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# Hypolipidemic drugs are inhibitors of phosphatidylcholine synthesis*

(cholinephosphotransferase/lysolecithin acyltransferase/liver microsomes/clofibrate/atherosclerosis)

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**ABSTRACT** **Clofibric acid (CPIB) and several other systemic hypolipidemic drugs are shown to block phosphatidylcholine synthesis by inhibiting cholinephosphotransferase (Cho*P*Tase; CDPcholine:1,2-diacylglycerol cholinephosphotransferase, EC 2.7.8.2) and particularly lysolecithin acyltransferase (LLAcylTase; acyl-CoA:1-acylglycero-3-phosphocholine *O*-acyltransferase, EC 2.3.1.23) of rat liver microsomes. Whereas millimolar drug concentrations are required to affect *de novo* lecithin synthesis catalyzed by Cho*P*Tase, reacylation of lysolecithin by LLAcylTase is inhibited at micromolar levels. Increasing effectiveness in Cho*P*Tase inhibition is observed in the series CPIB, SaH-42-348, tibric acid, S-321328, WY-14643, S-8527, and DH-990, with $IC_{50}$ ranging from 22 mM (CPIB) to 0.3 mM (DH-990). LLAcylTase inhibition by the hypolipidemic drugs follows the same general pattern, but $IC_{50}$ concentrations range from 9 mM (CPIB) to 40 μM (DH-990). The agents inhibit Cho*P*Tase ($K_i$, 25–0.25 mM) and LLAcylTase ($K_i$, 10–0.025 mM) noncompetitively. The data suggest that inhibition of phosphatidylcholine synthesis, particularly by the LLAcylTase pathway, may be related to a drug's effectiveness in decreasing serum triglyceride and cholesterol levels by blocking lipoprotein synthesis.**

Atherosclerosis manifests itself in changes in serum lipid and serum lipoprotein metabolism and in an apparent breakdown of the mechanisms that regulate normal lipid transport processes (1, 2). In an attempt to modulate or control lipid changes associated with the disease and possibly to reverse atherosclerosis symptoms, a number of hypolipidemic drugs have been tested and some have found clinical application.

Clofibrate (ethyl *p*-chlorophenoxyisobutyrate) has been studied and used extensively (3–5), but less is known about the biochemical and physiological effects of various other hypolipidemic agents such as tibric acid [2-chloro-5-(cis-3,5-dimethylpiperidinosulfonyl)benzoic acid] (4, 6–8), SaH-42-348[1-methyl-4-piperidyl bis-(*p*-chlorophenoxy)acetate] (9, 10), S-321328 [(4*R*)-4-(2,3,4,6,6α*β*,7,8,9,9a*α*,9b*β*-decahydro-6a*β*-methyl-3-oxo-1*H*-cyclopenta[*f*]-quinolin-7*β*-yl)valeric acid (11), WY-14643 ([4-chloro-(2,3-xylidino)-2-pyrimidinylthio]acetic acid) (4, 12–14), S-8527 (1,1-bis-[4′-(1″-carboxy-1″-methylpropoxy)phenyl]cyclohexane) (15–19), and DH-990 (2-[3,5-di-(*t*-butyl-4-hydroxyphenyl)thio]hexanoic acid) (4). The effectiveness of these agents generally has been attributed to their ability to interfere with certain phases of fatty acid (15, 18, 20–23), triglyceride (8, 23–31), and cholesterol (11, 14, 15, 32–39) metabolism, but the precise or prevalent mode of action of any of these drugs is not understood.

