A Comparative Study of the Inhibitory Activity of Clofibrate and Related Analogs on Lipoprotein Lipase *in Vitro*†

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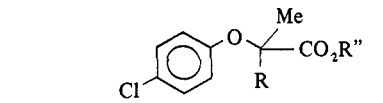
The antagonist activity of clofibrate (**1**) and some open-chain and cyclic analogs on human postheparin serum lipoprotein lipase (LPL), guinea pig postheparin serum LPL, and rat heart LPL *in vitro* is reported. For these studies we made use of a carbonyl-labeled triolein-^{14}C assay system. The results are compared to a titration assay for released free fatty acids and significant differences *in vitro* are demonstrated. Structure–activity relationships and the significance of the inhibitory effect observed *in vitro* are discussed.

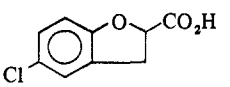
Abnormal lipid metabolism plays a major role in the development of atherosclerosis and coronary artery disease. Abnormal arterial wall lipid metabolism[1,2] and abnormal serum lipoprotein metabolism[3] have both been implicated. The latter, with the associated elevations in serum lipid levels, has been well established as a major risk factor in these diseases.[4] Although there is not yet adequate proof that lowering serum lipoprotein concentrations decreases the rate of deposition of lipid in arterial walls,[5] successful use of clofibrate [ethyl 2-(4-chlorophenoxy)-2-methylpropionate, **1**] is associated with the disappearance of cutaneous xanthomas in patients with hyperlipidemias types III and V.[6] Recent work has shown that the incidence of nonfatal myocardial infarction is decreased by **1** even with the persistence of varying degrees of hyperlipidemia in some treated individuals.[7]

Since **1** undergoes rapid hydrolysis *in vivo* and *in vitro*, the resulting acid **2** is presumed to be the active drug.[8,9] Studies *in vivo* and *in vitro* with **1** or **2** suggest that the latter may exert its effect by multiple modes of action (for applicable references through 1968, see ref 10). For example, it has been suggested that the effects of **1** or **2** may be indirect and related to a redistribution of thyroxine from plasma to liver,[11,12] to a direct effect on liver metabolism,[13-15] to inhibition of cholesterol biosynthesis,[16] to an effect on carbohydrate metabolism,[11,17] to a specific inhibition of acetyl CoA carboxylase,[18] to inhibition of hepatic triglyceride formation,[19] to inhibition of free fatty acid (FFA) mobilization from adipose tissue,[20-23] to inhibition of very low density lipoprotein (VLD) release,[24-26] and to increased VLD removal.[27,28]

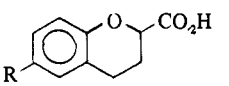
The enzyme, lipoprotein lipase (LPL), is a key enzyme of lipid transport in lipoproteins and could be crucial in any relationship of abnormal serum lipoprotein metabolism to atherogenesis. The function of LPL is to hydrolyze the triglycerides (TG) contained in chylomicrons and VLD. In this article we report our data on the inhibition *in vitro* of LPL by **1**, **2**, L-2-(4-chlorophenoxy)propionic acid (L-**3**), D-2-(4-chlorophenoxy)propionic acid (D-**3**), 2-(4-chlorophenoxy)-2-methylbutyric acid (**4**), 5-chloro-2,3-dihydro-2-benzofurancarboxylic acid (**5**), 6-chlorochroman-2-carboxylic acid (**6**), and chroman-2-carboxylic acid (**7**). Since **1** readily undergoes hydrolysis by serum and tissue esterases and the liberated acid **2** may be titrated along with FFA in titration methods for the determination of LPL activity, adaptation and utilization of an assay procedure which employs radiolabeled TG was essential to evaluate the effect of **1** *in vitro*. We have employed such an assay and have used it to assess the relative potency of the related carboxylic acids **3**–**7** on the LPL enzyme system.



