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Dissociation of Hypolipidemic and Antiplatelet Actions from Adverse Myotonic Effects of Clofibric Acid Related Enantiomers

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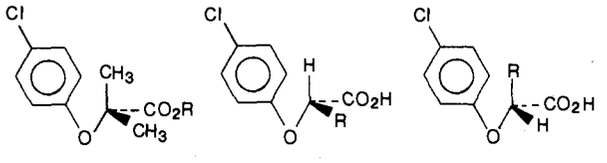
Enantiostructure–activity studies of chlorophenoxybutyric and propionic acids have provided evidence for the dissociation of serum cholesterol lowering and platelet antiaggregatory activities from the adverse chloride ion channel mediated myotonic effects of these compounds. *R*-(+) propionic and butyric acid enantiomers, unlike achiral clofibric acid and the *S*-(−) isomers, did not inhibit chloride conductance in rat extensor digitorum longus muscle fibers in vitro but, like clofibric acid and the *S*-(−) isomers, retained the serum cholesterol lowering activity in a cholesterol-fed rat model. Additionally, a stereoselective and greater inhibition was observed for the *R*-(+) isomers against adenosine diphosphate and arachidonic acid induced human platelet aggregation.

Clofibrate (**1**) is currently employed for the treatment of type III hyperlipoproteinemia, and the use of this drug is questioned owing to serious adverse side effects such as myotonia,[1] gall bladder disease, and possible carcinogenicity in humans.[2,3] Enantioselectivity of drugs important to many biological systems emphasizes the desirability of examining such structure–activity relationships.[4] To date, there are no reports of a dissociation of beneficial from toxic effects of (chlorophenoxy)alkyl acids based upon enantioselectivity. In this paper, we describe such dissociative effects of *R*-(+)- and (*S*)-(−)-propionic (**3**) and butyric (**4**) acid analogues of clofibric acid (**2**), the active metabolite[5] of **1**. Enantiomers (**3** and **4**) were resolved with brucine salts and their absolute configurations were determined by comparative CD analysis[6] of analytically pure samples. Pure isomers were employed in all biological experiments.



1, R = Et; **2**, R = H

(*S*)-(+)-**3**, R = Me; (*S*)-(−)-**4**, R = Et

(*R*)-(+)-**3**, R = Me; (*R*)-(+)-**4**, R = Et

Hypolipidemic activities of all enantiomers (**3**, **4**) and clofibric acid were assessed in cholesterol-fed male Sprague–Dawley rats.[7-10] We observed, in doses of 0.6 mmol/kg per day (Table I), a significant reduction in serum cholesterol concentrations with all compounds at both 4 and 7 days of treatment. However, the reduction in serum cholesterol at 4 days of treatment was greater with both enantiomers of **3** and clofibric acid than with (*R*)-(+)- or (*S*)-(−)-**4** treated groups. In this model, no enantioselectivity was observed. Liver cholesterol concentrations were not altered with any of the compounds tested, and serum triglyceride lowering was only observed with clo-

Table I. Influence of Clofibric Acid and Chiral Analogues 3 and 4 on Serum and Liver Cholesterol Concentrations[a]

drugs	serum cholesterol, mg/dL day−1	serum cholesterol, mg/dL day+4	serum cholesterol, mg/dL day+7	liver cholesterol, mg/g of tissue
control	268 ± 69.0	228 ± 57.4	267 ± 51.8	37.8 ± 5.17
clofibric acid	271 ± 75.7	113 ± 18.2[b]	149 ± 33.3[b]	41.9 ± 3.18
(*R*)-(+)-**3**	276 ± 61.4	116 ± 20.6[b,c]	191 ± 34.6[b]	40.8 ± 6.69
(*S*)-(−)-**3**	279 ± 64.0	107 ± 23.2[b,c]	184 ± 67.6[b]	45.4 ± 5.40
(*R*)-(+)-**4**	275 ± 65.1	158 ± 26.1[b,c]	184 ± 42.2[b]	34.8 ± 2.78
(*S*)-(−)-**4**	278 ± 64.8	174 ± 29.3[b,c]	193 ± 33.5[b]	37.2 ± 4.93