We previously reported (40, 41) that two of the key enzymes of phosphatidylcholine synthesis in mammalian systems—namely, cholinephosphotransferase (Cho*P*Tase; CDPcholine:1,2-diacylglycerol cholinephosphotransferase, EC 2.7.8.2) (42) and lysolecithin acyltransferase (LLAcylTase; acyl-CoA:1-acylglycero-3-phosphocholine *O*-acyltransferase, EC 2.3.1.23) (43)—are inhibited by centrophenoxine (CPO; *N*,*N*-dimethylaminoethyl *p*-chlorophenoxyacetate) and neophenoxine (NPO; (*N*,*N*-dimethylaminoethyl *p*-chlorophenoxyethyl ether). The structural similarity of CPO and clofibrate prompted us to study the effect of clofibric acid (CPIB; *p*-chlorophenoxyisobutyric acid) and several other hypolipidemic drugs on the two enzymes of choline phospholipid metabolism. We found that CPIB, tibric acid, SaH-42-348, S-321328, S-8527, WY-14643, and DH-990 all inhibited phosphatidylcholine synthesis in rat liver microsomes by the Cho*P*Tase and particularly by the LLAcylTase route. The drugs' effectiveness in inhibiting Cho*P*Tase and LLAcylTase correlated well with their known hypolipidemic potencies.

## MATERIALS AND METHODS

**Hypolipidemic Agents.** CPIB was from ICN Pharmaceuticals. S-8527 was kindly provided by Sumitomo Chemical (Osaka, Japan). Tibric acid was obtained from Pfizer. S-321328 was from Shionogi (Osaka, Japan). WY-14643 was received from the Wyeth Laboratories. DH-990 was from Dow. These compounds were converted to their sodium salts by neutralization with NaOH. SaH-42-348 was obtained from Sandoz Pharmaceutical and was used as such. The agents were freshly dissolved in buffers.

**Substrate.** 1,2-Diacyl-*sn*-glycerol was prepared from rat liver phosphatidylcholine by hydrolysis with phospholipase C from *Clostridium welchii* (41, 44). CDP[*methyl*-$^{14}$C]choline was purchased from Amersham and was diluted with unlabeled CDPcholine (Sigma). 1-Acyl-*sn*-glycero-3-phosphocholine was prepared from purified rat liver phosphatidylcholine by the action of phospholipase $A_2$ (*Ophiophagus hannah* venom, Miami Serpentarium Laboratories, Miami, FL) according to established procedures (45). [1-$^{14}$C]Oleoyl-CoA (58 mCi/mmol; 1 Ci = $3.7 \times 10^{10}$ becquerels; Dhom Products, Hollywood, CA) was diluted with unlabeled material (Sigma) before use.

**Enzyme Assays.** Microsomal fractions from liver of male Sprague–Dawley rats were prepared as described (40). Protein was measured by the method of Lowry *et al.* (46) with bovine serum albumin as a standard. All data given are means of at least three independent assays.

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Abbreviations: Cho*P*Tase, cholinephosphotransferase; LLAcylTase, lysolecithin acyltransferase; CPO, centrophenoxine; NPO, neophenoxine; CPIB, clofibric acid.

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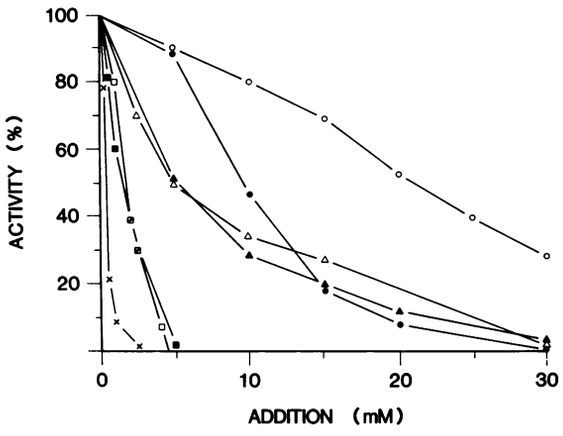


FIG. 1. ChoPTase activity of rat liver microsomes in the presence of various hypolipidemic agents: ○, CPIB; ●, SaH-42-348; △, S-321328; ▲, tibric acid; □, S-8527; ■, WY-14643; ×, DH-990.