1, R = Me; R" = Et
2, R = Me; R" = H
L-**3**, R = H; R" = H; L-(*S*)-(−)-isomer
D-**3**, R = H; R" = H; D-(*R*)-(+)-isomer
4, R = Et; R" = H; racemic



5, racemic



6, R = Cl; racemic
7, R = H; racemic

Experimental Section

The syntheses for experimental hypolipidemic drugs have been reported previously.[10,23,29,30] Clofibrate (**1**) was obtained in therapeutic form from Ayerst Laboratories, New York, N. Y.

Human LPL from Postheparin Serum. As our major source of LPL, human postheparin serum was obtained from subjects who had fasted for 16 hr. Heparin (Evans Medical Limited, Liverpool, England) in a dose of 10 units/kg was injected *via* an antecubital vein. After 10 min, blood was withdrawn into a glass syringe from an antecubital vein in the opposite arm and placed in a glass test tube. After clotting, the blood was centrifuged at 1000*g* for 10 min. The postheparin serum was removed and frozen until use.

Guinea pig postheparin serum was obtained from fasted (16 hr) guinea pigs (English variety) weighing 600–800 g. The guinea pigs were anesthetized with Et_2O; heparin, 20 units/kg, was injected into a mesenteric vein. During the 2–3-min period following injection of heparin, 10–20 ml of blood was rapidly withdrawn into a glass syringe from the abdominal aorta and placed in a glass test tube. The blood was allowed to clot; following centrifugation at 1000*g*, the postheparin serum was removed, pooled, and frozen until use.

Rat Heart (RH) LPL. RH acetone powders also were used as a LPL preparation since this is a good source of tissue enzyme.[31] Long Evans rats weighing 350 g and over were fasted for 16 hr and anesthetized with Et_2O. Two rat hearts were removed for the same preparation and placed in an ice-cold 250-ml Waring Blendor homogenizer. Ice-cold acetone (150 ml) was added and the hearts were homogenized for 30–60 sec. The finely homogenized suspension was filtered on Whatman No. 1 filter paper in a Büchner funnel. The precipitate was washed twice with 20 ml of acetone (0°) and twice with ether (20°), air-dried under reduced pressure for 5 min, and placed in a dessicator under reduced pressure for 2 hr. The RH acetone powders were extracted in 0.025 *M* NH_4Cl buffer, pH 8.6, at 0° and allowed to stand for 1 hr with frequent stirring. Each acetone powder was suspended in 7.0 ml of the NH_4Cl buffer. The particulate matter was removed by centrifugation at 1000*g* for 10 min at 4°, and the cloudy supernatants were removed and combined.

Radiolabeled TG Assay for LPL Activity. For these purposes,

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we have modified and adapted the previously reported technique of Schotz, *et al.*[32] The LPL enzyme activity was assayed as follows. Carbonyl-labeled triolein-^{14}C in heptane (1 μCi) (Amersham-Searle, Arlington Heights, Ill.) was dried in a test tube under a stream of N_2. Nonradiolabeled triolein (0.133 g) obtained from Sigma (St. Louis, Mo.) was weighed into the same test tube and 0.675 ml of 1.5% Triton-X-100 (Rohm and Haas, Dayton, Ohio), 5.25 ml of 15% bovine serum albumin (Sigma), pH 8.6 (FFA acceptor), 2.25 ml of 0.025 *M* NH_4Cl buffer, pH 8.6, and 1.425 ml of 1.35 *M* Tris-HCl buffer, pH 8.6, were added. The mixture was sonicated with a Biosonik IV microprobe (Bronwill Scientific, Inc., Rochester, N. Y.) for 4 min at maximum power output on ice. To 0.65 ml of the sonicated mixture were added 0.15 ml of postheparin serum of LPL enzyme extract and 0.2 ml of 0.01 *M* PO_4–0.005 *M* ethylenediamine tetraacetate (EDTA) buffer, pH 7.5, or 0.2 ml of a solution of the experimental drug in this buffer to give a final volume of 1.0 ml and a final concentration of drug expressed in terms of micromole per milliliter.

