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Mechanistic Studies on Metabolic Chiral Inversion of 4-(4-Methylphenyl)-2-methylthiomethyl-4-oxobutanoic Acid (KE-748), an Active Metabolite of the New Anti-rheumatic Agent 2-Acetylthiomethyl-4-(4-methylphenyl)-4-oxobutanoic Acid (KE-298), in Rats

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ABSTRACT. The chiral inversion properties of 4-(4-methylphenyl)-2-methylthiomethyl-4-oxobutanoic acid (KE-748), an active metabolite of 2-acetylthiomethyl-4-(4-methylphenyl)-4-oxobutanoic acid (KE-298), were compared with those of ibuprofen in rats. After administration of *R*(−)-[2α-^{2}H]KE-748, S(+)-KE-748 was present in the rat plasma, and the deuterium atoms of the S(+)-enantiomer were almost all replaced by hydrogen atoms. After administration of S(+)-[2α-^{2}H]KE-748, the deuterium content of S(+)-KE-748 in the plasma remained intact. In the *in vitro* study, using a cell-free system and rat liver homogenates, the chiral inversion of ibuprofen was apparent when both CoA and ATP were present; however, KE-748 was not inverted. In the study on isolated rat hepatocytes, the unidirectional chiral inversion from *R*(−)- to S(+)-enantiomer was observed for both ibuprofen and KE-748. When *R*(−)-ibuprofen was incubated with medium and long chain fatty acids (carbon chain length C_6 to C_{16}), using isolated hepatocytes, the chiral inversion decreased significantly. On the other hand, when *R*(−)-KE-748 was incubated with short and medium chain fatty acids (carbon chain length C_3 to C_8), chiral inversion was inhibited markedly. To induce hepatic microsomal long chain fatty acid CoA ligase, rats were treated with clofibric acid (CF rats). In both *in vitro* and *in vivo* experiments on CF rats, chiral inversion from *R*(−)- to S(+)-ibuprofen was enhanced significantly compared with that in controls, whereas the enhancement was not observed in the case of *R*(−)-KE-748. There was no influence of benzoic acid, a typical substrate on medium chain fatty acid CoA ligase in the mitochondrial matrix, on chiral inversion of *R*(−)-ibuprofen, using isolated hepatocytes. In contrast, the chiral inversion from *R*(−)- to S(+)-KE-748 was strongly inhibited in the presence of benzoic acid. These results indicate that chiral inversion of *R*(−)-KE-748 may proceed via formation of the CoA-thioester intermediate with loss of the 2α-methine proton, in a manner similar to that seem with *R*(−)-ibuprofen. However, the enzymes needed to form CoA-thioester of *R*(−)-KE-748 differ from those for *R*(−)-ibuprofen. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;2:179–187, 1997.

KEY WORDS. KE-298; anti-rheumatic drug; chiral inversion; CoA ligase; ibuprofen; rat

KE-298 [2-acetylthiomethyl-4-(4-methylphenyl)-4-oxobutanoic acid], an anti-rheumatic drug newly synthesized by the Taisho Pharmaceutical Co., Ltd., has shown potent activity in immunopharmacological studies with experimental animals [1, 2]. The phase III clinical study of KE-298 is underway in Japan. KE-298 contains a single chiral center in its chemical structure (Fig. 1). It is an equimolar mixture of S(+)- and *R*(−)-enantiomers. Unchanged KE-298 proved to be a minor component in the plasma of rats given KE-298; the major components were KE-758 and KE-748 (Fig. 1) [3]. In ongoing work, we found that these metabolites had an equal pharmacological potency to unchanged KE-298 as determined in *in vitro* tests (interleukin-1 antagonist effect). We reported that the potent in vivo pharmacological potency of S(+)-KE-298, compared with *R*(−)-KE-298, in adjuvant arthritis induced in rats might be attributable to the stereoselective pharmacokinetic properties of the metabolites [4, 5]. In addition, unidirectional metabolic chiral inversion from *R*(−)- to S(+)-enantiomers of KE-748 occurred in rats, whereas no inversion was detected for KE-298 and KE-758 [5]. Metabolic chiral inversion is an important reaction required to understand the pharmacokinetics of some optical drugs. There

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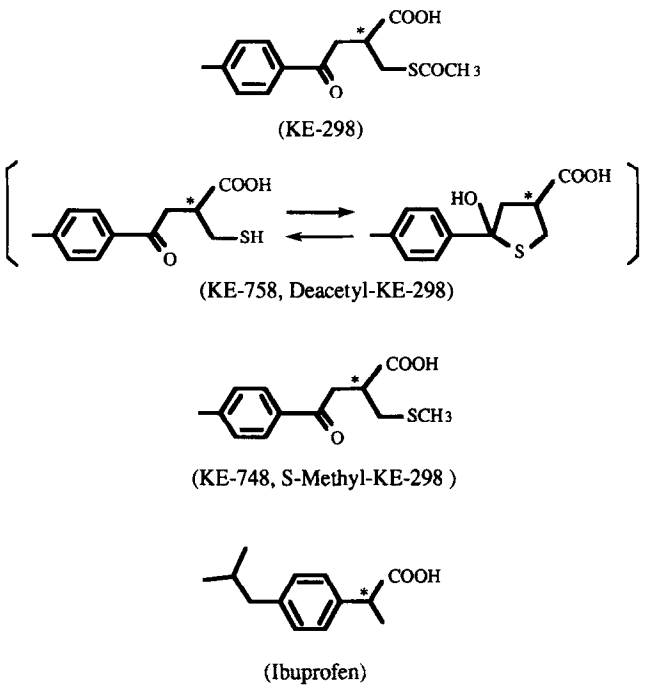