ChoPTase activity was determined according to Coleman and Bell (47) as modified in our laboratory (41, 48). The assay, carried out in the presence of bovine serum albumin (1 mg/ml), used 100 nmol of rat liver 1,2-diacyl-*sn*-glycerol, 100 μM CDP[*methyl*-$^{14}$C]choline (2.5 mCi/mmol), and 30 μg of protein in a final volume of 200 μl (37°C, 10 min). The labeled phosphatidylcholine formed was isolated and quantified as described (48).

LLAcylTase activity was measured by the procedure described by Subbaiah *et al.* (49) as modified in our laboratory (50). The assay system contained 100 μg of microsomal protein, 0.4 μmol of 1-acyl-*sn*-glycero-3-phosphocholine, 15 μmol of $MgCl_2$, 160 μmol of phosphate buffer (pH 7.4), and 50 nmol of [1-$^{14}$C]oleoyl-CoA (1,400 dpm/nmol) in a final volume of 0.4 ml. Incubations were carried out at 37°C for 5 min and were terminated by vortexing with 3 ml of isopropyl ether. The radioactivity associated with phosphatidylcholine was determined after extraction with isopropyl ether/*n*-butanol, 60:40 (vol/vol) (50).

## RESULTS

The effect of several hypolipidemic drugs on ChoPTase and LLAcylTase activities of rat liver microsomes was studied. ChoPTase and LLAcylTase activities were determined by following incorporation of CDP[*methyl*-$^{14}$C]choline or [1-$^{14}$C]-oleoyl-CoA, respectively, into phosphatidylcholine.

**Inhibition of ChoPTase Activity.** CPIB inhibited ChoPTase-catalyzed *de novo* synthesis of phosphatidylcholine in a concentration-dependent manner. Yet, this inhibition required millimolar drug concentrations, and 22 mM CPIB caused only 50% inhibition (Fig. 1). SaH-42-348, which is known to lower serum lipids more effectively than does clofibrate (9, 10), proved to be a better inhibitor ($IC_{50}$, 10 mM) of ChoPTase, particularly at higher concentrations. S-321328 (11), in turn, was more effective ($IC_{50}$, 5 mM) than CPIB or SaH-42-348. Tibric acid, which has been clinically successful in the treatment of hypertriglyceridemia and nonfamilial hypercholesterolemia (6), exerted an inhibitory effect similar to that of S-321328. It completely blocked ChoPTase activity at 30 mM and produced 50% inhibition at 5 mM.

The most effective inhibitors of ChoPTase activity were S-8527, WY-14643, and DH-990. S-8527 decreases serum lipids in rodents (at 1 mg/kg per day) 20–30 times more effectively than does clofibrate (15, 17, 18) and inhibits hepatic lipoprotein

