—Regular Articles—

Enantioselective Uptake of BOF-4272, a Xanthine Oxidase Inhibitor with a Chiral Sulfoxide, by Isolated Rat Hepatocytes

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(Received May 15, 2001; Accepted August 24, 2001)

The transport mechanisms of the enantiomers of BOF-4272, a new drug for the treatment of hyperuricemia, were studied using freshly prepared rat hepatocytes. BOF-4272 consists of S(−) and R(+) enantiomers due to a chiral center in the sulfoxide moiety. The uptake of these BOF-4272 enantiomers by hepatocytes was found to be temperature and dose dependent. The temperature-dependent uptake of the S(−) and R(+) enantiomers showed saturation kinetics. The K_m values for the S(−) and R(+) enantiomers were 59.3 and 25.7 μM, respectively, which was a significant difference ($p<0.05$). However, the maximal uptake rate was comparable for both enantiomers. Metabolic inhibitors such as antimycin, oligomycin, rotenone, carbonylcyanide *m*-chlorophenyl hydrazone, and carbonyl cyanide-*p*-(trifluromethoxy)-phenylhydrazone significantly inhibited uptake of the R(+) enantiomer, but had little effect on uptake of the S(−) enantiomer. Ouabain (an inhibitor of Na^+/K^+-ATPase) and *p*-nitrobenzylthioinosine (NBMPR, a nucleoside transporter inhibitor) showed no significant effects on the uptake of either enantiomer. Organic anions such as taurocholate and cholate reduced the uptake of both enantiomers. These results suggest that the hepatic uptake of both BOF-4272 enantiomers is not due to simple diffusion but also involves carrier-mediated uptake. We suggest that the carrier-mediated uptake of BOF-4272 enantiomers includes both NBMPR-insensitive facilitated diffusion and an active transport system in liver plasma membrane, and that the enantioselective uptake of BOF-4272 is due to differences in affinity for the active transporter.

Key words——enantioselective uptake; xanthine oxidase inhibitor; chiral sulfoxide; hepatocytes; BOF-4272

INTRODUCTION

BOF-4272, a derivative of pyrazolotriazine, is a new drug that has been developed for the treatment of hyperuricemia and ischemic reperfusion injury (Fig. 1).[1—4] BOF-4272 inhibits the *de novo* biosynthesis of uric acid by blocking the xanthine oxidase/xanthine dehydrogenase system, which catalyzes the last step of purine catabolism.[2,3] The mechanism of inhibition by BOF-4272 was elucidated in an *in vitro* study using milk xanthine oxidase/xanthine dehydrogenase.[5]

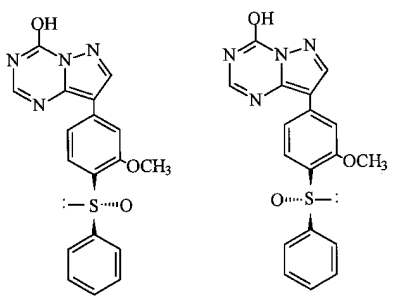

Fig. 1. Chemical Structure of BOF-4272

The structures of the S(−) and R(+) enantiomers of BOF-4272 are shown on the left and right, respectively.

BOF-4272 significantly reduces the concentration of free radicals generated by xanthine oxidase and consequently inhibits cell necrosis.[6] It was found in previous *in situ* studies that the hepatic elimination of BOF-4272 was quite substantial at 37°C.[7—9] It was also demonstrated in a previous *in situ* study that the hepatic extraction ratio of the R(+) enantiomer of BOF-4272 was significantly greater than that of the S(−) enantiomer in the presence of bovine serum albumin in rats.[10] Recently, we have found that the plasma concentration of the S(−) enantiomer of BOF-4272 after the intravenous and oral administration of racemic BOF-4272 in rats was significantly higher than that of R(+)enantiomer, and that the liver concentration after oral administration of racemic BOF-4272 in rats was significantly higher for the R(+) enantiomer than for the S(−) enantiomer of BOF-4272 (in preparation). The stereoselectivity of plasma concentrations of racemic BOF-4272 is suggested to be due to differences in the hepatic uptake of the two BOF-4272 enantiomers, and/or in their biotransformation in the rat liver. In the present study, we investigated the *in vitro* uptake of

BOF-4272 by isolated rat hepatocytes to elucidate the hepatic transport mechanism of the enantiomers of BOF-4272.