The reaction mixture was incubated for 30 min at 37°; the reaction was terminated by the addition of 5 ml of a solution containing 2-propanol (40 parts), 2 *N* H_2SO_4 (1 part), and heptane (10 parts). The lipids were extracted by addition of 2.0 ml of H_2O and 3.0 ml of heptane, followed by shaking for 3 min and centrifugation. The heptane phase (3 ml), which contained FFA and any unhydrolyzed TG, was removed. KOH (3 ml, 0.1 *N*) was added, shaken, and centrifuged. The KOH phase (2 ml) containing the released FFA was removed *via* a syringe attached to a long needle and placed in a β scintillation counting vial. To this solution was added 15 ml of Instagel (Packard Instrument Co., Inc., Downers Grove, Ill.). The radiolabeled FFA's released by LPL hydrolysis were counted utilizing a β liquid scintillation counter. One unit of LPL activity was expressed as 1.0 μmol of FFA released/ml of enzyme/hr.

Assay of LPL Activity by Titration. LPL activity was assayed in triplicate in order to compare the results obtained utilizing titration *vs.* the radioactive method in the presence of 1 *in vitro*. The TG substrate consisted of unlabeled triolein utilized in exactly the same way as described above in the absence of triolein-^{14}C. The assay mixture was incubated for 30 min at 37°. Released FFA's were extracted by the method of Dole,[33] as modified by Trout and coworkers.[34] The FFA's were then titrated by a modification of the method of Salaman and Robinson.[35] One unit of LPL activity is equivalent to 1.0 μmol of FFA released/ml of postheparin serum/hr. Titrations were made on aliquots removed at 0 and 30 min from the assay system; the reaction was shown previously to be linear over a 60-min period.

Results

The effect of increasing concentrations of **1** on human LPL activity was studied using both the titrimetric and radiolabeled TG methods. As the concentration of **1** progressively was increased, a dose-dependent decrease in human LPL activity *in vitro* was observed (Table I). This was demonstrated with both assay methods. At each concentration of **1** used, LPL activity, as determined by titration, indicated a higher value than the one determined by the radiolabeled TG method. The titrimetric method may give somewhat higher values than the radiolabeled TG method because of some extraction of previously unesterified FFA. However, the higher values obtained utilizing the titration method probably result in part from the titration of **2** formed by the hydrolysis of **1** in the *in vitro* assay by serum esterases. The hydrolysis product of **1**, namely **2**, would appear in the same extractions as FFA isolated in the assay system and be titrated. While both methods suggest that **1** in high *in vitro* concentrations has an inhibitory effect on human LPL activity, the radiolabeled method is the most reliable. The rate of hydrolysis of **1** does not influence the final determination of FFA in the radiolabeled assay since it only measures released and labeled long-chain FFA. The results employing guinea pig LPL, with the TG substrate activated by human VLD to further increase enzyme activity, emphasize this point (Table II). In this case increasing concentrations of **1** resulted in a marked "apparent increase" in LPL activity by the titrimetric method which is not explainable on the basis of extraction of previously unesterified FFA. When LPL activity was measured utilizing radiolabeled TG, the same dose-dependent increase in LPL activity was observed with guinea pig LPL as with human LPL. The "apparent increase" in guinea pig LPL activity when determined by the tritrimetric method probably represents an increased rate of hydrolysis of **1** by guinea pig serum esterases not present in sufficient concentration

Table I. Comparison of the Titration and Radiolabeled TG Methods for Determination of Human Lipoprotein Lipase Activity in the Presence of Clofibrate (1)

Concn of 1, μmol/ml	Expt no.[a]	Titration method[b]	Radiolabeled TG method[b]
None	1	29.8, 30.5	24.7, 22.6
	2	27.5, 27.4	15.6, 17.6
4.67	1	26.9, 26.7	20.3, 18.2
	2	24.1, 24.6	13.0, 10.1
46.7	1	20.6, 21.8	9.3, 9.4
	2	19.0, 19.2	5.9, 4.3
467	1	11.5, 14.9	4.6, 2.6
	2	9.8, 11.3	2.1, 1.7

[a]Two separate experiments were carried out in duplicate. Since different postheparin LPL preparations were employed, the results were not averaged. [b]Values are given in duplicate for each experiment.