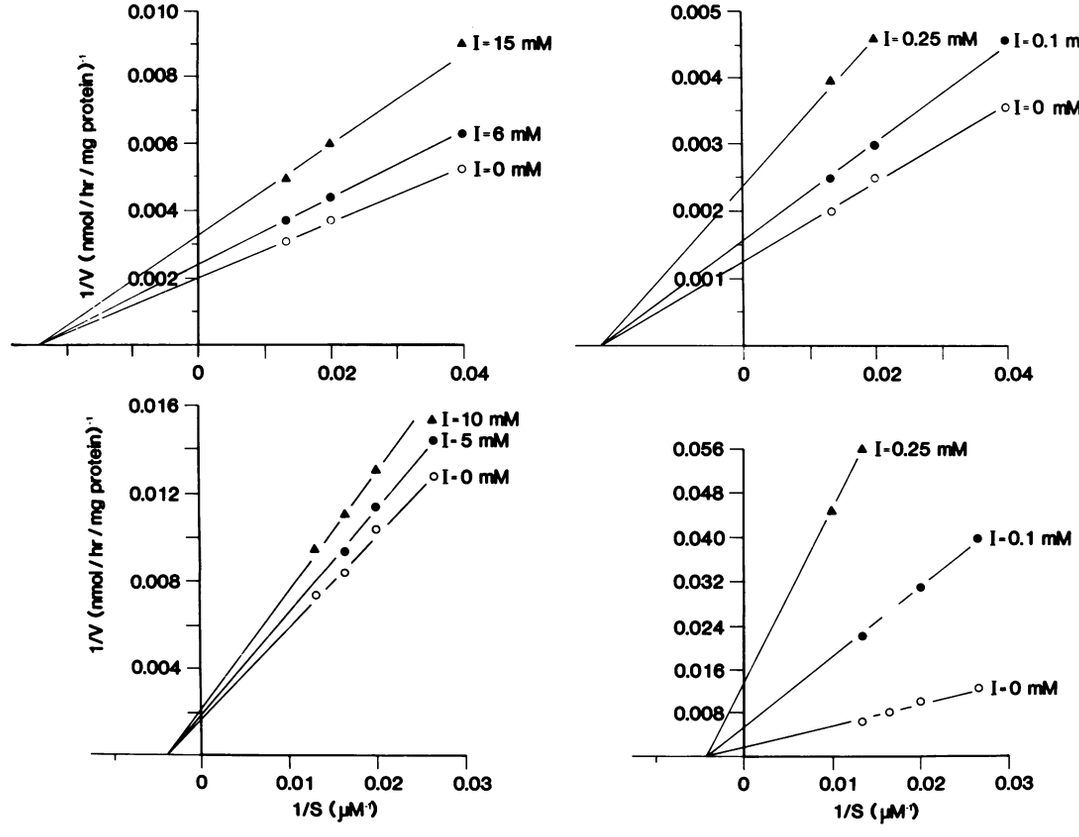


FIG. 2. Double-reciprocal plots of ChoPTase inhibition with CDPcholine as substrate (*Upper*) and LLAcylTase inhibition with oleoyl-CoA as substrate (*Lower*) by CPIB (*Left*) or DH-990 (*Right*). I, inhibitor concentration.

Table 1. $IC_{50}$ and $K_i$ values for inhibition of Cho*P*Tase and LLAcylTase by various hypolipidemic drugs

| Drug added | $IC_{50}$, mM | | $K_i$, mM | |
|---|---|---|---|---|
| | Cho*P*Tase | LLAcylTase | Cho*P*Tase | LLAcylTase |
| CPIB | 22 | 9 | 25 | 10 |
| SaH-42-348 | 10 | 3.2 | 2 | 0.6 |
| S-321328 | 5 | 0.8 | — | — |
| Tibric acid | 5 | 0.4 | 3.2 | 0.8 |
| S-8527 | 1.7 | 0.13 | 2.2 | 0.8 |
| WY-14643 | 1.4 | 0.10 | 1.5 | 0.65 |
| DH-990 | 0.3 | 0.04 | 0.25 | 0.025 |

synthesis (19); it showed an $IC_{50}$ of 1.7 mM and complete Cho*P*Tase inhibition was attained at <5 mM (Fig. 1). WY-14643, an effective hypocholesterolemic agent at low levels (1 mg/kg per day) (12), exerted significant Cho*P*Tase inhibition ($IC_{50}$, 1.4 mM) and showed a concentration dependence similar to that of S-8527. DH-990, which lowers serum lipid levels most effectively but has considerable hepatomegalic side effects (4), was the most potent Cho*P*Tase inhibitor we tested. It blocked cholinephosphate transfer to diglyceride almost completely at 1 mM, and 50% inhibition was attained at 0.3 mM. In fact, the Cho*P*Tase inhibitory effect of DH-990 at 0.4 mM was greater than that of CPIB at 30 mM.