[a] Hypocholesterolemic activity of these drugs was tested in male Sprague–Dawley rats prefed a high cholesterol semisynthetic diet.[7] After 1 week on this reeimen, blood was withdrawn from the orbital plexus of ether-anesthetized animals, and serum cholesterol concentrations were determined.[8] On the basis of these values, rats were distributed through stratification randomization into six groups with six rats per group. Rats received 0.3 mmol/kg body weight, twice daily of the respective drug through intragastric administration with 0.25% methylcellulose vehicle for 1 week. Control rats received the same amount of vehicle only. Body weights (which ranged from 232 to 306 g) among treatment groups were not significantly different at day−1, day+4 or day+7. After 1 week of drug treatment, blood was collected by exsanguination from the abdominal aorta, and livers were removed. Liver cholesterol concentrations were determined in lipid extracts.[8-10] Values are the mean ± SD of six rats. Day+4 and day+7 represent 4 and 7 days of drug treatment, respectively. Day−1 represents initial values before drug treatment. [b] Control (day+4 or day+7) vs. drug treatment group, $P < 0.01$. [c] 4 vs. 3, $P \leq 0.0001$; *R*-(+) vs. *S*-(−), not significant using a two-way ANOVA.

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Table II. Inhibition of Platelet Aggregation by Enantiomers of 3 and 4 and Clofibric Acid[a]

	IC_{50}, μM				
inducer	(*R*)-(+)-**3**	(*R*)-(+)-**4**	(*S*)-(−)-**3**	(*S*)-(−)-**4**	**2**
ADP	201 ± 66	686 ± 65	998 ± 89	1176 ± 89	1238 ± 234
AA	253 ± 69	721 ± 113	1118 ± 115	1601 ± 73	>4000

[a] Human platelet-rich plasma was prepared from drug-free volunteers as previously described.[11,12] Aggregation was monitored in an aggregometer using the minimum concentration of inducer necessary to produce a maximum change in transmittance with arachidonic acid (AA, 300–500 μM) or adenosine diphosphate (ADP, 1–3 μM) after 4 or 6 min, respectively. When present, drugs were preincubated with platelets for 1 min prior to the addition of ADP or AA. Data are the mean IC_{50}'s ± SEM (n = 4–10 donors) for inhibitions of maximal aggregation after sample incubation. Values for ADP represent inhibition of the second phase of aggregation.

fibric acid [day−1, 128 ± 19.6 mg/dL; day+4, 112 ± 15.3 mg/dL; day+7, 97.4 ± 17.7 mg/dL].

Effects of all compounds on human platelet aggregation in vitro were determined turbidometrically,[11,12] and the results are shown in Table II. Both arachidonic acid (AA) induced aggregation and the second wave of adenosine diphosphate (ADP) induced aggregation are mediated by prostaglandin biosynthesis. The concentration of clofibric acid that inhibited prostaglandin-dependent aggregation by 50% (IC_{50}) is comparable to concentrations that are found in human subjects treated for hyperlipidemia.[13] All isomers of **3** and **4** were more effective inhibitors of ADP-induced aggregation than **2** and, unlike **2**, also inhibited aggregation caused by AA. The relative potency in this series is (*R*)-(+)-**3** > (*R*)-(+)-**4** ≥ (*S*)-(−)-**3** > (*S*)-(−)-**4** ≥ **2**. Similar results were found for inhibition of ADP- or AA-induced serotonin secretion from platelets (data not shown). Two-way analyses of variance on the optical isomers revealed highly significant differences ($p < 0.001$) in both ADP- and AA-induced aggregation due to changes in both absolute configurations as well as α-alkyl group size. Interactions between these effects were also highly significant, indicating a synergistic relationship favoring the *R*-(+) compound with the α-methyl group. This compound, (*R*)-(+)-**3**, was at least sixfold more potent than **1**, which was reported to reverse platelet hyperaggregatory activity in patients.[14]