METHODS

Animals The animals used were 7-week-old male Wistar rats weighing between 200 and 250 g, purchased from Charles River Japan (Kanagawa, Japan).

Materials The BOF-4272 used in this study was synthesized at Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan). Antimycin, oligomycin, rotenone, ouabain, sodium taurocholate (TCA), probenecid (PBC), *p*-chloromercuriphenylsulfonic acid (PCMBS), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), carbonyl cyanide-*p*-(trifluoromethoxy)-phenylhydrazone (FCCP), *p*-nitrobenzylthioinosine (NBMPR), carbonylcyanide m-chlorophenyl hydrazone (CCCP), bovine serum albumin (fraction V) (BSA), and trypsin inhibitor (type II-S; soybean) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium cholate (CA), *p*-chloromercuribenzoic acid (PCMB), and collagenase (from *Clostridium histolyticum*) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Trypan blue was purchased from Flow Laboratories Ltd. (Irvine, UK). Eagle's minimum essential medium (MEM) Nissui type 1 was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals and reagents used were of the highest grade available from commercial sources.

Preparation of Isolated Hepatocytes Liver perfusion was performed as described by Moldeus et al.[11] Briefly, the rat liver was perfused at 37℃ for 4 min with Ca^{2+}- and Mg^{2+}-free Hanks' solution (pH 7.4) containing 0.5 mM ethylene glycol-*bis* (*β*-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) and for 6 min with Hanks' solution (pH 7.4) containing 0.1% collagenase and 0.05% trypsin inhibitor. Hepatocytes were liberated from the perfused liver by blunt dissection and dispersed by gentle shaking in Ca^{2+}- and Mg^{2+}-free Hanks' solution (pH 7.4). After filtration through gauze, the hepatocytes were separated from nonparenchymal cells by centrifugation three times, with the cells washed and resuspended in Ca^{2+}- and Mg^{2+}-free Hanks' solution (pH 7.4) each time. Finally, the cells were resuspended in Eagle's MEM (pH 7.4) containing 0.5% bovine serum albumin (BSA) and 2 mM L-glutamate. Cell suspensions with a viability greater than 90% as assessed by trypan blue dye exclusion were used for experiments. The number of cells was counted using a Coulter counter. For incubation, the cell suspensions were diluted to a final concentration of 2×10^6 viable cells/ml.

Uptake Studies After preincubation of 1 ml of cell suspension containing 2×10^6 viable cells/ml for 5 min at 4℃ or 37℃, the reaction was started by the addition of 1 ml of the incubation medium containing BOF-4272 preincubated for 5 min at 4℃ or 37 ℃. The reaction time was fixed at 5 min except in the time-course experiments. When the effects of inhibitors, sulfhydryl (SH) -modifying reagents (PCMBS, PCMB), DIDS, organic anions, or BOF-4272 distomer were tested, these reagents were added to the medium 5 min before the addition of BOF-4272. After incubation for a suitable period, 1.5 ml of the reaction mixture was transferred to another tube and was centrifuged at 10,000 rpm for 10 s in a Hinac centrifuge CR15T (Hitachi Koki Co., Ltd., Tokyo, Japan) at 4℃ to stop the reaction by separating the cells from the incubation medium. After centrifugation, the supernatant was transferred to another tube for measurement of lactate dehydrogenase (LDH) activity. The cells were washed twice with 1.5 ml of ice-cold phosphate-buffered saline (PBS) (−) and dissolved by adding 1.5 ml of 0.1% Triton X-100. After centrifugation, 0.5 ml of the supernatant was transferred to two other tubes, with one tube used to determine protein content and LDH activity and other used as the high-performance liquid chromatography (HPLC) sample. The HPLC sample was supplemented with 3 ml of ethyl acetate and agitated. After centrifugation, the ethyl acetate layer was transferred to another tube and dried under reduced pressure. The mobile phase was added to the dried sample for analysis of BOF-4272 and agitated. The dissolved sample was filtered through a membrane filter (pore size, 0.2 μm). An aliquot of 50 μl of the filtrate was injected onto the HPLC system for analysis of BOF-4272.

