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Effects of clofibric acid on the biliary excretion of benoxaprofen glucuronide and taurine conjugate in rats

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Benoxaprofen (BOP) is a 2-methyl propionic acid derivative with anti-inflammatory activity. BOP has an asymmetric carbon, and receives chiral inversion from *R* to *S in vivo*. BOP is metabolized to glucuronide (BOP-G) and taurine conjugate (BOP-T). The configuration of BOP-G is mainly *S*, and that of BOP-T is *R*. Chiral inversion of *R* to *S* of the propionic acid moiety and amino acid conjugation of carboxyl compounds proceed *via* an acyl CoA intermediate. It is known that fibrates, used in hyperlipidemia, induce acyl CoA synthetase and increase CoA concentration. We administered racemic BOP (10 mg/kg body weight) to rats (CFA+) pre-administered clofibric acid (CFA, 280 mg/kg/day), and studied BOP, BOP-G, and BOP-T enantiomer concentrations in plasma and bile up to 12 h after administration. The findings were compared with those in rats (CFA-) that had not received CFA. Furthermore, we studied the amounts of BOP-G enantiomer produced by glucuronidation *in vitro* using microsomes pretreated with CFA. The amounts of (*S*)-BOP-G in CFA+ rats were 2.7-fold larger than that in CFA- rats. Although (*R*)-BOP-T was excreted in CFA- rats, BOP-T could not be detected in CFA+ rats. Plasma clearance values of racemic BOP and (*S*)-BOP in CFA+ rats were 5-fold and 6-fold larger than those in CFA- rats, respectively. (*S*)-BOP-G formation activities were higher than (*R*)-BOP-G formation activities in both CFA+ and CFA- microsomes. These findings suggest that CFA increases biliary excretion of (*S*)-BOP-G and facilitates plasma elimination of BOP, and further suggests that CFA predominantly induces chiral inversion to *S* rather than metabolic reaction to (*R*)-BOP-T, resulting in an increase of (*S*)-BOP-G.

1. Introduction

Anti-inflammatory 2-methyl propionic acid derivatives (profens) exhibit stereoselective chiral inversion *in vivo* from *R* to *S* (Caldwell et al. 1988; Iwakawa et al. 1991), and the *S* enantiomer exhibits an active anti-inflammatory effect (Evans 1992). Chiral inversion of profens progresses with 3 reaction steps. (*R*)-Acyl-CoA thioester intermediate is formed from the *R* form by acyl-CoA ligase in the first reaction, chiral inversion of (*R*)-acyl-CoA to (*S*)-acyl-CoA intermediate by epimerase occurs in the second reaction, and the *S*-antipode (free acid) is formed by hydrolysis in the third reaction (Hall and Quan 1994). All 2-methyl propionic acid derivatives, i.e., profens, sold in Japan (ketoprofen, flurbiprofen, ibuprofen (IBP), oxaprofen, tiaprofenic acid, pranoprofen, loxoprofen sodium, alminoprofen, and zaltoprofen), excluding naproxen (*S*-form), are used in the racemic form and converted to the *S*-antipode *in vivo* (Evans 1992).

Benoxaprofen [2 - (4- chlorophenyl)-α-methyl-5-benzoxazole acetic acid, BOP] (Fig. 1) is a 2-methyl propionic acid derivative (profen compound) with anti-inflammatory activity (Cashin et al. 1977; Tsurumi et al. 1980; Tsurumi et al. 1982).

We previously reported the presence of its taurine conjugate (BOP-T) in rat bile after treatment with, BOP. We also found that

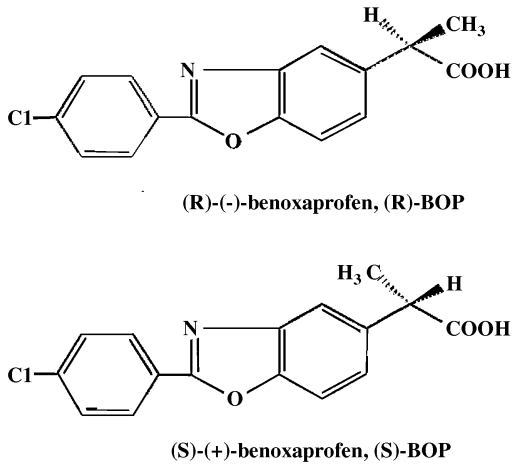


Fig. 1: Structures of benoxaprofen enantiomers.

the absolute configuration of the main metabolite, glucuronic acid conjugate (BOP-G), is *S*, while that of BOP-T is *R* (Mohri et al. 1998), and that BOP-G is mainly derived from (*S*)-BOP, while BOP-T is derived from (*R*)-BOP. We also showed that chiral inversion from *R* to *S* of the parent compound, BOP, is

closely related to the absolute configurations of these metabolites (Mohri et al. 2005). BOP was recalled from the market and no longer used clinically because of its lethal hepatotoxicity (Duthie et al. 1982; Goudie et al. 1982). However, BOP is the sole model compound of stereoselective amino acid conjugation. It is known that fibrates (fibric acid derivatives) enhance hepatic acyl-CoA synthetase activity involved in fatty acid metabolism and increase the co-substrate, CoA, content (Alegret et al. 1994; Horie et al. 1986). Knights et al. reported that clofibric acid (CFA) treatment promoted formation of an intermediate metabolite of IBP, *R*-ibuprofenyl-CoA, increasing the *R*-to-*S* chiral inversion rate in an experiment using rat liver homogenate (Knights et al. 1991; Shirley et al. 1994).