FIG. 1. Chemical structures of KE-298, KE-758, KE-748, and ibuprofen. Asterisk denotes asymmetric center.

are reports on the mechanism of chiral inversion, e.g. chiral inversion of the thalidomide analogue EM12 [6] occurred spontaneously, and reactions of tolperison [7] and latamoxef [8] were evidenced by interactions with plasma protein. The most intriguing aspect in these reports is the mechanism of chiral inversion of 2-arylpropionates such as ibuprofen [9, 10], ketoprofen [9], and fenoprofen [11–13]. The available evidence suggested that the reaction proceeded via the formation of the acyl CoA-thioester intermediate [14–18]. It was also reported that the 2α-methine proton was exchanged during the chiral inversion of ibuprofen [19–21].

To better comprehend the mechanism of chiral inversion of KE-748, we compared the chiral inversion properties of KE-748 with those of ibuprofen.

MATERIALS AND METHODS

Chemicals

Racemic KE-748 was synthesized according to Kameo *et al.* [1]. Ibuprofen was purchased from Wako Pure Chemical Industries (Tokyo, Japan). The S(+)- and R(−)-enantiomers (optical purity of over 99%) of KE-748 and ibuprofen were prepared from corresponding racemic compounds, using HPLC systems on a chiral stationary phase (chiral-HPLC), as described below. Other chemicals and solvents were obtained from standard commercial sources (Sigma Chemical Co., St. Louis, MO, U.S.A., and Wako Pure Chemical Industries).

Synthesis of 2α-Deuterium-Labeled KE-748

2α-Deuterium-labeled KE-748 ([2α-^{2}H]KE-748, Fig. 2) was synthesized, as follows: itaconic anhydride, nitroethane, and toluene were stirred for 30 min at room temperature to yield 2-methylene-4-oxobutanoic acid **(I)**. Potassium thioacetate suspended with a solution of deuterium chloride in deuterium oxide, and deuterio-thioacetic acid **(II)** were purchased. **I** was suspended in toluene, and **II** was added. The stirred mixture was heated at 60°, and a solution of triethylamine in toluene was added via a syringe at 3-min intervals over a 45-min period. The mixture was stirred for 3 hr at 60°. After cooling the product on ice, it was acidified with 3 N hydrochloride, extracted into ethyl acetate, and purified by recrystallization, which then became deuterio-KE-298 **(III)**. **III** was dissolved in 3 N potassium hydride and left to stand for 30 min at room temperature. After adding methyl iodide, the solution was stirred for 1 hr. The desired product, [2α-^{2}H]KE-748, was purified and separated as S(+)-[2α-^{2}H]KE-748 and R(−)-[2α-^{2}H]KE-748 with an optical purity of over 99% using chiral-HPLC. Deuterium incorporation of S(+)-[2α-^{2}H]KE-748 and R(−)-[2α-^{2}H]KE-748 was calculated to be 60 and 55 atom%, respectively, as determined by GC/MS analysis.

Animals and Preparations of Liver Homogenates and Hepatocytes

Male Wistar rats, weighing about 180 g and obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) were housed under standard conditions of temperature and light cycle, and had free access to food and water for more than a week before the start of the experiments. The livers of ether-anesthetized rats were flushed with saline via the portal vein and excised immediately. The livers were then homogenized in 4 vol. of buffer (50 mM Tris–HCl, pH 7.4, 0.25 M sucrose, and 50 mM $MgCl_2$), using a Potter–Elvehjem tissue grinder. Hepatocytes were isolated according to Moldeus *et al.* [22]; the livers were perfused for 15 min with 0.12% collagenase solution prior to extraction of

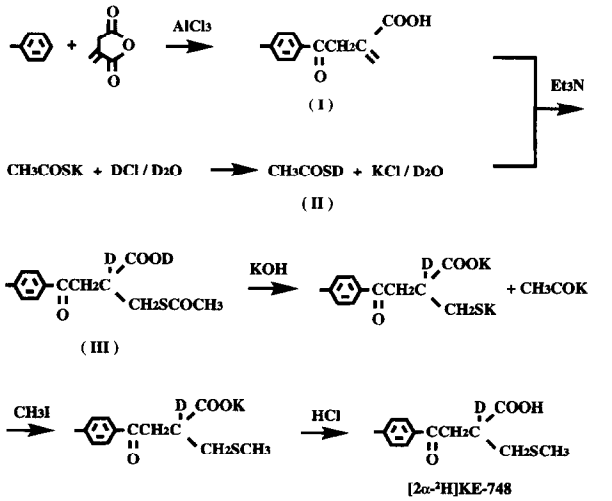


FIG. 2. Scheme depicting preparation of the 2α-deuterium-labeled KE-748. Compounds (I) to (III) are described in the text.

cells. The viability of cells in the final preparation exceeded 95%, as assessed by the exclusion of trypan blue dye.

Deuterium/Hydrogen Exchange Studies

S(+)-[2α-^{2}H]KE-748 and *R*(−)-[2α-^{2}H]KE-748 were administered orally to rats in a dose of 5 mg/kg; these compounds were suspended in 5% gum arabic solution just before administration. Whole blood samples taken from anesthetized rats were withdrawn from the inferior aorta into heparinized containers at 15, 30, 60, and 120 min after administration of the drugs. Plasma was separated by centrifugation at 2000 *g* for 10 min at 4°. KE-748 in plasma was extracted, and S(+)- and *R*(−)-KE-748 were separated, using chiral-HPLC. Prepared enantiomers were methylated, and the [^{2}H]/[^{1}H] ratio was determined by GC/MS.