Chromatography BOF-4272 concentrations were measured using an HPLC system (CCP & 8010 Series, Tosoh Co., Tokyo, Japan) with a stationary phase of TSKgel Silica-60 (250×4.6 mm i.d., Tosoh Co.) on the extraction column and two pieces of CHIRALCELL OD (50×4.6 mm i.d., Daicel Chemical Industries, Ltd., Tokyo, Japan) on the chiral

Table 1. Kinetic Parameters of BOF-4272 in Rat Hepatocytes

	K_m (μM)	V_{max} (pmol/mg/min)
S(−) enantiomer	59.3±23.7	350±192
R(+) enantiomer	25.7±13.0*	384±180

Data are mean±SD of four separate experiments. * $p<0.05$ vs. S(−) enantiomer.

separation column. The concentrations of BOF-4272 enantiomers were determined using a fluorescence photometer (Tosoh Co.) with excitation and emission wavelengths of 319 and 402 nm, respectively. The integrated data analyzer was a C-R4AX Chromatopac (Shimadzu Co., Kyoto, Japan). The flow rate and the column temperature were 0.6 ml/min and 40°C, respectively. The mobile phase was a mixture of *n*-hexane, ethanol, and formic acid (650 : 350 : 4, v/v).

Protein Content The protein content of the samples was estimated by the method of Lowry et al.[12] using BSA as the standard.

⊿LDH Latency The LDH activity of the samples was measured according to the method of Bergmeyer and Bernt.[13] The LDH latency was calculated from the LDH activity of the samples dissolved with Triton X-100 and the total activity (LDH activity of the supernatant of the reaction sample plus that of the samples dissolved with Triton X-100). ⊿LDH latency was calculated as a percentage relative to the LDH latency of controls.

Data Analysis The kinetic parameters (K_m, V_{max}) of uptake were calculated from the concentration-dependence data by non linear least-squares analysis using the MULTI program,[14] by use of equation 1:

$$v = V_{max} X_s / (K_m + s) \quad (1)$$

where v and s are temperature-dependent uptake rate and concentration of substance, and V_{max} and K_m are maximum uptake rate and Michaelis constant, respectively.

All experimental results are given as mean±SD, and the statistical analysis in Table 1 was performed using the paired Student's *t* test (two-tailed) with a significance level of $p<0.05$.

RESULTS AND DISCUSSION

Figure 2 shows the time course of the uptake of BOF-4272 enantiomers by isolated hepatocytes at a substrate concentration of 5 μM. The top panel shows uptake of each enantiomer at 37°C and at 4°C. The hepatic uptake of the R(+) enantiomer at 37°C gradually increased with time and reached a plateau up to 10 min. However, the hepatic uptake of the S(−) enantiomer at 37°C increased with time up to only 2 min. On the other hand, the hepatic uptake of either BOF-4272 enantiomer at 4°C reached a plateau within 1 min and then remained at the same level. The bottom panel of Fig. 2 shows the temperature-dependent uptake, which is calculated by the difference between hepatic uptake at 37°C and that at 4°C. The temperature-dependent uptake of the R(+) enantiomer shows high velocity and a high saturation point as compared with the S(−)enantiomer. However, the time to reach equilibrium in the temperature-dependent uptake of the R(+) enantiomer is five times longer than that of the S(−) enantiomer (Fig. 2, bot-