Assuming that fibrates enhance formation of (*R*)-BOP-CoA intermediate metabolites, promoting metabolism to (*R*)-BOP-T, we orally administered CFA (280 mg/kg) dissolved in polyethylene glycol to Sprague-Dawley rats for 3 successive days, followed by intravenous administration of BOP (10 mg/kg), and collected blood and bile for 12 h (CFA-pretreated (CFA+) group). Polyethylene glycol alone was orally administered for 3 successive days to control rats (CFA-untreated (CFA-) group). The pharmacokinetics of BOP enantiomers in plasma were investigated in the CFA+ and CFA- groups, respectively. The biliary enantiomer levels of BOP and its metabolites (BOP-G and BOP–T) were measured in each group and the biliary excretion rates were determined. It was revealed that CFA promoted metabolic reaction: (*R*)-BOP-CoA → (*S*)-BOP-CoA → (*S*)-BOP → (*S*)-BOP-G, rather than (*R*)-BOP-CoA → (*R*)-BOP-T.

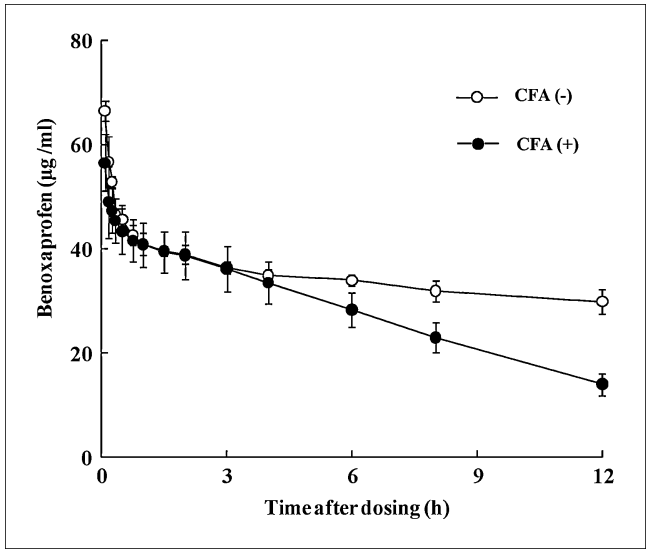


Fig. 2: Plasma concentration-time profiles for racemic BOP (dose: 10 mg/kg body weight, n = 6). Polyethylene glycol 400 (◯) 1.0 ml/kg body weight or CFA (●) 280 mg/kg body weight was administered orally for consecutive 3 days before BOP administration.

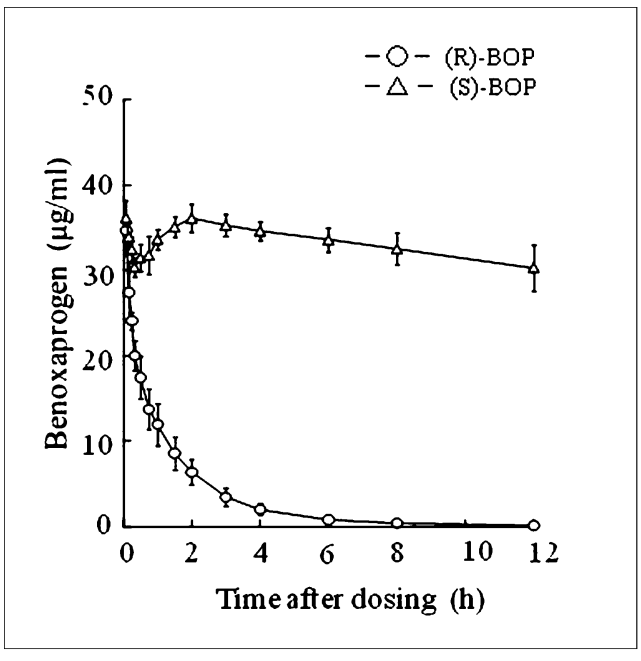
2. Investigations and results

2.1. Influence of CFA on elimination of racemic BOP in rats

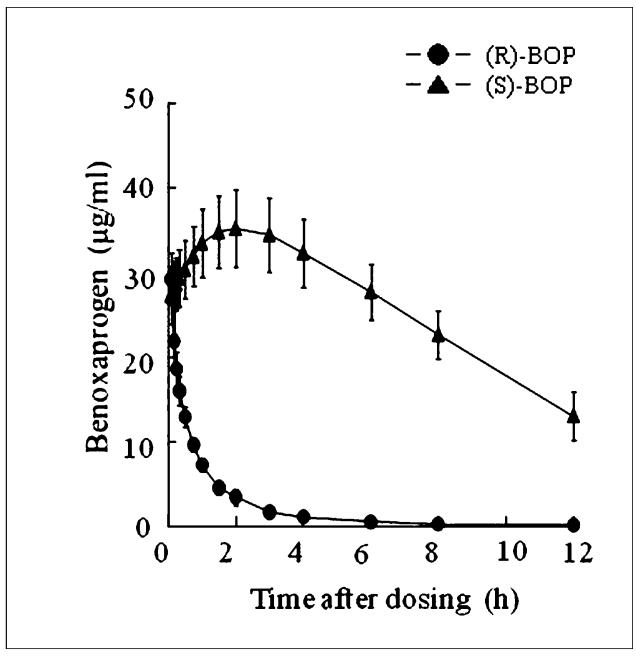
The plasma concentration-time curves of racemic BOP in the CFA+ and CFA- groups are shown in Fig. 2. BOP was eliminated from plasma more rapidly in the CFA+ group than that in the CFA- group. The area under the plasma concentration-time curve (AUC_{0-12h}) and mean residence time (MRT) were significantly lower in the CFA+ group than those in the CFA- group ($p < 0.01$). In contrast, the elimination rate constant (Kel) and total body clearance (CLtot) values were 6 and 5 times greater in the CFA+ group than those in the CFA- group, respectively ($p < 0.01$) (Table 1). These findings indicated that CFA treatment promoted elimination of BOP from plasma.