Table III. Stereoselective Block of Membrane Chloride Conductance in Rat Skeletal Muscle Fibers[a]

compound	IC_{50}, μM
clofibric acid	205.4 ± 0.8[b]
(*R*)-(+)-**3**	>230[c]
(*S*)-(−)-**3**	12.0 ± 0.7
(*R*)-(+)-**4**	>230[c]
(*S*)-(−)-**4**	10.3 ± 0.9

[a] Membrane cable parameters were measured in normal and in chloride-free physiological medium after addition of serval concentrations of the compounds by using the square-pulse microelectride method described previously.[17] Chloride conductance was calculated as the difference between the membrane conductance in normal solution minus the conductance in chloride-free solution determined at each drug concentration. The resultant conductance vs. concentration curves were fit to a single-site binding equation with a nonlinear least-squares method from which the IC_{50}'s were determined. The IC_{50}'s ± SD for the drugs were calculated from the variance–covariance matrix. Charles River Sprague–Dawley rats were maintained on a normal diet prior to sacrifice. [b] For each concentration, the average ± SD was determined with 7–79 fibers from 2–16 muscles. [c] No effects were seen up to this concentration for both *R*-(+) isomers.

The myotonic effects of these compounds were studied by evaluating chloride conductance[15,16] in rat extensor digitorum longus (EDL) muscle fibers in vitro. As shown in Table III, chloride conductance was markedly reduced by low concentrations of (*S*)-(−)-**3** (IC_{50} = 12 μM) and (*S*)-(−)-**4** (IC_{50} = 10 μM) whereas the *R*-(+) enantiomers were ineffective at concentrations ranging from 5 to 230 μM. Clofibric acid also gave a concentration-dependent inhibition of chloride conductance with an IC_{50} of 205 μM in rat EDL muscle fibers.

The enantiospecificity observed in muscle fibers using the chiral desmethyl isomers of clofibric acid indicates interaction with a specific chloride ion channel receptor. In platelets, the marked increase in potency of *R*-(+) enantiomers also likely reflects interaction with specific enzymes or receptors, whereas lack of enantioselectivity in the cholesterol-fed rat model suggests multiple modes of interaction.

These data demonstrate that employment of chiral clofibrate-related analogues provide for a greater separation of beneficial serum cholesterol lowering and platelet antiaggregatory activities from adverse properties (blockade of membrane chloride conductance in skeletal muscle fibers) in this class of therapeutic agents.[17,18] Thus,

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(6) (*R,S*)-2-(4-Chlorophenoxy)propionic acid (**3**) was prepared according to: (a) Witiak, D. T.; Ho, T. C.-L.; Hackney, R. E.; Conner, W. E. *J. Med. Chem.* **1968**, *11*, 1086. (*R,S*)-2-(4-Chlorophenoxy)butyric acid (**4**) was prepared according to: (b) Sjoberg, B. *Ark. Kemi* **1960**, *15*, 451. Optically pure (*S*)-(−)-**3** and (*S*)-(−)-**4** brucing salts crystallized from MeOH and (*R*)-(+)-**3** and (*R*)-(+)-**4** brucine salts were obtained from the mother liquors. (*S*)-(−)-**3**, mp 103–104 °C ($CHCl_3$, petroleum ether), exhibited $[\alpha]^{20}_D$ −34.9° (*c* 1.9, MeOH) (lit.[4a] mp 104–105 °C, $[\alpha]^{25}_D$ −34.9°). (*R*)-(+)-**3**, mp 103–104 °C ($CHCl_3$, petroleum ether), exhibited $[\alpha]^{20}_D$ +35° (*c* 5.0, MeOH) (lit.[4a] mp 104–105 °C, $[\alpha]^{25}_D$ +34.1°). (*S*)-(−)-**4**, mp 88–90 °C (petroleum ether), exhibited $[\alpha]^{20}_D$ −43° (*c* 4.7, MeOH) and (*R*)-(+)-**4**, mp 88–90 °C (petroleum ether), exhibited $[\alpha]^{20}_D$ +41.5° (*c* 5.0, MeOH). Synthetic details and CD/ORD analyses will be published in conjunction with other work (V. Tortorella, personal communication).