In Vitro *Study Using Cell-Free Systems*

A mixture containing 2 mL of liver homogenates, 0.1 mL of ATP (final concentration 3 mM), 0.1 mL of CoA (0.4 mM) and 0.3 mL of the stock solution for either *R*(−)-ibuprofen or R(−)-KE-748 (0.2 mM), was incubated at 37° for 30 min in a shaking water bath. The *S*/*R* enantiomeric ratios of KE-748 and ibuprofen in the medium were measured by chiral-HPLC.

Influence of Various Fatty Acids

Isolated rat hepatocytes were suspended (5×10^5 cells/mL) in Hanks' buffer with 0.5% fatty acid-free BSA containing various carbon chain length (C_3 to C_{16}) fatty acids (propionic, butyric, hexanoic, octanoic, lauric and palmitic acids) (0.1 mM). The media were incubated with *R*(−)-KE-748 or *R*(−)-ibuprofen (0.025 mM) for 120 min at 37°, and then the levels of the S(+)- and *R*(−)-enantiomers for both drugs in the media were measured.

Influence of Long Chain Fatty Acid CoA Ligase Induction

Hepatic microsomal long chain fatty acid CoA ligase activity was induced, according to Knights *et al.* [23]. Briefly, rats were given orally clofibric acid (CF rat, 280 mg/kg/day) or the vehicle (vehicle rat, 5% gum arabic) for 5 days. Food and water were provided *ad lib.*

IN VIVO. *R*(−)-KE-748 or *R*(−)-ibuprofen was given orally in a dose of 5 mg/kg to CF rats and vehicle rats. Whole blood samples from ether-anesthetized rats were withdrawn from the inferior aorta into heparinized containers 30 min after drug administration. Plasma was separated by centrifugation at 2000 *g* for 10 min at 4°. The levels of S(+)- and *R*(−)-enantiomers of the drugs in the plasma were measured.

IN VITRO. Hepatocytes were isolated from CF- and vehicle-administered rats. Suspensions of hepatocytes (5×10^6 cells/mL) in Tris–HCl buffer, pH 7.4, were incubated at 37° with *R*(−)-KE-748 or R(−)-ibuprofen (0.1 mM) for 5, 10, 15, 30, 60, and 90 min. The time–course levels of S(+)- and *R*(−)-enantiomers of both drugs were fitted to a kinetic model, and metabolic rate constants, including the rate constant for the chiral inversion, were calculated as described below.

Influence of Medium Chain Fatty Acid CoA Ligase Inhibitor

Hepatocytes were suspended (5×10^5 cells/mL) in Hanks' buffer with 0.5% fatty acid-free BSA containing benzoic acid (0.1 mM), a typical substrate of medium chain fatty acid CoA ligase, in the mitochondrial matrix [24]. The media containing *R*(−)-KE-748 or *R*(−)-ibuprofen (0.025 mM) were incubated at 37° for 5, 10, 15, 30, 60, and 90 min. The time–course levels of S(+)- and *R*(−)-enantiomers of the drugs were measured and fitted to a kinetic model.

Extraction and Analytical Procedures

KE-748 and ibuprofen in plasma *in vivo* were extracted with benzene, under acidic conditions with 3 N hydrochloride. The enzymatic reactions *in vitro* were terminated by the addition of 2 N sodium hydride followed by acidification with 3 N hydrochloride; then the drugs were extracted with benzene. The extracts were centrifuged at 2000 *g* for 10 min; then the supernatants were decanted and evaporated to dryness *in vacuo*. The residue was dissolved in a 100-μL aliquot of methanol and injected into the chiral-HPLC system. The chiral-HPLC methods were validated for the determination of enantiomers of KE-748 and ibuprofen in plasma. The relative standard deviations were less than 10% in the concentration range of 0.1 to 25 μg/mL. The levels in rat hepatocytes were measured using the same procedure. Quantification was made using pure racemic standards (*S*/*R* ratio: 1/1) introduced into blank plasma and incubation medium over the range of 0.2 to 50 μg/mL. The levels were quantified from peak areas, with reference to respective enantiomers. In deuterium exchange studies, separated enantiomers were methylated in methanol with diazomethane, and evaporated to dryness under a steam of nitrogen. After adding a small amount of methanol to each residue, aliquots of the reconstituted samples were injected into the GC/MS system.

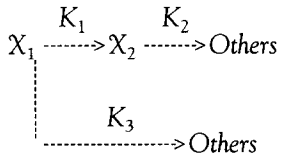
CHIRAL-HPLC CONDITIONS. S(+)- and *R*(−)-enantiomers of KE-748, including [2α-^{2}H]KE-748, were analyzed by the chiral-HPLC system using a CHIRALCEL OD column (4.6 mm i.d. × 250 mm; Daicel Chemical Industries, Tokyo, Japan). The column was a chiral stationary phase made of cellulose tris(3,5-dimethylphenyl-carbomate) coated on silica gel. The flow rate of the mobile phase was 1.0 mL/min, the column temperature was 40°, and the effluent was monitored at 252 nm. When using a mobile

phase with *n*-hexane:2-propanol:formic acid (4000:120:1, by vol.), S(+)-KE-748 and R(−)-KE-748 were separated with retention times of 26 and 31 min, respectively. The S(+)- and R(−)-enantiomers of ibuprofen were analyzed using a TSKgel Enantio-OVM column (4.6 mm i.d. × 150 mm; Tosoh, Tokyo, Japan), which was coated with an ovomucoid glycoprotein as the chiral stationary phase. The flow rate of the mobile phase was 0.9 mL/min, the column temperature was 25°, and the effluent was monitored at 220 nm. Using a mobile phase with 20 mM K_2PO_4 buffer (pH 4.6)/ethanol (10:1, v/v), S(+)- and R(−)-ibuprofen were separated with retention times of 35 and 27 min, respectively.