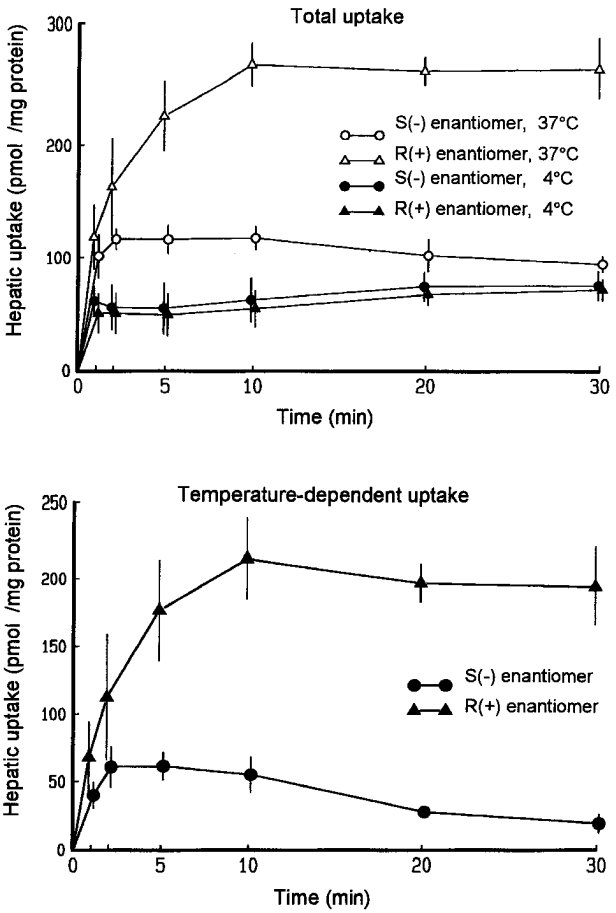

Fig. 2. Time Course of Uptake of BOF-4272 Enantiomers at 5 μM by Isolated Hepatocytes

The top panel shows total uptake at 37°C and at 4°C. The bottom panel shows temperature-dependent uptake (the difference between hepatic uptake at 37°C and that at 4°C). Experiments were performed in duplicate. Data are mean±SD of three separate experiments.

tom panel). The hepatic uptake rate of BOF-4272 at 37℃ increased as a function of the BOF-4272 concentration in the medium, exhibiting a curvilinear pattern and enantioselectivity (data not shown). On the other hand, the hepatic uptake rate at 4℃ increased in proportion to the BOF-4272 concentration in the medium, and neither the saturation pattern nor enantioselectivity was observed in the uptake of either BOF-4272 enantiomer at 4℃ (data not shown).

Figure 3 shows the relationship between the initial temperature-dependent hepatic uptake rate of BOF-4272 enantiomers and the substrate concentration. The closed circles and triangles are the points where the hepatic uptake rate at 4℃ was subtracted from that at 37℃, and the lines are the curves of the Michaelis-Menten equation as predicted by MULTI. The kinetic parameters obtained by fitting the data for the BOF-4272 enantiomers to the Michaelis-Menten equation using MULTI are listed Table 1. The K_m of the R(+) enantiomer was significantly lower than that of the S(−) enantiomer ($p<0.05$), whereas the Vmax was comparable for the two enantiomers. The data show the higher affinity of the R(+) enantiomer to the temperature-dependent hepatic uptake system than that of the S(−) enantiomer, indicating that the uptake rate of the R(+) enantiomer by the hepatocytes was faster than that of the S(−) enantiomer.

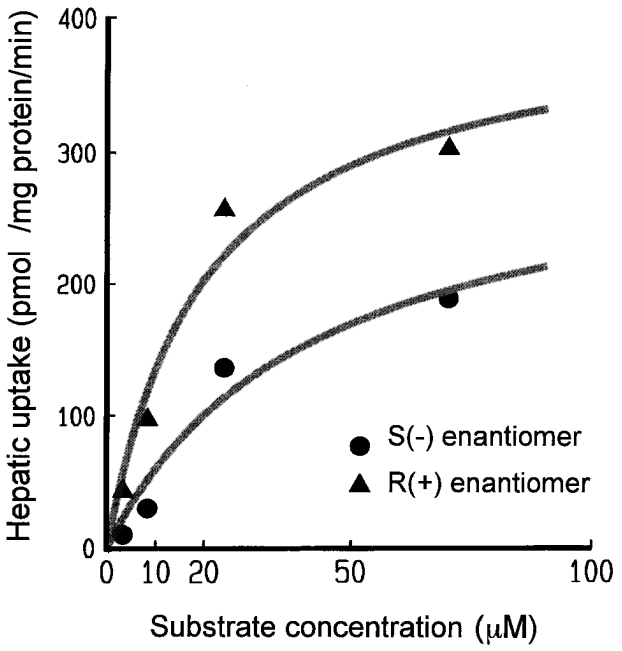

Fig. 3. Effect of Initial Substrate Concentration on Temperature-dependent Uptake of BOF-4272 Enantiomers by Isolated Hepatocytes

Experiments were performed in duplicate. Data show typical saturation kinetics obtained in four separate experiments.