2.2. Influence of CAF on elimination of BOP enantiomers in rats

The plasma concentration-time curves of BOP enantiomers in the CFA+ and CFA- groups are shown in Fig. 3. In both groups, (*R*)-BOP was rapidly eliminated from plasma, while (*S*)-BOP appeared in plasma after administration of racemic BOP. The plasma (*S*)-BOP level started to increase 10 and 20 min after administration in the CFA+ and CFA- groups, respectively.



(A)



(B)

Fig. 3: Plasma concentration-time profiles for BOP enantiomers (dose: racemic BOP 10 mg/kg body weight, each n = 6). (A) polyethylene glycol 400 1.0 ml/kg body weight or (B) CFA 280 mg/kg body weight was administered orally for consecutive 3 days before BOP administration.

Table 1: Pharmacokinetic parameters of racemate or enantiomers of BOP in clofibric acid treated or untreated rats

Parameter	Rac.-BOP		(R)-BOP		(S)-BOP	
	Untreated	Treated	Untreated	Treated	Untreated	Treated
AUC0–12 h(mg h/ml)	0.42 ± 0.01	0.34 ± 0.03*	0.04 ± 0.007	0.03 ± 0.003*	0.40 ± 0.02	0.31 ± 0.03*
MRT(h)	53.2 ± 18.6	9.16 ± 1.96*	1.82 ± 0.16	2.42 ± 0.63	65.9 ± 23.1	9.10 ± 2.31*
CL_{tot} (ml/h)	1.37 ± 0.55	6.79 ± 0.53*	62.3 ± 12.0	109.2 ± 8.41*	1.19 ± 0.58	7.55 ± 0.89*
Vd(l)	65.6 ± 4.9	61.5 ± 9.1	112.1 ± 14.1	261.9 ± 57.0*	68.7 ± 3.6	67.3 ± 10.2
K_{el}(1/h)	0.02 ± 0.01	0.12 ± 0.03*	0.31 ± 0.02	0.17 ± 0.05*	0.02 ± 0.009	0.13 ± 0.03*

Treated: clofibric acid treated rats; CFA (+), Untreated: clofibric acid untreated rats; CFA (−).
*Significantly different CFA (+) from clofibric acid untreated rats CFA (−) ($p < 0.01$) (each n = 6).

(*S*)-BOP was eliminated more rapidly in the CFA+ group than that in the CFA- group. T_{max} and C_{max} of (*S*)-BOP were 2 h and 36.1 μg/ml in the CFA+ group and 2 h and 35.3 μg/ml in the CFA- group, respectively, with no significant differences between the groups ($p = 0.613$).

Table 1 shows the pharmacokinetic parameters (0–12 h) of (*R*)-BOP and (*S*)-BOP. The $AUC_{0–12h}$ values of (*R*)-BOP and (*S*)-BOP were significantly smaller in the CFA+ group than those in the CFA- group [(*R*)-BOP: 32.6%, (*S*)-BOP: 20.9%]. The CL_{tot} values of (*R*)-BOP and (*S*)-BOP were significantly greater in the CFA+ group than those in the CFA- group [(*R*)-BOP: 1.8 times, (*S*)-BOP: 6.3 times]. The volume of distribution (Vd) of (*R*)-BOP in the CFA+ group was significantly greater (2.3 times) than that in the CFA- group.

2.3. Influence of CFA on biliary and urinary excretion of BOP and its metabolites

The cumulative biliary excretion rates (% of dose) of each enantiomer of BOP and its metabolites in the CFA+ and CFA- groups for 12 h after BOP administration are shown in Fig. 4. In the CFA+ group, the cumulative excretion rates of (*S*)-BOP-G and (*R*)-BOP-G were 28.1 ± 4.2 and 2.1 ± 0.8% and those of (*S*)-BOP and (*R*)-BOP were 3.3 ± 2.6 and 0.6 ± 0.5%, respectively, but no BOP-T was detected. In the CFA- group, those of (*S*)-BOP-G and (*R*)-BOP-G were 10.6 ± 1.5 and 1.8 ± 0.5%, those of (*S*)-BOP-T and (*R*)-BOP-T were 0.3 ± 0.04 and 1.8 ± 0.5%, and those of (*S*)-BOP and (*R*)-BOP were 0.3 ± 0.2 and 0.03 ± 0.03%, respectively. The cumulative excretion rate of (*S*)-BOP-G was significantly increased (2.7 times) in the CFA+ group compared to that in the CFA- group ($p < 0.05$), however no significant difference was noted in the cumulative biliary excretion rate of (*R*)-BOP-G between the 2 groups. The biliary excretion rates of (*S*)-BOP and (*R*)-BOP were 11 and 20 times increased in the CFA+ group, compared to those in the CFA- group, respectively ($p < 0.05$).

In both the CFA+ and CFA- groups, the cumulative biliary excretion rate of (*S*)-BOP-G was greater than that of (*R*)-BOP-G (CFA+ group: 13.4 times, CFA- group: 5.9 times). Regarding biliary taurine conjugates, the cumulative excretion rate of (*R*)-BOP-T was 6 times greater than that of (*S*)-BOP-T in the CFA- group.

The enantiomer ratios (*S*/*R* ratio) of BOP, BOP-G, and BOP-T in bile and its total ratio in the CFA+ and CFA- groups are shown in Table 2. The total S/R ratio of BOP and its metabolites excreted in bile was 3.7 times greater and the ratio of BOP-G was 2.2 times greater in the CFA+ group than those in the CFA- group, whereas the ratio of BOP was 1.9 times greater in the CFA- group than that in the CFA+ group.

