite produced by rat hepatocytes was the 7-keto form. In fact, when the 7-keto form was used as an expected intermediate substrate, it was stereospecifically re-

duced to the alcohol having the [R]-configuration, supporting the above hypothesis. Also the results indicate that the direction of chiral inversion of RS-8359 may be controlled by stereospecific reduction of the ketone group. Thus, the [R]-enantiomer is not thought to be inverted to the [S]-enantiomer, because the [R]-enantiomer is oxidized to the ketone form, but can again be readily reduced to the [R]-enantiomer. The extent of chiral inversion was small in WI rats.¹²⁾ As no marked differences were observed in the reduction rate of the 7-keto derivative between WI and SD rats, the major reason for the strain differences in the chiral inversion may be due to the extent of the oxidation reaction. The experiments in WI rats, using the radiolabeled pseudoracemic RS-8359, showed that 12.8% of the [R]-enantiomer in plasma after administration of the racemate was derived from the [S]-enantiomer. The value was in fair agreement with the chiral inversion rate (15.8%) estimated after administration of each enantiomer. These results indicate the possibility of a partial contribution by [S] to the [R] chiral inversion to maintain the plasma concentration of the [R]-enantiomer when the racemate was administered to rats.

Besides rats, [S] to [R] chiral inversion occurred at a low but significant rate in mice (3.8%) and monkeys (4.2%), but was very low in dogs (0.8%). From these results, there are large species differences in the chiral inversion of this compound. In addition to dehydrogenase, the complex combination of aldehyde oxidase, glucuronosyl transferase, and cytochrome P-450 responsible for the metabolism of RS-8359¹⁴⁾ may contribute to the species differences in the chiral inversion.

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