The effects of various inhibitors on the initial hepatic uptake rate of BOF-4272 at 5 μM are shown in Fig. 4. The hepatic uptake rate of the R(+) enantiomer was about 2.2 times higher than that of the S(−) enantiomer in controls. Metabolic inhibitors such as antimycin, oligomycin, rotenone, CCCP, and FCCP (an inhibitor of oxidative phosphorylation)[15] significantly reduced the uptake rate of the R(+) enantiomer, but had little effect on the uptake rate of the S(−) enantiomer. Ouabain, an inhibitor of Na^+/K^+-ATPase,[15] had no significant effect on the uptake rate of either enantiomer. This indicates that the uptake system of both enantiomers is ATP dependent and Na^+ independent. Since the structure of BOF-4272 resembles that of xanthine, BOF-4272 inhibits the *de novo* biosynthesis of uric acid by blocking the xanthine oxidase/xanthine dehydrogenase system.[2,3] However, NBMPR, an inhibitor of the plasma membrane equilibrative (Na^+-independent, facilitated diffusion) nucleoside transporter,[16] had no significant effect on the uptake rate of either enantiomer.

The decrease in LDH latency observed with metabolic inhibitors such as antimycin, oligomycin, rotenone, CCCP, and FCCP suggests hepatic injury resulting from inhibition of the energy supply (Table 2). However, the inhibition of the hepatic uptake was not due to a lethal cytotoxic effect of inhibitors, because cell viability, as estimated by LDH latency, was

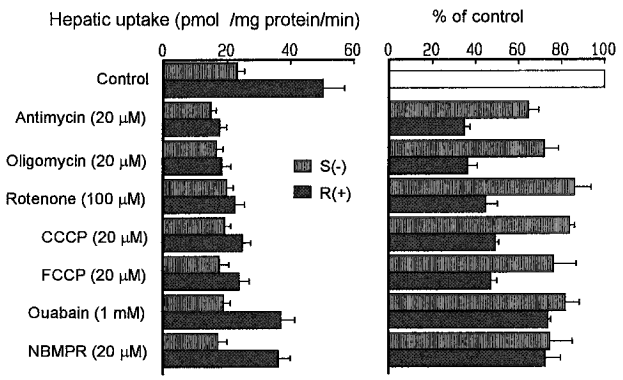

Fig. 4. Effects of Inhibitors on the Initial Uptake Rate of BOF-4272 at 5 μM

The left and light panels show the hepatic uptake rate and percent uptake relative to the control, respectively. Experiments were performed in duplicate. Data are mean ± SD of three separate experiments. The initial uptake rates of the S(−) and R(+) enantiomers in controls were 23.1 ± 2.3 and 50.3 ± 6.8 pmol/mg protein/min, respectively.

Table 2. Cell Viability in Rat Hepatocytes

Group	Concentration (μM)	ΔLDH latency (% of control)
Control		100
Antimycin	20	55.3±0.7
Oligomycin	20	77.8±5.1
Rotenone	100	61.7±7.7
CCCP	20	50.8±3.0
FCCP	20	48.3±10.2
Ouabain	1000	93.4±4.8
NBMPR	100	92.9±4.9
PCMBS	100	92.4±1.7
PCMB	100	94.3±12.2
DIDS	100	93.3±4.5
TCA	50	92.1±3.7
CA	50	95.3±3.5
PBC	50	98.8±7.1

Experiments were performed in duplicate. Data are mean±SD of three separate experiments.

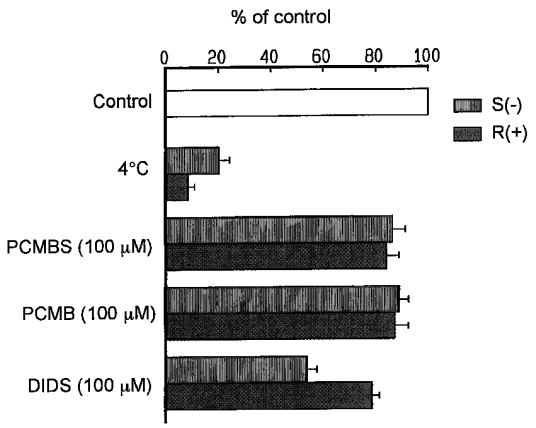

Fig. 5. Effects of Temperature, SH-modifying reagents, and DIDS on the Initial Uptake Rate of BOF-4272 at 5 μM

Experiments were performed in duplicate. Data are mean±SD of three separate experiments. The initial uptake rates of the S(−) and R(+) enantiomers in controls were 23.1±2.3 and 50.3±6.8 pmol/mg protein/min, respectively.

about 50% or more of that of the control.