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through an understanding of enantiostructure multiactivity relationships of significant pharmaceuticals, we should improve therapeutic indices and promote enhanced benefit to risk ratios. In this specific case, since hyperlipidemia and platelet hyperactivity are both implicated in atherosclerosis, *R*-(+) desmethyl isomers of clofibric acid may provide leads for the development of more efficacious and safer drugs for the treatment of coronary artery disease.

(18) Thus, it would be desirable to assess the enantiomers for serum triglyceride lowering activity in other animal models.

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Stereospecificity of the Chloride Ion Channel: The Action of Chiral Clofibric Acid Analogues

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2-(*p*-Chlorophenoxy)isobutyric acid (clofibric acid (1) or CPIB) is a drug known to block chloride membrane conductance (G_{Cl}) in rat striated muscle. In the present study chiral analogues of CPIB (2-(*p*-chlorophenoxy)propionic acid (2) and 2-(*p*-chlorophenoxy)butyric acid (3)) have been tested to evaluate the influence of chirality on Cl ion flux in the channel. The results showed that the chloride channel conductance strongly depends on the absolute configuration: in fact, the *S*-(−) isomers of the tested compounds strongly decreased the G_{Cl} of skeletal muscle membrane, whereas the *R*-(+) isomers were virtually ineffective. These data allow the hypothesis that, like other ion channels present in various biological systems, the chloride channel of skeletal muscle membrane could also have a stereospecific binding site (or receptor) regulating chloride ion flux.

In skeletal muscle 65–85% of the resting membrane conductance is due to chloride ions.[1,2] This large chloride conductance (G_{Cl}) stabilizes the resting membrane potential of mammalian muscle. In fact, muscles with abnormally low G_{Cl} can become hyperexcitable and produce trains of action potentials as observed in some forms of hereditary myotonia of goats[3] or humans and in myotonia produced by certain drugs.[4-7]

In spite of the important role of resting G_{Cl} in mammalian muscle excitability, relatively few studies are available on the molecular mechanism underlying this function.

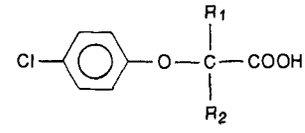
Recently we showed that 2-(*p*-chlorophenoxy)isobutyric acid (1) (CPIB), an in vivo metabolite of clofibrate,[8] specifically decreases membrane G_{Cl} of rat skeletal muscle.[9,10] There are no reports in the literature on stereospecificity of the chloride channel in skeletal muscle. Experiments were performed to evaluate the capability of chiral analogues of CPIB to interfere with chloride channel conductance and to produce myotonia.

Results and Discussion

In a preceding paper we presented evidence that 2-(*p*-chlorophenoxy)isobutyric acid (1) (clofibric acid or CPIB) is capable of blocking in vivo and in vitro G_{Cl} in rat extensor digitorum longus (EDL) muscle fibers.[9] Recently, it was found[10] that the CPIB concentration required for 50% block ($IC_{50} \pm SD$) of G_{Cl} was 205 ± 0.8 μM.

In order to interfere more specifically with chloride channel conductance, a series of CPIB chiral derivatives with an asymmetric carbon atom directly linked to the carboxy group has been synthesized, resolved, and tested.

(*RS*)-2-(*p*-Chlorophenoxy)propionic acid[11] (2) and (*RS*)-2-(*p*-chlorophenoxy)butyric acid[12] (3) were synthesized by means of known methods and they were resolved into the corresponding enantiomers by fractional crystallization of their brucine salts. Optically pure (*S*)-(−)-2 and (*S*)-(−)-3 were obtained by crystallizing the diastereomeric mixture from ethanol. (*R*)-(+)-2 and (*R*)-(+)-3 were isolated from the mother liquors of the preceding crystallization.



1: $R_1 = R_2 = CH_3$
2: $R_1 = CH_3$, $R_2 = H$
3: $R_1 = C_2H_5$, $R_2 = H$

The absolute configuration of **2** was known[11] whereas the configuration of the compound **3** was established by

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