No BOP and its metabolites were found in the urine.

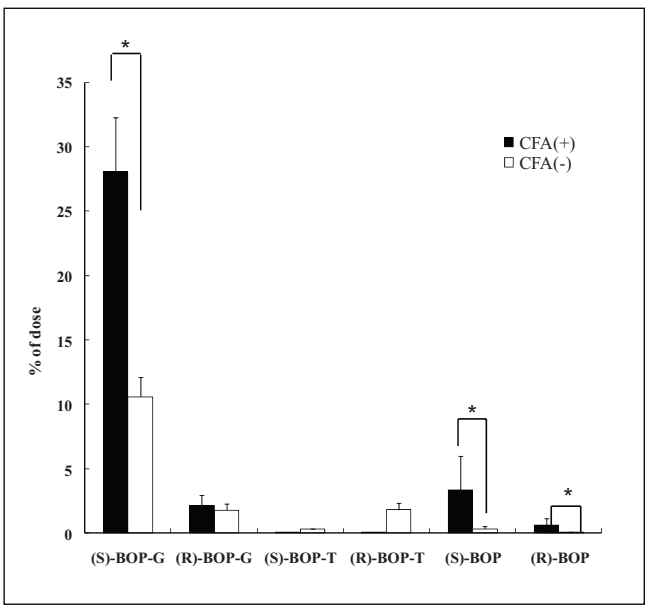


Fig. 4: Effects of pretreatment with CFA on cumulative biliary excretion (% of dose) of BOP-G, BOP-T, and BOP enantiomers after a single administration of racemic BOP (dose: 10 mg/kg body weight). CFA(+) and CFA(−) represent pretreatment and untreatment with CFA, respectively (each n = 6). Each point and vertical bar represent the mean and s.d., respectively. *Significantly different CFA(+) from clofibric untreated rats CFA(−) ($p < 0.05$).

2.4. Influence of CFA on BOP glucuronidation activity of rat liver microsomes

The rat liver microsomal BOP-G formation activity levels using racemic BOP as a substrate in the CFA+ and CFA- groups are shown in Fig. 5.

The (*R*)-BOP-G- and (*S*)-BOP-G-forming activity levels of CFA+ microsome were 0.11 ± 0.006 and 0.23 ± 0.006 nmol/mg/min, and those of CFA- microsomes were 0.21 ± 0.01 and 0.38 ± 0.008 nmol/mg/min, respectively. The activity levels were significantly lower by 47.6 and 39.5% in the CFA+ microsomes than those in the CFA- microsomes, respec-

Table 2: Enantiomeric ratio of BOP, BOP-G, and BOP-T in bile after i.v. administration of racemic BOP (10 mg/kg body weight)

CFA	S/R ratio			
Treatment	BOP	BOP-G	BOP-T	total
CFA(−)	9.99	5.94	0.16	3.10
CFA(+)	5.31	13.13	–	11.32

CFA(−); Rats were administered polyethylene glycol 400 (1.0 ml/kg body weight) for 3 days before BOP i.v. administration.
CFA(+); Rats were administered clofibric acid (280 mg/kg body weight) for 3 days before BOP i.v. administration.

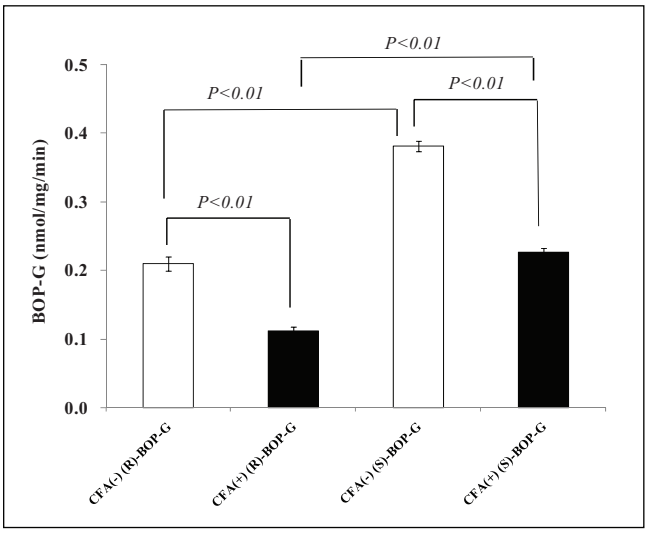


Fig. 5: Effects of CFA on the BOP glucuronidation activities in rat hepatic microsomes. Each point and vertical bar represent the mean and s.d. (each n = 3), respectively.

tively ($p < 0.01$). The (*S*)-BOP-G-forming activity level was higher than (*R*)-BOP-G-forming activity level in both microsomes (CFA+ microsomes: 2.0 times, CFA- microsomes: 1.8 times).

3. Discussion

The objective of this study was to investigate the influence of CFA on pharmacokinetics and metabolism of BOP. Plasma and bile were collected for 12 h from the rats pretreated and untreated with CFA after single intravenous bolus administration of 10 mg/kg BOP. And then plasma BOP and biliary BOP and its metabolites (BOP-G and BOP-T) were analyzed by HPLC using C18 octadecylsilane (ODS) and optical isomer separation columns.