The effects of temperature, SH -modifying reagents, and DIDS, a potent inhibitor of the anion exchanger,[17] on the initial hepatic uptake rate of BOF-4272 at 5 μM are shown in Fig. 5. PCMBS and PCMB (a poorly permeable SH reagent and a highly permeable SH reagent, respectively)[18] had no significant effects on the uptake rate of either enantiomer. DIDS inhibited the hepatic uptake of the S(−) enantiomer to a greater degree than it inhibited the hepatic uptake of the R(+) enantiomer.

BOF-4272 is present as an organic anion in the

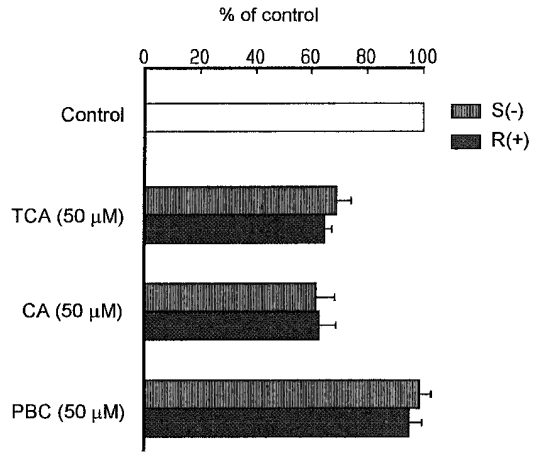

Fig. 6. Effects of Organic Anions on the Initial Uptake Rate of BOF-4272 at 5 μM

Experiments were performed in duplicate. Data are mean±SD of three separate experiments. The initial uptake rates of the S(−) and R(+) enantiomers in controls were 23.1±2.3 and 50.3±6.8 pmol/mg protein/min, respectively.

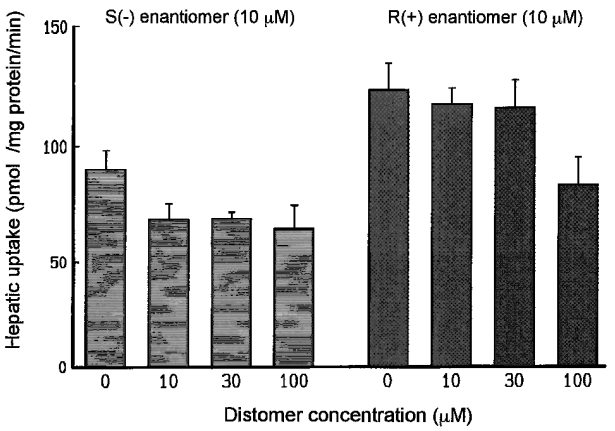

Fig. 7. Effects of Enantiomer-Enantiomer Interactions on the Hepatic Uptake of BOF-4272

Experiments were performed in duplicate. Data are mean±SD of three separate experiments.

body. We therefore investigated the effects of organic anions on the initial hepatic uptake rate of BOF-4272 at 5 μM (Fig. 6). Organic anions such as TCA and CA reduced the uptake of both enantiomers. However, there were no significant differences in the percent uptake of the two enantiomers in the presence of these organic anions. PBC and β-lactam antibiotics were transported by the same carrier-mediated transport system through the plasma membrane and was actively uptaken into hepatocytes,[18] but neither of the enantiomers was affected by PBC.

The effects of enantiomer-enantiomer interactions on the hepatic uptake of BOF-4272 are shown Fig. 7. The influence of distomer on the hepatic up-

take rate of the S(−) enantiomer was greater than that on the hepatic uptake rate of the R(+) enantiomer.

In conclusion, we found that the hepatic uptake of both BOF-4272 enantiomers is not due to simple diffusion but involves carrier-mediated uptake. We suggest that the carrier-mediated uptake of BOF-4272 enantiomers includes both NBMPR-insensitive facilitated diffusion and active transport, and that the enantioselective uptake of BOF-4272 is due to differences in affinity for the active transporter.

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