Table II. Comparison of the Titration and Radiolabeled TG Methods for Determining Guinea Pig Lipoprotein Lipase Activity in the Presence of Clofibrate (1)

Concn of 1, μmol/ml	VLD protein added, mg	Titration method[a]	Radiolabeled TG method[b]
None	None	27.2 ± 0.6[b]	16.3 ± 0.2[b]
None	0.02	32.5 ± 0.4	22.3 ± 0.9
4.67	0.02	41.4 ± 0.9	18.8 ± 0.6
46.7	0.02	59.8 ± 2.5	9.0 ± 0.8
467	0.02	88.2 ± 2.0	2.9 ± 1.0

[a]Values represent micromoles of FFA released per milliliter of postheparin serum per hour. [b]Values represent the mean of four determinations ± S.E. Data are from the results obtained in two experiments in duplicate using the same postheparin preparation.

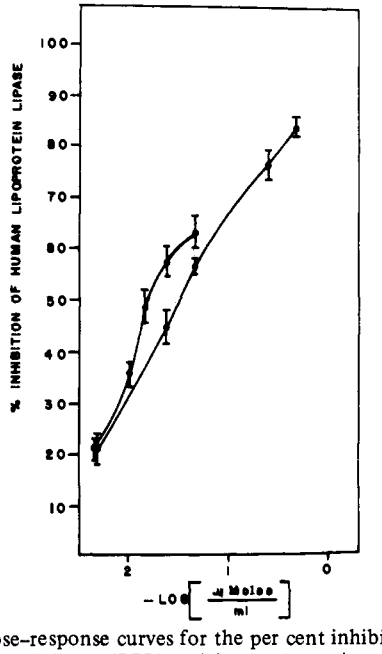

Figure 1. Dose–response curves for the per cent inhibition of human lipoprotein lipase (LPL) activity *in vitro* using carbonyl-labeled triglyceride: upper curve = 2-(4-chlorophenoxy)-2-methylpropionic acid (**2**); lower curve = ethyl 2-(4-chlorophenoxy)-2-methylpropionate (**1**).

Table III. Inhibition of Human Lipoprotein Lipase by Analogs of 2-(4-Chlorophenoxy)-2-methylpropionic Acid (2)

Compd no.	Concn, μmol/min	% inhibition[a]
2	14.0	48.8 ± 3.3[b]
L-3	15.6	42.8 ± 3.5
D-3	15.6	20.5 ± 1.0
4	12.7	44.3 ± 1.6
5	15.6	33.8 ± 1.7
6	14.0	58.7 ± 1.4
7	17.5	33.7 ± 2.3

[a]Values are calculated as a per cent of the maximal response observed in the absence of inhibitors. [b]Values represent the mean of six determinations ± S.E. Data are from the results obtained in three experiments in duplicate.

Table IV. Comparison of the Titration and Radiolabeled TG Methods for Determination of Rat Heart Lipoprotein Lipase Activity in the Presence of Clofibrate (1)

Concn of 1, μmol/ml	VLD protein added, mg	Titration method[a]	Radiolabeled TG method[a]
None	None	2.47 ± 0.08[b]	0[b]
None	0.02	14.7 ± 0.2	5.3 ± 0.7
4.67	0.02	21.1 ± 0.4	4.5 ± 0.7
46.7	0.02	23.4 ± 0.5	2.9 ± 0.3
467	0.02	29.0 ± 0.8	2.1 ± 0.2

[a]Values represent micromoles of FFA released per milliliter of rat heart enzyme extract per hour. [b]Values represent the mean of four determinations ± S.E. Data are from the results obtained in two experiments in duplicate using a rat heart acetone powder extract.

in human serum, thereby affording increased amounts of 2 which is titrated along with FFA.