3.1. Influence of CFA on plasma pharmacokinetics of BOP

Scheuerer et al. (1998a, b) reported that the CL_{tot} value of (*R*)-IBP was increased in rats and humans pretreated with clofibrate (CF) compared to those without CF pretreatment. They discussed that the cause was due to an enhancement of the (*R*)- to (*S*)-IBP chiral inversion rate by CF pretreatment, whereas CF pretreatment did not change the CL_{tot} value of (*S*)-IBP in rats, although it was increased in humans (1.6 times increased compared to that in the CFA- group). They indicated that oxidizing enzyme induction is also involved in the increased CL_{tot} values of (*R*)- and (*S*)-IBP, in addition to the promotion of chiral inversion by CF, because CF induces oxidizing enzyme of P-450 (CYP), in addition to CoA ligase and CoA hydrolase (Scheuerer et al. 1998a). The effect of CF on pharmacokinetics of IBP in humans in their study was consistent with our findings (Fig. 3 and Table 1).

The appearance of (*S*)-BOP in plasma and increased CL_{tot} value in the CFA+ group were due to chiral inversion of (*R*)-BOP to (*S*)-BOP. The CL_{tot} values of both enantiomers were significantly increased by CFA pretreatment ((*R*)-BOP: 1.75 times, (*S*)-BOP: 6.34 times), particularly marked in (*S*)-BOP (Bopp et al. 1979; Simmonds et al. 1980). (*S*)-BOP produced by chiral inversion was metabolized to (*S*)-BOP-G, which may have acted as a driving force.

The Vd of (*R*)-BOP was increased in the CFA+ group, and this finding was consistent with that of IBP reported by Scheuerer et al (1998a). They observed that the IBP level rose in fat and lung, brain, and liver tissues in the CF-treated group. CFA may also promote incorporation of BOP into tissue. They also found that the expression level of the main enzyme involved in chiral inversion, long-chain acylCoA synthetase, was increased by CF treatment, but the epimerase expression level was not changed (Scheuerer et al. 1998a). (*R*)-BOP-CoA formation was enhanced by CFA treatment, but the rate of conversion from (*R*)-BOP-CoA to (*S*)-BOP-CoA was limited in that reaction, which may have increased the apparent Vd of (*R*)-BOP.

3.2. Influence of CFA on metabolism and biliary excretion of BOP

The ratios of optical isomers of BOP and its metabolites excreted in bile to the dose were investigated. In the CFA- group, the biliary excretion rates of (*S*)-BOP-G (10.6%), (*R*)-BOP-G (1.8%), (*R*)-BOP-T (1.8%), and (*S*)-BOP-T (0.3%) were consistent with those in our previous report (Mohri et al. 1998). In contrast, in the CFA+ group, the excretion rate of (*S*)-BOP-G was markedly increased compared to that in the CFA- group (2.65 times), but the BOP-T level was lower than the detection limit (Fig. 4), clarifying that CFA increased biliary excretion of (*S*)-BOP-G but inhibited BOP-T formation. These may have been due to an increased level of (*S*)-BOP, the preferential substrate of glucuronidation, while the level of (*R*)-BOP, selective substrate of BOP-T formation, was decreased by CFA-enhanced chiral inversion.

The *S*/*R* ratio of biliary BOP-G in the CFA+ group was 2.2 times greater than that in the CFA- group (Table 2). It was clarified that CFA pretreatment enhanced (*S*)-BOP-G formation.

We previously reported that more BOP-G was excreted as the *S*- than *R*-form in bile in untreated rats, and BOP-G was derived from (*S*)-BOP (Mohri et al. 1998; Mohri et al. 2005). Spahn et al. also found that (*S*)-BOP-G was mainly excreted in urine in humans (Spahn et al. 1989).

3.3. Influence of CFA on BOP-G formation activity of rat liver microsomes

When BOP was administered to CFA-pretreated rats, biliary excretion of (*S*)-BOP-G was induced. To clarify whether this phenomenon was due to UDP-glucuronosyltransferase (UGT) activity enhanced by CFA pretreatment, each BOP-G enantiomer formation activity was investigated using rat liver microsomes of CFA-pretreated rats. However, the microsomal BOP-G formation activity level was significantly lower in the CFA+ microsomes than that in the CFA- microsomes (Fig. 5), i.e., CFA pretreatment might reduce the activity of UGT molecular species responsible for BOP *N*-glucuronidation in the liver. Li et al. (2008) administered 2-phenylpropionic acid (2-PPA) to rats pretreated with oral fibrates (CFA, fenofibrate, and gemfibrozil) for 7 days, and measured the intrahepatic levels of 2-PPA CoA and 2-PPA acylglucuronide after 2 h. All 3 fibrates markedly elevated the acyl-CoA synthetase activity and intrahepatic 2-PPA CoA levels. In contrast, pretreatment with fenofibrate and gemfibrozil significantly reduced the intrahepatic 2-PPA acylglucuronide level, and clofibric acid pretreatment also slightly reduced the intrahepatic level of 2-PPA acylglucuronide.

Prueksaritanont et al. (2005) reported that fibrate pretreatment (CFA, fenofibric acid, and gemfibrozil) increrased the UGT1A1 mRNA level to a 2–3 times higher than that in the control group, but the UGTs activity level for chrysin, which does not