GC/MS CONDITIONS. Chemical ionization mass spectral data were obtained from a model TSP-70 triple-stage quadruple mass spectrometer (Finnigan MAT, San Jose, CA, U.S.A.). The GC utilized a fused silica capillary column (30 m × 0.25 mm i.d.) coated with the bonded stationary phase DB-1 (J & W Scientific, Folsom, CA, U.S.A.). The injector temperature was 250°, and the column temperature was increased 30°/min, from 150° to 300°. Helium was used as the carrier gas in the GC column, at a head pressure of 6 psi. The ion source temperature was 200°. Methane was used as the chemical ionization reagent gas in the ion source at a pressure of 10 Torr. Analyses were carried out in the selected ion monitoring mode, and the $[^2H]/[^1H]$ ratio was determined by measurement of the respective base ion peak species of the methyl ester of [2α-^{2}H]KE-748 and unlabeled KE-748 ($[M\text{-}CH^3O]^+$; *m/z* 236 and 235, respectively).

Kinetic Model and Analysis

Concentration–time data of enantiomers of KE-748 and ibuprofen in the medium used in the *in vitro* study were fitted to a kinetic model (see below) using a nonlinear, extended least squares fitting procedure (Simplex method [25]), running on an NEC (Tokyo, Japan) personal computer.



$$K = K_1 + K_3$$

X_1 and X_2 designate the quantities of R(−)- and S(+)-enantiomers, respectively. The rate constant for the chiral inversion from R(−)- to S(+)-enantiomer is expressed as K_1, and the metabolic rate constants from R(−)- and S(+)-enantiomers to other metabolites are expressed as K_3 and K_2, respectively. K is the rate constant for elimination of the R(−)-enantiomer, and t is time. I is the initial concentration of the drug. This kinetic model is described by the equation:

$$dX_1/dt = -KX_1$$
$$dX_2/dt = K_1X_1 - K_2X_2$$
$X_1(0) = I$; $X_2(0) = 0$. The solution is:
$$X_1 = I[\exp(-Kt)]$$
$$X_2 = IK_1/(K - K_2)[\exp(-K_2t) - \exp(-Kt)]$$

The significance of differences was evaluated by Student's *t*-test.

RESULTS

Deuterium/Hydrogen Exchange Studies

When R(−)-[2α-^{2}H]KE-748 was given orally to rats in a dose of 5 mg/kg, both S(+)- and R(−)-KE-748 appeared in the plasma (Table 1). After administration of S(+)-[2α-^{2}H]KE-748, only S(+)-KE-748 was detected. These enantiomers were isolated from plasma, purified using chiral-HPLC, and analyzed by GC/MS. Figure 3 shows a quantitative loss of the deuterium atom during chiral inversion. After administration of R(−)-[2α-^{2}H]KE-748, the deuterium atoms of S(+)-KE-748 in plasma were almost all exchanged for hydrogen atoms, and the deuterium content of R(−)-KE-748 was decreased slightly (at 15 min, 6%; at 2 hr, 8%). On the other hand, after administration of S(+)-[2α-^{2}H]KE-748, the deuterium content of S(+)-KE-748 in plasma was unchanged, compared with that before administration.

In Vitro *Study by the Cell-Free System*

The requirement of ATP and CoA for chiral inversion of KE-748, using rat liver homogenates, was compared with that of ibuprofen. Figure 4 shows the enantiomeric ratio following incubation at 37° for 30 min with R(−)-ibuprofen or R(−)-KE-748. When R(−)-ibuprofen was incubated with neither or either cofactor, S(+)-ibuprofen was not detected. But, after incubation with both ATP and CoA, 13% of the total ibuprofen was detected as the S(+)-enantiomer. On the other hand, even when R(−)-KE-748 was incubated with both ATP and CoA, the amounts of S(+)-KE-748 were insignificant.

TABLE 1. Plasma levels of KE-748 enantiomers after oral administration of R(−)-[2α-^{2}H]KE-748 and S(+)-[2α-^{2}H]KE-748 to rats

Time (min)	Concentration (μg/mL)			
	R(−)-[2α-^{2}H]KE-748		S(+)-[2α-^{2}H]KE-748	
	R(−)	S(+)	R(−)	S(+)
15	8.3 ± 1.5	2.7 ± 0.4	ND*	13.3 ± 1.3
30	3.1 ± 0.4	2.4 ± 0.1	ND	21.8 ± 3.4
60	0.7 ± 0.1	0.9 ± 0.2	ND	7.9 ± 0.9
120	0.2 ± 0.1	0.5 ± 0.0	ND	2.9 ± 0.2

Results are expressed as the plasma level of both R(−) and S(+)-KE-748 after oral administration of R(−)-[2α-^{2}H]KE-748 and S(+)-[2α-^{2}H]KE-748 (5 mg/kg). Each value is the mean ± SEM of three animals.

* ND: not detected.