The dose-response curves for the inhibition *in vitro* of human LPL by 1 and 2 are shown in Figure 1. The results obtained in these studies using 1 at a concentration range of 4.67-467 μmol/ml show that this drug significantly inhibits the LPL enzyme system at these concentrations and that the inhibition is dose dependent. A similar dose-response curve for the per cent inhibition of human LPL was obtained using the free acid 2, but with 2 the inhibitory effect was somewhat greater. The maximum concentration of 2 used was 46.7 μmol/ml; at higher concentrations, such carboxylic acids exceed the buffer capacity of the assay system and the pH drops below the pH optimum for LPL (pH 8.6). Therefore, this upper limit was placed in order to avoid the complication of LPL inhibition by a pH effect alone. Possibly the increased inhibition by 2, which is statistically significant ($p < 0.05$), reflects the necessity of 1 to undergo hydrolysis to the active hypolipidemic agent 2. Since all of the ester 1 may not undergo hydrolysis during the incubation period, the lower inhibitory effect of 1 may simply be due to a lower concentration of 2 in the incubation medium.

The inhibitory effects of various carboxylic acid analogs of 2 on human LPL activity *in vitro* are compared in Table III. To obtain these data the same pool of frozen human postheparin serum was used as the source of human LPL. Frozen postheparin serum has been shown to maintain a stable level of LPL activity for several weeks.‡ Stereoselective LPL inhibitory activity was observed for the desmethyl analogs of 2. The L-3 isomer exhibited an inhibitory effect at 15.6 μmol/ml, nearly equal to the antagonist effect of 2. However, the D-3 isomer was approximately one-half as potent at this concentration. The *dl*-ethyl homolog of 2, namely 4, exhibited about the same inhibitory effect as L-3 or 2. The dihydrobenzofuran 5, which is a *dl* cyclic analog of 3, was slightly less effective as an antagonist of human LPL. However, when the five-membered ring of 5 is increased to a six-membered ring, thereby resulting in 6-chlorochroman-2-carboxylic acid (6), the most potent antagonist of all the compounds studied was obtained. Removal of the Cl atom from 6 results in the deschlorochroman 7 which shows a decreased inhibitory effect. Chroman 7 exhibits an inhibitory effect at 17.5 μmol/ml, equivalent to the effect of dihydrobenzofuran 5 assayed at 15.6 μmol/ml.

In addition to our studies with serum LPL, it was of interest to determine whether 1 blocked tissue LPL activity. RH-LPL extract was prepared as described in the Experimental Section. When this enzyme preparation was assayed utilizing TG substrate alone, no LPL activity was obtained. Therefore, human VLD's prepared by methods previously described[36] were added to the assay system as TG substrate activator in a concentration of 0.02 mg of VLD protein/ml. With this tissue enzyme source activated by human VLD, increasing concentrations of 1 caused a marked "apparent increase" in LPL activity by the titrimetric method which is not explainable on the basis of extraction of previously unesterified FFA (Table IV), just as was observed with guinea pig postheparin serum. However, when LPL activity was measured utilizing radiolabeled TG, the same dose-dependent decrease in LPL activity was observed with RH-LPL as with human and guinea pig LPL. The "apparent increase" in RH-LPL activity when determined by the titrimetric method probably represents an increased rate of hydrolysis of 1 by RH tissue esterases, resulting in an increased amount of 2 which is extracted and titrated along with FFA.