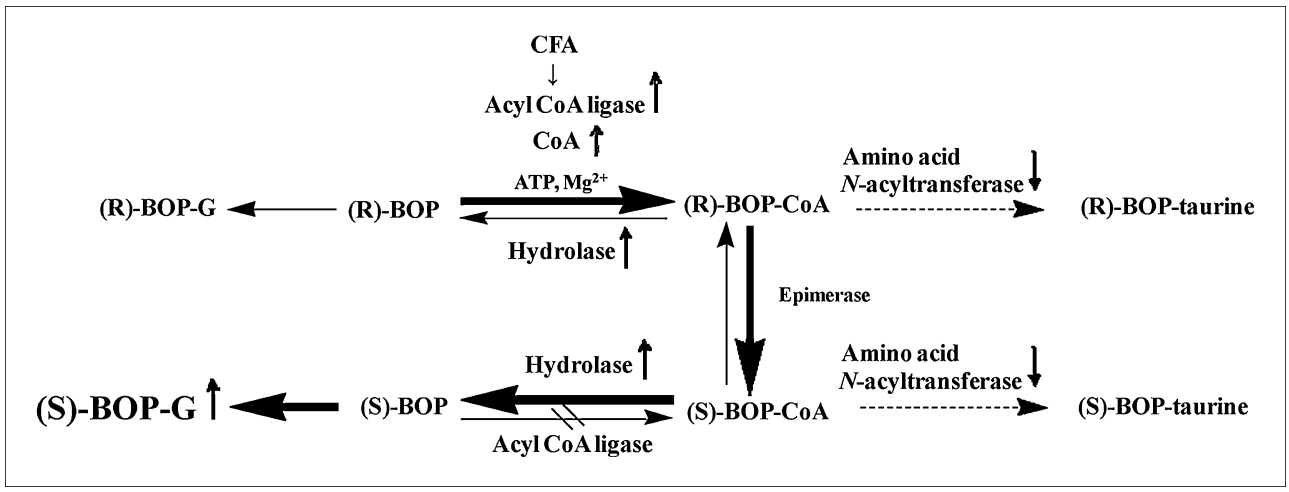


Fig. 6: Estimated mechanism of BOP metabolism in rats pretreated with CFA.

show UGT isoform-specificity, was similar to that in the control group. In addition, reduced UGTs activity for glucuronidation of non-steroidal antiinflammatory drugs (pirprofen, flurbiprofen, and IBP) in liver microsomes of rats treated with CF for 5 days (Magdalou et al. 1990) and slightly reduced 2-PPA acylglucuronide formation by CFA pretreatment (Fournel-Gigleux et al. 1988) have been reported. Our findings were consistent with these reports. Furthermore, (*S*)-BOP was more likely to be conjugated with glucuronic acid than (*R*)-BOP (Fig. 5), which was also consistent with previously reported findings (Mohri et al. 1998, 2005; Spahn et al. 1989).

3.4. Influence of CFA on BOP-T formation

We previously suggested that (*R*)-BOP is conjugated mainly with taurine (Mohri et al. 1998, 2005). However, no biliary excretion of BOP-T was detected in the CFA+ group, although it was observed in the CFA- group (Fig. 4). *R*-Forms of 2-methyl propionic acid derivatives are metabolized to (*R*)-CoA intermediates by acyl-CoA synthetase, converted to (*S*)-CoA intermediates by epimerase and then hydrolyzed to *S*-forms by hydrolase. On the other hand, in amino acid conjugation, (*R*)-CoA intermediates are directly metabolized to amino acid conjugates by amino acid *N*-acyltransferase. (*R*)-CoA thioester intermediates in these series of reactions are competitive substrates of chiral inversion by epimerase and amino acid conjugation (Knights and Drogemuller 2000; Li et al. 2008).
Grillo et al. recently reported stereoselectivity of the CoA form (FLX-CoA) and glutathione conjugate (FLX-SG) of an analogue of BOP, flunoxaprofen (FLX), in an *in vitro* experiment using rat hepatocytes. The amounts of FLX-CoA and FLX-SG formation significantly increased when (*R*)-FLX was used as a substrate, compared to the use of (*S*)-FLX, and these formations were completely inhibited by lauric acid (Grillo et al. 2010), demonstrating that FLX-SG was formed *via* (*R*)-FLX CoA, and indicating that (*R*)-FLX-SG-forming capacity is dependent on the (*R*)-FLX CoA level.
Gregus et al.(1998) investigated the influence of CF on glycine conjugation of benzoic acid (hippuric acid) *in vivo* and *in vitro* using rats. Benzoic acid is metabolized to hippuric acid *via* benzoic acid-CoA by mitochondrial benzoyl-CoA synthetase and benzoyl-CoA/glycine *N*-acyltransferase using ATP, CoA, and glycine in hepatocytes. In rats fed a diet containing fenofibrate or bezafibrate, in addition to an increase in the liver weight, the CoA content per liver weight increased 8–10 times, and the blood and urinary hippuric acid levels rose. In the *in vitro* experiment, the liver microsomal benzoyl-CoA hydrolase activity was enhanced but benzoyl-CoA/glycine *N*-acyltransferase activity was reduced in rats fed fenofibrate or bezafibrate, resulting in hippuric acid formation decreased in liver slices from bezafibrate-fed rats.
Gregus et al. reported that the increase in hippuric acid formation induced by fibrate treatment resulted from changes in the balance among factors involved in amino acid conjugation (increases in the CoA level and benzoyl-CoA hydrolase activity and reduction of glycine *N*-acyltransferase activity).
The cause of the disappearance of BOP-T formation was considered to be by the balance of enzymatic activities induced by CFA between CoA epimerase catalyzing chiral inversion from (*R*)-BOP-CoA to (*S*)-BOP-CoA (no change in activity) and taurine *N*-acyltransferase catalyzing taurine conjugation from (*R*)-BOP-CoA to (*R*)-BOP-T (reduce in activity). The reaction may progress toward (*S*)-BOP-CoA, rather than toward (*R*)-BOP-T, because the affinity of CoA epimerase for (*R*)-BOP-CoA and its V_{max} value were greater than those of taurine *N*-acyltransferase (Fig. 6).