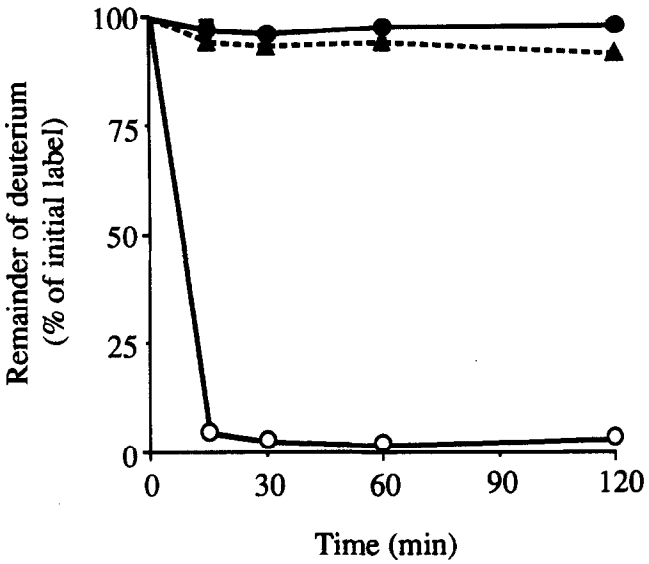


FIG. 3. Loss of 2α-deuterium by KE-748. Results are expressed as the deuterium content in plasma of (▲) *R*(−)- and (○) *S*(+)-enantiomer after oral administration of *R*(−)-[2α-^{2}H]KE-748 to rats, and of (●) *S*(+)-enantiomer following administration of *S*(+)-[2α-^{2}H]KE-748 (5 mg/kg). Each point is the mean ± SEM of three animals.

Effect of Various Fatty Acids

In the study on isolated rat hepatocytes, the unidirectional chiral inversion from *R*(−)- to *S*(+)-enantiomer was observed for both ibuprofen and KE-748. When *R*(−)-ibuprofen (0.025 mM; 52 μg/mL) was incubated with hepatocytes for 120 min, the levels of *R*(−)-ibuprofen and *S*(+)-ibuprofen were 18.6 ± 0.5 and 6.4 ± 0.3 μg/mL, respectively. After incubation of *R*(−)-KE-748 (0.025 mM; 63 μg/ml), *R*(−)-KE-748 and *S*(+)-KE-748 levels were 11.5 ± 0.5 and 2.0 ± 0.2 μg/mL, respectively. Figure 5 shows the influence of various fatty acids (carbon chain length C_3 to C_{16}) on the chiral inversion of *R*(−)-ibuprofen and *R*(−)-KE-748 by isolated rat hepatocytes. When *R*(−)-ibuprofen was incubated with C_6 to C_{16} fatty acids for 120 min at 37°, the chiral inversion was decreased markedly; the levels of *S*(+)-ibuprofen in the medium were about ¼ to ⅙ over levels in the absence of the fatty acids described above. The levels of *R*(−)-ibuprofen increased by adding long chain fatty acids. There was little effect of C_3 and C_4 fatty acids on chiral inversion from *R*(−)- to *S*(+)-ibuprofen. On the other hand, when *R*(−)-KE-748 was incubated with C_{12} to C_{16} fatty acid, the influence on the levels of *S*(+)-KE-748 was nil. In contrast, chiral inversion of *R*(−)-KE-748 was inhibited by adding C_3 to C_8 fatty acids; the levels of *S*(+)-KE-748 were decreased markedly over those found in the absence of fatty acids. The levels of *R*(−)-KE-748 increased by adding short chain fatty acids.

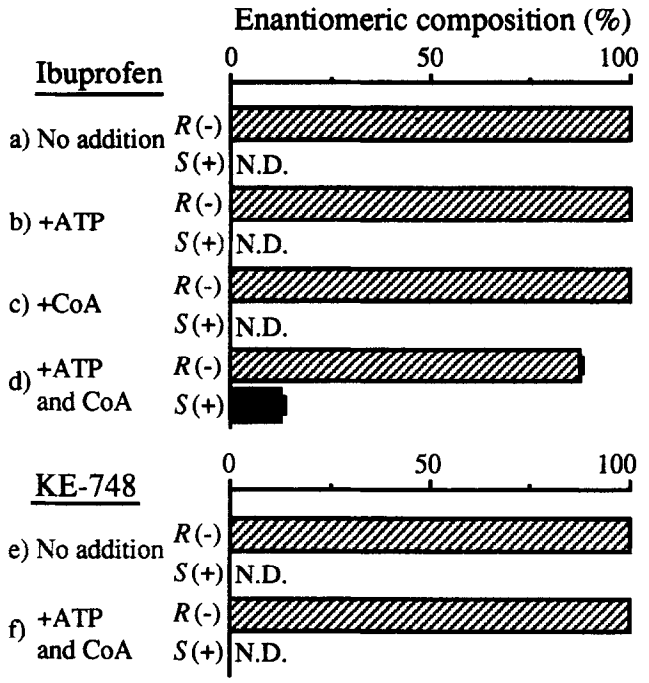


FIG. 4. Cofactor requirement for the chiral inversion of ibuprofen and KE-748, using a cell-free system. *R*(−)-Ibuprofen was incubated for 30 min at 37° in rat liver homogenates to which was added (a) no cofactor, (b) ATP, (c) CoA, or (d) both ATP and CoA. *R*(−)-KE-748 was incubated under the same conditions with (e) no cofactor and (f) both ATP and CoA. Each value is the mean ± SEM of three experiments. N.D. = not detected.