Discussion

The results presented in this paper demonstrate that 1 or 2 exerts a dose-dependent inhibition of human serum LPL, guinea pig serum LPL, and RH-LPL *in vitro* at concentrations which start four times greater than the therapeutic concentration (approximately 1.0 μmol/ml) of 1 *in vivo*. This is important to the understanding and interpretation of studies of 1 and related carboxylic acids and esters in experimental animals, especially when the effect of large doses on lipid metabolism is evaluated. TG hydrolysis by the key enzyme of circulating lipoprotein metabolism (LPL) could be markedly inhibited by these or related drugs at sufficiently high concentrations. Such a block might significantly alter the observed effect of such analogs on serum lipid levels since lipolysis of VLD and chylomicrons would be diminished. Theoretically, this could be significant to the prevention of atherosclerosis if low density lipoprotein (LPL) infiltration of the arterial intima is a major pathogenic factor; VLD breakdown would be inhibited with a resultant decrease in LDL formation.[37] Such infiltration of the arterial intima by LDL has been shown to occur.[38,39]

In this regard, recent studies have shown an effect by 1 in the prevention of ischemic heart disease which does not appear related to serum lipid levels.[7,40,41] While inhibition of VLD conversion to LDL by 1 is an attractive hypothesis to consider with regard to these exciting epidemiological results, further speculation is not warranted owing to the high concentrations of analogs required to block this reaction *in vitro*. In addition, *in vivo* and *in vitro* results have failed to correlate in other tests with these analogs. For example, whereas there seems to be a correlation of D- and L-3 desmethyl isomers of 1 or 2 in terms of inhibition of cho-

‡T. F. Whayne, unpublished results.

lesterol biosynthesis *in vitro* and their hypocholesterolemic activity *in vivo*,[10,30] no such apparent correlation exists when unpublished results recently obtained *in vivo* are compared with analog inhibition of lipolysis in rat adipose tissue *in vitro*.[23,29] With regard to the effect of **1** on VLD metabolism, the results *in vivo* have not been consistent with LPL inhibition and drug treatment has been shown to decrease VLD while having variable effects on LDL or even increasing their concentration.[42]

The differences in results obtained using the radiolabeled TG and the titrimetric assays for LPL activity are of particular significance. Previous investigators, studying the effects of **1** *in vivo* or *in vitro* on LPL activity, assayed released FFA by the titration method of Dole.[33] Since the hydrolysis product of **1**, namely **2**, could appear during the course of incubation *in vitro* for the LPL assay, this may account for some of the differences in results previously reported. For example, whereas Grafnetter and Geizerová[43] reported that heart LPL activity was strongly inhibited *in vivo* and that postheparin serum LPL and epididymal fat tissue activity were inhibited *in vitro*, other investigators[44,45] have published data supporting the activation of LPL by **1** or **2**.

Our data emphasize the importance of the radiolabeled TG method for determination of LPL activity in order to explore further the effect of **1** or **2** or related analogs on this key enzyme of lipid metabolism. This applies specifically to those assays in which **1** is added *in vitro* since hydrolysis of **1** would introduce an error of apparent increased LPL activity into the titration method. This was especially marked in the case of guinea pig postheparin serum (Table II) where a very potent serum esterase must be operative. With the radiolabeled TG method, this problem is obviated since any ^{14}C-FFA released must be derived from the enzymatic hydrolysis of ^{14}C-TG by LPL.

While it is possible that very high concentrations of **1**, **2**, or related analogs may exert a nonspecific type of interaction on the lipase enzymes *in vitro* owing to the detergent nature of these substances, all of the activity of analogs studies (Table III) cannot be accounted for on this basis. For example, desmethyl analogs L-**3** and D-**3** would be expected to have identical detergent properties, but in this assay the L-**3** isomer is approximately twice as potent as D-**3**. This information, coupled with the observation that DL-**8** exerts the greatest inhibition of all analogs studied, suggests that L-**8** alone or analogs of L-**8** may exert an even greater inhibitory effect and have potential therapeutic use in the treatment of ischemic heart disease.

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