3.5. Influence of CFA on (S)-BOP-G formation

CFA promoted chiral inversion of (*R*)-BOP to (*S*)-BOP, increasing the preferential substrate of UGT, (*S*)-BOP, which was supported by the finding that the total *S*/*R* ratio of the metabolites and unchanged form excreted in bile was 3.65 times increased in the CFA+ group compared to that in the CFA- group (Table 2). Therefore, it was speculated that significant biliary excretion of (*S*)-BOP-G resulted from a high substrate level, even though CFA reduced the activity of (*S*)-BOP-G formation enzyme, UGT2B1 (Ritter 2000) (Fig. 5).
This study clarified that CFA increased biliary excretion of (*S*)-BOP-G while inhibiting (*R*)-BOP-T formation, and these phenomena were suggested to be due to CFA-induced promotion of chiral inversion from (*R*)-BOP to (*S*)-BOP. It is interesting that the fibrate influenced stereoselective conjugation reactions of the 2-methyl propionic acid derivative, particularly, the influence of CFA on taurine conjugation, as first reported in this study.

4. Experimental

4.1. Chemicals and reagents

Tetrabutylammonium hydrogen sulfate (TBA) and Lubrol WX were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Clofibric acid [2-(*p*-chlorophenoxy)-2-methylpropionic acid] (CFA) and naproxen were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo).

Polyethylene glycol 400 (PEG 400), tris (hydroxymethyl) aminomethane (Tris), and saccharo-1,4-lactone were purchased from Nakarai Tesque Inc. (Kyoto). Sucrose, disodium ethylenediaminetetraacetate (EDTA), glycerin, dimethylsulfoxide (DMSO), magnesium chloride ($MgCl_2$), UDP-glucuronic acid (UDPGA), and ammonium sulfate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Urethane was purchased from ICN Biomedicals, Inc. (Ohio). Japanese Pharmacopoeia heparin sodium injection was purchased from Mochida Pharmaceutical Co., Ltd. (Tokyo). Benoxaprofen (BOP) was provided by Eli Lilly (Indianapolis, IN) before recall from the market. Methanol (MeOH), acetonitrile (MeCN), and tetrahydrofuran (THF) of HPLC grade were purchased from Wako Pure Chemical Industries. Double distilled water was used. Naproxen methylester (NAP-Me, IS) was synthesized as previously reported. Reference (*R*)-BOP and (*S*)-BOP were prepared by HPLC using an optical resolution column as previously reported. (*R*)-BOP-T and (*S*)-BOP-T were synthesized from (*R*)- and (*S*)-BOP enantiomers, respectively, as previously reported (Mohri et al. 1998). Reference (*R*)-BOP-G and (*S*)-BOP-G were biosynthesized from (*R*)- and (*S*)-BOP enantiomers, respectively, following a reported method (Spahn et al. 1989). For all other reagents, the commercially available highest grades were used.

4.2. Animals and drug administration

All animal procedures were approved by Meiji Pharmaceutical University Committee for Ethics of Experimentation and Animal Care. Twelve male Sprague-Dawley rats (six rats per each group) (Sankyo Labo Service Co., Tokyo), weighing 250–300 g, were used throughout the study. The rats were housed in stainless steel cages in groups of 3, in a temperature-controlled (23–25 °C) room with a 12-hr light/dark cycle. The rats were allowed free access to standard rat chow (Sankyo) and water for 1 week before the experiments. Thirty %(W/V) CFA solution (CFA/PEG 400) was prepared and orally administered to rats at a dose of 280 mg/kg body weight at 12:00 once a day for successive 3 days prior to the experiments. To the control group, PEG 400 was orally administered at 1.0 ml/kg body weight at 12:00 once a day for successive 3 days prior to the experiments. Each animal was anesthetized with 20% (w/v) urethane (1 g/kg body weight, i.p.). The femoral vein was cannulated with PE-10 tubing (Clay Adams, Parsippany, NJ) for instillation of saline solution and drug administration. The femoral artery was cannulated with PE-50 tubing (Clay Adams), for collection of blood samples, and a heparin-lock was established, using 100 units/ml heparin in saline. An abdominal incision was made and the common bile duct was cannulated with PE-10 tubing (Clay Adams), for collection of bile samples, and closed with surgical clips. During experimental procedures, body temperatures were maintained at 38 ± 0.5 °C with a heating lamp, to prevent hypothermic alterations of bile flow. Saline was injected into the rats through the cannula to supplement body fluids. A dosing solution was prepared by dissolving 20 mg of racemic BOP with 1 M NaOH (200 µl) and ethanol (EtOH) (200 µl), and the total volume was adjusted to 1.5 ml by adding purified water. At 24 h after the final administration of CFA or PEG 400, a single bolus of racemic BOP was injected into the femoral vein at a dose of 10 mg/kg body weight to each rat. Blood samples (each approximately 0.2 ml) were collected from the femoral artery at 0, 5, 10, 15, 20, 30, and 45 min and 1, 1.5, 2, 3, 4, 6, 8, and 12 h after administration of BOP. The collected blood was immediately centrifuged at 15,000 g for 15 min at 4 °C, and plasma was separated. To avoid hydrolytic cleavage of acyl-glucuronide, bile was collected in 2-mL plastic tubes containing 100 µl of 10% H_3PO_4 aqueous solution every 30 min. Urine samples were collected continuously for 12 h through a PE-50 tubing bladder cannula into 5-ml glass tubes. Saline supplements were administered to the rats through the femoral cannula, at volumes equivalent to blood and bile collection volumes (each approximately 0.2−0.5 ml). Bile and urine outputs were measured by weight. The plasma, bile, and urine samples collected were stored at −80 °C until analysis.