Influence of Long Chain Fatty Acid CoA Ligase Induction

IN VIVO. The enantiomeric plasma levels at 30 min after oral administration of *R*(−)-ibuprofen and *R*(−)-KE-748 in a dose of 5 mg/kg to CF rats (treated with clofibric acid) and vehicle rats (treated with 5% gum arabic) are shown in Table 2. When *R*(−)-ibuprofen was administered to CF

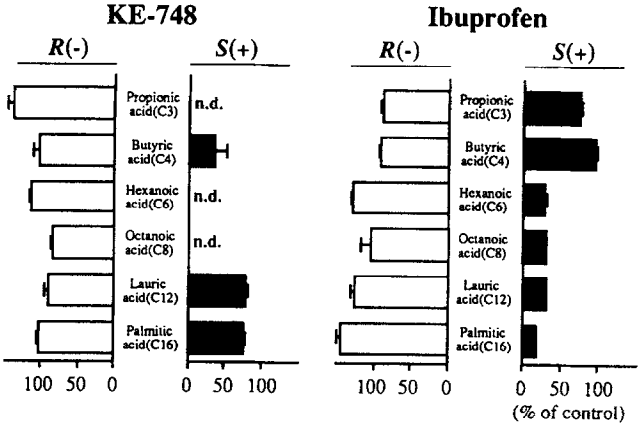


FIG. 5. Effect of fatty acids on chiral inversion of ibuprofen and KE-748 in isolated rat hepatocytes. *R*(−)-Ibuprofen and *R*(−)-KE-748 were incubated for 120 min at 37° in media with various fatty acids added. Each value is the mean ± SEM of three experiments. Control value: the levels of *R*(−)- and *S*(+)-enantiomers when *R*(−)-enantiomer was incubated without fatty acids. When *R*(−)-ibuprofen (0.025 mM; 52 μg/mL) was incubated with hepatocytes for 120 min, the levels of *R*(−)-ibuprofen and *S*(+)-ibuprofen were 18.6 ± 0.5 and 6.4 ± 0.3 μg/mL, respectively. After incubation of *R*(−)-KE-748 (0.025 mM; 63 μg/mL), *R*(−)-KE-748 and *S*(+)-KE-748 levels were 11.5 ± 0.5 and 2.0 ± 0.2 μg/mL, respectively.

TABLE 2. Effect of clofibric acid on enantiomeric ratio of ibuprofen and KE-748 in rat plasma *in vivo*

Substrate	Animal	Concentration (μg/mL) R(−)	Concentration (μg/mL) S(+)	(S/R ratio)
R(−)-Ibuprofen	Vehicle rats	4.90 ± 1.11	2.86 ± 0.24	(0.58)
	CF rats	3.15 ± 0.45	6.25 ± 2.31	(1.98)
R(−)-KE-748	Vehicle rats	18.93 ± 0.71	11.09 ± 0.31	(0.59)
	CF rats	1.52 ± 0.24	0.89 ± 0.09	(0.59)

Rats were treated with clofibric acid (CF rats, 280 mg/kg/day, p.o.) or vehicle (vehicle rats, 5% gum arabic) for 5 days. Results are expressed as the plasma level of both R(−)- and S(+)-enantiomers at 30 min after oral administration of the R(−)-enantiomer (5 mg/kg). Each value is the mean ± SEM of three animals.

rats, the level of S(+)-ibuprofen was over twice that for vehicle rats, and the level of R(−)-ibuprofen was lower than that for vehicle rats. Consequently, the S/R enantiomeric ratio of ibuprofen for CF rats was 3.4 times that for vehicle rats (1.98 vs 0.58). On the other hand, when R(−)-KE-748 was administered to CF rats, the levels of both R(−)- and S(+)-KE-748 were considerably lower than those for vehicle rats, while nearly equal S/R enantiomeric ratios were seen for the two groups (0.59).

IN VITRO. Hepatocytes were isolated from CF and vehicle rats, and cell viability (>95%) was found to be similar to that of normal rat cells. For both ibuprofen and KE-748, when the R(−)-enantiomer was incubated with CF rat hepatocytes, the levels of both the S(+)- and R(−)-enantiomers were higher than those for vehicle rat hepatocytes. Therefore, the time–course data were fitted to a kinetic model. The results of analysis indicated that the chiral inversion rate constant (K_1) of R(−)-ibuprofen during incubation with CF rat hepatocytes was approximately 10 times that for vehicle rat hepatocytes (Table 3). On the other hand, the K_1 for R(−)-KE-748 was not significantly higher than that for vehicle rat hepatocytes. Other metabolic rate constants from S(+)- and R(−)-KE-748 (K_2 and K_3) in CF rat hepatocytes were lower than the corresponding constants for vehicle rat hepatocytes, as was the case for R(−)-ibuprofen.

Effect of Medium Chain Fatty Acid CoA Ligase Inhibitor

The effects of benzoic acid, a typical substrate of medium chain fatty acid CoA ligase in the mitochondrial matrix, on the chiral inversion of ibuprofen and KE-748 by isolated rat hepatocytes are shown in Table 4. When R(−)-ibuprofen was incubated with or without benzoic acid, no significant influence of benzoic acid was found on the chiral inversion rate constant (K_1) or other metabolic rate constants (K_2 and K_3). In contrast, the chiral inversion rate of R(−)-KE-748 was inhibited significantly by adding benzoic acid; K_1 was less than 1/10 that seen in the absence of the inhibitor.

DISCUSSION

We have demonstrated that the unidirectional chiral inversion from R(−)- to S(+)-enantiomer of KE-748, an active metabolite of KE-298, occurs in rats [5]. As metabolic chiral inversion is an important reaction in the pharmacokinetics of some optically active drugs, the present study was designed to compare chiral inversion properties of KE-748 with those of ibuprofen.