**Kinetics of Cho*P*Tase Inhibition.** The mode of drug-induced Cho*P*Tase inhibition was followed as function of CDPcholine concentration. Double-reciprocal plots of reaction velocities versus CDPcholine concentrations demonstrated that all agents tested inhibited the Cho*P*Tase reaction noncompetitively with respect to CDPcholine. Fig. 2 depicts representative plots for CPIB, the least active agent, and DH-990, the most potent. Inhibition constants, $K_i$, determined from the plot of the slopes against inhibitor concentrations, ranged from about 25 mM for CPIB to 0.25 mM for DH-990. $K_i$ and $IC_{50}$ values for the agents tested are compiled in Table 1.

**Inhibition of LLAcylTase Activity.** The hypolipidemic drugs showed pronounced and characteristic differences in their inhibitory potency on the acylation of lysolecithin by rat liver microsomal LLAcylTase. All agents tested inhibited LLAcylTase far more effectively than they inhibited Cho*P*Tase.

CPIB exerted the weakest inhibitory effect on LLAcylTase activity (Fig. 3) as was the case for Cho*P*Tase. Approximately 9 mM CPIB was required to decrease LLAcylTase activity by 50%, compared to an $IC_{50}$ concentration of 22 mM for

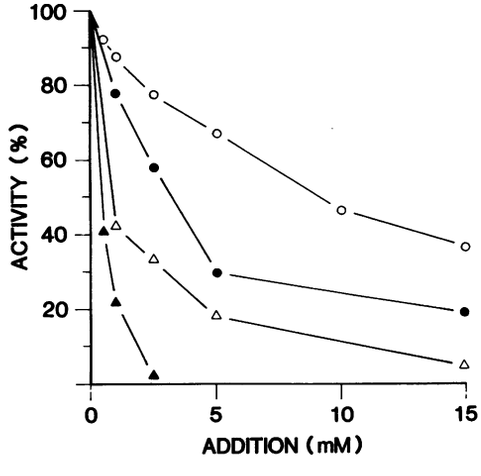


FIG. 3. LLAcylTase activity of rat liver microsomes in the presence of CPIB (○), SaH-42-348 (●), S-321328 (△), or tibric acid (▲).

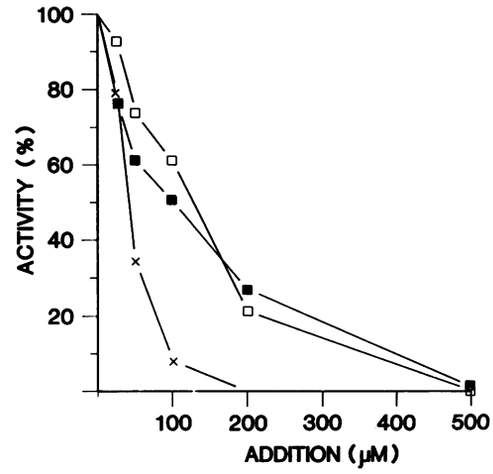


FIG. 4. LLAcylTase activity of rat liver microsomes in the presence of S-8527 (□), WY-14643 (■), or DH-990 (×).

Cho*P*Tase. SaH-42-348 and S-321328 showed $IC_{50}$ values of 3.2 and 0.8 mM, respectively, whereas tibric acid caused 50% inhibition at 0.4 mM and complete inhibition at less than 3 mM.

The most effective Cho*P*Tase inhibitors—S-8527, WY-14643, and DH-990—were also most effective against LLAcylTase. The three agents completely prevented reacylation of lysolecithin at <500 μM (Fig. 4). The $IC_{50}$ concentrations were 130 μM for S-8527, 100 μM for WY-14643, and 40 μM for DH-990.