4.3. HPLC apparatus and chromatographic conditions

The HPLC system comprised a PU-1580 Intelligent pump (JASCO Corporation, Tokyo), FP-1520 Intelligent fluorescence detector (JASCO), and AS-1555 Intelligent sampler (JASCO), and data was analyzed using the Bowin computer program (JASCO). The excitation and fluorescence wavelengths were set at 315 and 365 nm, respectively. Analysis was performed at room temperature (23 °C). For measurement of the plasma racemic BOP level, a reverse-phase column, Capcell Pak C18 column (4.6 mm i.d. × 25 cm; particle size, 5 µm; Shiseido) attached with a Capcell Pak C18 guard column (4.6 mm i.d. x 1 cm; particle size, 5 µm; Shiseido Co., Tokyo) was used. The mobile phase was 10 mM TBA/MeCN/THF (100:35:35 v/v), and the flow rate was 1.3 ml/min. The plasma and biliary levels of BOP and BOP metabolite enantiomers were measured using an optical isomer separation HPLC column, Sumichiral OA 3300 (4.6 mm i.d. × 25 cm; Sumika Chemical Analysis Service, Osaka), attached with a guard column (4.6 mm i.d. × 1 cm; Sumika). The mobile phases were 0.04 M ammonium acetate/MeOH (for separation of plasma BOP enantiomers), 0.06 M ammonium acetate/MeOH (for separation of biliary BOP-G enantiomers), and 0.01 M ammonium acetate-supplemented MeOH/MeCN/H_2O (85:15:5, v/v) (for separation of biliary BOP and BOP-T enantiomers). In *in vitro* BOP-G formation experiments, (*R*)-BOP-G and (*S*)-BOP-G were measured using a SUPERIOREX ODS column (4.6 mm i.d. x 25 cm; particle size, 5 µm; Shiseido), attached with a guard column (PEGASIL ODS, 4.6 mm i.d. x 1 cm, Senshu Scientific Co., Ltd., Tokyo). The mobile phase was composed of 10 mM TBA and MeOH, and flowed at a gradient concentration (0→20 min: 10 mM TBA:MeCN = 60:40 v/v → 72:28 v/v; 20→21 min: 72:28 v/v → 40:60 v/v; 21→30 min: 40:60 v/v; 30→31 min: 40:60 v/v → 60:40 v/v; and 31→40 min: 60:40 v/v). The mobile phase flow rate was 1.5 ml/min.

4.4. Assay procedures for the biological samples and pharmacokinetic analysis

Ten µl of plasma or bile collected from rats was combined with 30 µl of IS (100 µg/ml), 180 µl of MeCN, and 30 µl of distilled water and mixed for 30 s. After centrifugation at 15,000 g for 15 min, 5 µl of the supernatant was directly injected onto the HPLC.

Samples for calibration curves were prepared by dissolving BOP with control plasma and bile, and the final concentration was adjusted to a range of 1–100 µg/ml. The BOP-G and BOP-T levels were calculated from the BOP calibration curve, and corrections were made as previously reported (Mohri et al. 1998). Pharmacokinetic analysis of BOP was performed employing the moment analysis method (Yamaoka et al. 1978) using a computer program, MULTI.

4.5. Preparation of rat hepatic microsomes

A 30% (W/V) CFA solution was orally administered at 280 mg/kg for successive 3 days to male Sprague-Dawley rats (body weight: 250–350 g). To the control group, PEG 400 was orally administered at 1.0 ml/kg for successive 3 days. Under ether anesthesia, the liver was immediately excised from the rats. After wet weight measurement, the liver was perfused with ice-cold 1.15% KCl solution to remove blood, and 20% homogenate was prepared with 10 mM Tris HCl (pH 7.4)−0.25 M sucrose solution (sucrose-Tris HCl buffer). The homogenate was centrifuged at 600 g for 10 min and then 10,000 g for 10 min. The supernatant was further centrifuged at 105,000 g for 1 h. The pellet was homogenized by 10 strokes in 10 volumes of sucrose-Tris HCl buffer. This suspension was centrifuged at 105,000 g for 1 h, and the precipitate was resuspended with 1 ml of 100 mM Tris HCl (pH 7.4)-10 mM EDTA −20% glycerol buffer as a microsomal fraction and stored at −80 °C until use.

4.6. Determination of optimal reaction conditions for the conjugation enzymes of BOP-G formation

Firstly, to establish conditions to maximize rat liver microsomal BOP-G formation activity, the reaction was performed under various conditions [microsomal solubilizer (0−0.5%), pH of incubation solution (6–8), $MgCl_2$ (0–10 mM), saccharolactone (0–8 mM), UDPGA (0–8 mM), BOP (0–1 mM), and incubation times (0–60 min)].

Rat liver microsomes (0.5 mg) and Lubrol (final concentration 0.15%) were added into a 1.5-ml plastic tube and kept on ice for 30 min. $MgCl_2$ (final concentration: 8 mM), saccharolactone (final concentration: 4 mM), and UDPGA (final concentration: 4 mM) were added to Tris-HCl buffer (pH 6.9) (final concentration: 80 mM) and preincubated at 37 °C for 5 min. The enzymatic reaction was started by adding BOP to the reaction solution (final concentration: 0.6 mM), followed by incubation at 37 °C for 30 min, and the reaction was stopped by adding 200 µl of EtOH. The reaction solution was then combined with 1 mg/ml NAP-Me DMSO solution (10 µl), 1 M HCl (20 µl), and ammonium sulfate (0.5 g), and vigorously mixed for 1 min. After centrifugation at 16,000 g for 10 min, the supernatant was injected onto the HPLC to analyze BOP-G enantiomers. Under the optimal conditions established above, sufficient amounts of the substrate and co-substrate were present, and the correlation between the incubation time and BOP-G formation was liner for 40 min.

4.7. Statistical analysis

All data were presented as the mean ± s.d. The significance of between-group differences was analyzed employing the unpaired Student's t-test. For comparison among 3 or more groups, two-way layout analysis of variance was employed. The significance level was set at $p < 0.05$.

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