An important molecular mechanism of chiral inversion was studied by Nakamura *et al.* [19], who reported that the 2α-methine proton in the chemical structure of ibuprofen was exchanged during chiral inversion. When 2α-

TABLE 3. Effect of clofibric acid on the metabolic rates of ibuprofen and KE-748, determined using isolated rat hepatocytes

Substrate	Hepatocytes	Rate constant (1/min × 10^{-3}) K_1	K_2	K_3
R(−) Ibuprofen	Vehicle rats	4.0 ± 0.6	39 ± 3	43 ± 3
	CF rats	38.3 ± 3.5*	13 ± 5†	27 ± 7
R(−)-KE-748	Vehicle rats	5.6 ± 0.5	37 ± 3	57 ± 8
	CF rats	7.7 ± 0.9	15 ± 2*	42 ± 3

Isolated hepatocytes from CF rats and vehicle rats (final concentrations 5 × 10^6 cells/mL. Tris-HCl buffer, pH 7.4) were incubated at 37° with R(−)-enantiomers of KE-748 and ibuprofen (final concentration 0.1 mM). The rate constant of the chiral inversion from R(−)- to S(+)-enantiomer (K_1) and the rate constants of transformation from each R(−)- and S(+)-enantiomer to other metabolites (K_3 and K_2, respectively) were calculated, using a kinetic model. Each value is the mean ± SEM of three experiments.

*,† Statistically significant difference between groups denoted by *$P < 0.01$ and †$P < 0.05$.

TABLE 4. Effect of benzoic acid on the metabolic rates of ibuprofen and KE-748, determined using isolated rat hepatocytes

Substrate	Benzoic acid	Rate constant (1/min × 10^{-3})		
		K_1	K_2	K_3
R(−)-Ibuprofen	Without	2.8 ± 0.1	1.9 ± 0.4	0.5 ± 0.2
	With	2.5 ± 0.2	2.0 ± 0.7	0.5 ± 0.3
R(−)-KE-748	Without	1.0 ± 0.1	0.9 ± 0.4	3.8 ± 0.3
	With	<0.1*	0.7 ± 0.5	1.4 ± 0.1*

Benzoic acid (0.1 mM) was preincubated with hepatocytes (final concentration 5 × 10^6 cells/mL Hanks' buffer with 0.5% fatty acid-free BSA, pH 7.4) at 37°C for 1 min prior to addition of substrate (0.025 mM). The rate constant of the chiral inversion from R(−)- to S(+)-enantiomer (K_1) and the rate constants of transformation from each R(−)- and S(+)-enantiomer to other metabolites (K_3 and K_2, respectively) were calculated, using a kinetic model. Each value is the mean ± SEM of three experiments.

* Statistically significant difference from the control group, $P < 0.01$.

deuterium-labeled R(−)-[2α-^{2}H]KE-748 was administered to rats, the deuterium atoms of S(+)-KE-748, the inverted product, in plasma were practically replaced by hydrogen atoms, and the deuterium content of R(−)-KE-748 was decreased slightly. These observations were the same as for ibuprofen [19–21]. Nakamura *et al.* [19] postulated that R(−)-ibuprofen is first activated to R(−)-ibuprofen-CoA by CoA ligase, and then is racemized and hydrolyzed to free ibuprofen. Thus, our present data indicate that the chiral inversion from R(−)- to S(+)-KE-748 proceeds via the formation of an intermediate with loss of the 2α-methine proton, and there is some feedback from the intermediate to R(−)-KE-748. In general, the unidirectional chiral inversion from the R(−)- to the S(+)-enantiomer of 2-arylpropionates such as ibuprofen has been noted. However, S(+)-ibuprofen also underwent chiral inversion to a small extent, but was detectable in rats, indicating that the CoA-ligase acting on ibuprofen may not be completely stereospecific for the R(−)-enantiomer [20, 21]. After administration of S(+)-[2α-^{2}H]KE-748 to rats, R(−)-KE-748 was not detected in the plasma, and the deuterium content of S(+)-KE-748 in the plasma remained intact. However, since there seems to be a large difference in this reverse inversion with regard to species and tissues, further studies will be needed to investigate these problems in the case of KE-748.

In the *in vitro* cell-free system using rat liver homogenates, the chiral inversion from R(−)- to S(+)-ibuprofen was observed during incubation with both ATP and CoA, findings that support previous data of Knihinicki *et al.* [14]. However, the chiral inversion of R(−)-KE-748 was not evident, under the same conditions. These results indicate that the spontaneous chiral inversion of KE-748 does not occur. Similar to the case with KE-748, the chiral inversions of benoxaprofen and hydratropic acid were not observed in a cell-free system, although they were seen to be inverted *in vivo* [26–28]. The complete explanation of these causes has not been found yet, but an intriguing aspect was reported in a previous study by Mayer *et al.* [29] using 2-dimethylaminoethanethiol 2-phenylpropionate as a model acyl thioester. They postulated that the affinity of the proton at the chiral center was highly dependent on the polarity of the environment, and it may relate to racemization. Many organelles were broken during homogenization, and the environment of the cell-free system was more aqueous than when cell membranes were intact. Therefore, the balance of enzymatic reaction may be insufficient to induce the chiral inversion of R(−)-KE-748.