**Kinetics of LLAcylTase Inhibition.** The effect of the drugs on LLAcylTase activity was followed as function of oleoyl-CoA and lysolecithin concentration. With either substrate, double-reciprocal plots showed that LLAcylTase inhibition was noncompetitive for all the drugs. Representative double-reciprocal plots for the least and the most potent inhibitor are shown in Fig. 2. $K_i$ values ranged from 10 mM (CPIB) to 25 μM (DH-990) (Table 1).

## DISCUSSION

In the present study we have shown that nonsequestrant hypolipidemic drugs inhibit Cho*P*Tase and LLAcylTase activities of rat liver microsomes. Because of the great affinity of such drugs for the microsomal membrane (41), and because of the drugs' particular potency in respect to LLAcylTase, agents such as S-8527, WY-14643, and DH-990 should become useful as modulators of choline phospholipid metabolism and of membrane properties in general. In fact, we have found that incorporation of $^{14}C$-labeled choline into lysolecithin of cultured hepatoma cells can significantly be stimulated by use of these LLAcylTase inhibitors (unpublished data).

Our observation that CPIB, SaH-42-348, S-321328, tibric acid, S-8527, WY-14643, and DH-990 all have the ability to inhibit the enzymes of two major routes of phosphatidylcholine synthesis and to lower serum lipid levels may suggest that their potency as Cho*P*Tase and LLAcylTase inhibitors may be related to their effectiveness as hypolipidemic drugs. Although subcellular concentrations of the drugs have not been measured under *in vivo* conditions, it is intriguing to note that the drugs' hypolipidemic potencies increase in the same order and to a similar extent as do Cho*P*Tase and LLAcylTase inhibitions in our *in vitro* system (see $IC_{50}$ and $K_i$ values listed in Table 1). For example, SaH-42-348 has been reported to be 8–9 times more effective than CPIB in decreasing serum lipids (9, 10) whereas S-8527 (15–18) and WY-14643 (12, 38), particularly at low drug concentrations, were up to 180 times more effective than CPIB.

Despite the important role of phospholipids in lipoprotein structures, current knowledge of the effect of hypolipidemic drugs on phospholipid metabolism is scanty at best (23, 28, 30, 51). However, it has been shown that CPIB does decrease serum phosphatidylcholine levels (23, 28, 51) in experimental animals and that lysolecithin levels can become increased as a result of such drug treatment (51).

Inhibition of ChoPTase and LLAcylTase may also explain one of the side effects of these drugs—namely, hepatomegaly—which entails significant triglyceride and cholesterol deposition in the liver. Decreased utilization of diacylglycerol and acyl-CoA in phosphatidylcholine synthesis would be expected to supply the necessary precursors for triglyceride formation. In fact, hypolipidemic drugs have been shown to cause an increase of acyl-CoA in the liver (52–54), and there is considerable evidence that CPIB and similar drugs actually stimulate hepatic triglyceride synthesis (21, 23–25, 28, 29).

Several of the hypolipidemic agents have previously been shown to affect *de novo* cholesterol synthesis by inhibiting the rate-limiting enzyme hydroxymethylglutaryl-CoA reductase (11, 15, 33, 36, 39). Yet, cholesterol circulation appears to be affected more severely by the drugs' ability to decrease cholesterol transport from the liver and to cause cholesterol deposition in the liver (14, 15, 38).

Drug-induced triglyceride and cholesterol retention in the liver could readily be explained by impaired lipoprotein synthesis. Impaired lipoprotein synthesis, in turn, is likely to be reflected in decreased serum lipoprotein levels (15, 19, 24, 25, 38). Moreover, lipoprotein synthesis and secretion appear to depend on phosphatidylcholine synthesis and on efficient lysophosphatidylcholine reacylation. The latter requirement has been well documented for intestinal lipoprotein production (55–58) and also appears to hold true for liver cells (unpublished data). Hence, blockage of phosphatidylcholine synthesis, particularly through inhibition of the LLAcylTase pathway, could be an underlying cause of the drugs' effectiveness in decreasing serum lipid levels by interfering with lipoprotein assembly and release.

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