In the *in vitro* system using isolated rat hepatocytes, we noted the unidirectional chiral inversions from the R(−)- to the S(+)-enantiomer of both ibuprofen and KE-748. R(−)-Ibuprofen was a substrate for fatty acid CoA ligase, and CoA-thioester intermediate on the chiral inversion was formed [24]. The effects of various fatty acids, substrates of CoA ligases, on the chiral inversion of R(−)-ibuprofen and R(−)-KE-748 were also investigated using isolated rat hepatocytes. The chiral inversion of R(−)-ibuprofen was inhibited by adding medium and long chain fatty acids. On the other hand, that of R(−)-KE-748 was inhibited by adding short and medium chain fatty acids rather than long chain fatty acids. These results suggest that the enzymes responsible for the formation of CoA-thioester of R(−)-KE-748 may differ from those of R(−)-ibuprofen.

Aas [30] reported that long chain fatty acids are converted into the CoA-derivatives by fatty acid CoA ligases in the microsome and in the outer membrane of mitochondria. Activity of the microsomal long chain fatty acid CoA ligase in the liver is increased when rats are treated with clofibric acid (CF rats) [23, 31, 32]. In the present *in vivo* and *in vitro* experiments using CF rats, the chiral inversion of R(−)-ibuprofen was enhanced significantly compared with that in the vehicle rats. However, enhancement of the chiral inversion of R(−)-KE-748 was not observed. These results suggest that the chiral inversion of R(−)-ibuprofen is strongly influenced by long chain fatty acid CoA ligase in microsomes, whereas chiral inversion of R(−)-KE-748 may be almost independent of its enzyme. Medium chain fatty acids, which inhibited the chiral inversion of both R(−)-ibuprofen and R(−)-KE-748, activate three different enzymes, one soluble in the mitochondrial matrix, one bound to outer membrane of mitochondria, and one soluble in the microsome [30]. Benzoic acid is a substrate for medium chain fatty acid CoA ligase in the mitochondrial matrix

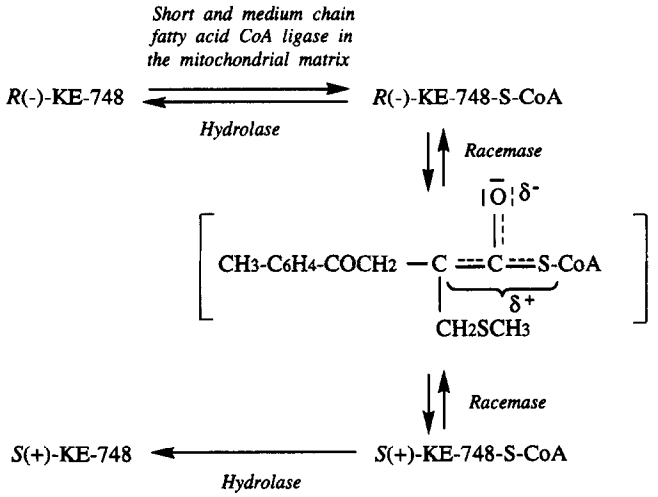


FIG. 6. Proposed scheme for the chiral inversion mechanism of KE-748 (adapted from Nakamura *et al.* [19] and Chen *et al.* [20]).

[24, 33]. In the present *in vitro* experiments using hepatocytes, the chiral inversion of R(−)-ibuprofen was not inhibited by adding benzoic acid, thereby supporting data of Muller *et al.* [24]. Thus, chiral inversion of R(−)-ibuprofen may depend on medium chain fatty acid CoA ligases in the extramitochondria rather than in the mitochondrial matrix. On the contrary, the chiral inversion of R(−)-KE-748 was strongly inhibited in the presence of benzoic acid, which means that the chiral inversion of R(−)-KE-748 may be catalyzed by medium chain fatty acid CoA ligase in the mitochondrial matrix. Short chain fatty acids, which inhibited only the chiral inversion of R(−)-KE-748, were seen to be activated in the mitochondrial matrix [30]. Therefore, the chiral inversion of R(−)-KE-748 may possibly be influenced by short chain fatty acid CoA ligase in the mitochondrial matrix.

Extramitochondrial fatty acid CoA ligases are destined for lipid synthesis [34]. Since ibuprofen was a substrate for this enzyme, incorporation into adipose tissue was observed after administration of ibuprofen to rats [28, 35, 36]. Such a reaction has potential toxicological significance since it would interfere with lipid biochemistry [37]. However, after administration of [^{14}C]KE-298 to rats, the uptake of radioactive metabolites, including KE-748, into adipose tissue was not observed [3]. This phenomenon agreed with our present observations that the chiral inversion of R(−)-KE-748 was catalyzed by enzymes in the mitochondrial matrix rather than by extramitochondrial enzymes.

In conclusion, the chiral inversion of R(−)-KE-748 may involve formation of the CoA-thioester intermediate, and a 2α-methine proton may be lost during inversion of the thioester, as was the case for R(−)-ibuprofen. Hilal and El-Aaser [38] reported that the proton affinity of the α-carbon in thioester was lower than that in oxygen ester. The exchange of a 2α-methine proton in the chemical structure of CoA-thioester for either R(−)-KE-748 or R(−)-ibuprofen may relate to delocalization of the π-electron. However, enzymes required to form CoA-thioester of R(−)-KE-748 may differ from those for R(−)-ibuprofen; the chiral inversion of R(−)-ibuprofen depends on the extramitochondrial medium and long chain fatty acid CoA ligases, whereas that of R(−)-KE-748 is catalyzed by the short chain and medium chain fatty acid CoA ligases in the mitochondrial matrix. Consequently, we propose the chiral inversion mechanism of R(−)-KE-748, as shown in Fig. 6.

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