Inhibitors of neutral cholesteryl ester hydrolase

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Abstract *p*-Nitrophenyl N-butyl, N-octyl, and N-dodecyl carbamates and a newly synthesized diethyl phosphate compound were studied as potential inhibitors of the cholesteryl ester hydrolases of Fu5AH rat hepatoma cells. Whole homogenates of Fu5AH cells were used as an enzyme source for the assay of cholesteryl ester hydrolase activity. All four compounds led to marked inhibition (70–80%) of neutral cholesteryl ester hydrolase activity (assayed at pH 7) at concentrations where the activity of acid cholesteryl ester hydrolase (assayed at pH 4) was unaffected. Cholesteryl ester hydrolysis was also evaluated in intact cultured cells induced to accumulate cholesteryl esters in cytoplasmic lipid droplets by exposure to cholesterol-rich phospholipid dispersions. Hydrolysis was then assessed during subsequent incubations in the presence of an inhibitor of cholesterol esterification. All compounds caused significant inhibition of cholesterol ester hydrolysis with the diethyl phosphate being the most effective. At a concentration that caused >90% inhibition of the hydrolysis of cytoplasmic cholesteryl esters, the compound had only a minimal effect on lysosomal hydrolysis of cholesteryl esters. These results suggest that diethyl phosphates and N-alkylcarbamates may be of value in future studies on the substrate specificities, regulation, and physiological role(s) of cholesteryl ester hydrolases. —**Harrison, E. H., D. W. Bernard, P. Scholm, D. M. Quinn, G. H. Rothblat, and J. M. Glick.** Inhibitors of neutral cholesteryl ester hydrolase. *J. Lipid Res.* 1990. **31:** 2187–2193.

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The accumulation of cholesteryl esters in lipid droplets is a hallmark of the foam cells characteristic of the atherosclerotic lesion (1–3). Several cultured cell systems have been described that have proved useful for studying the mechanisms and regulation of cellular cholesteryl ester accumulation, hydrolysis, and clearance. In two of these in particular, viz. mouse peritoneal macrophages (4) and Fu5AH rat hepatoma cells (5), it has been demonstrated that the accumulated cholesteryl esters undergo a constant turnover. Thus, the cholesterol esters are enzymatically hydrolyzed and the released free cholesterol, if not removed from the cells by extracellular acceptors, is then reesterified by the enzyme acyl CoA:cholesterol acyltransferase (ACAT). The availability of an effective and specific inhibitor of ACAT, Sandoz compound 58035 (6), has proven extremely useful in elucidating the details of cellular cholesteryl ester turnover (5, 7, 8). The availability of specific inhibitors for the hydrolytic arm of the cholesteryl ester cycle should likewise be of value.

In cells there are at least two distinct enzymes capable of catalyzing the hydrolysis of cholesterol esters (9–11). The better characterized enzyme is the lysosomal, acid cholesteryl esterase (acid lipase) involved in the hydrolysis of cholesteryl esters delivered to cells via receptor-mediated endocytosis of lipoproteins (10). This enzyme can be nonspecifically inhibited by agents that raise the intralysosomal pH (12) and appears not to be involved in the hydrolysis of cholesteryl esters in the cytoplasmic lipid droplets (13), which is thought to be catalyzed by a neutral cholesteryl ester hydrolase.

We now report the results of investigations of the efficacy of *p*-nitrophenyl N-alkyl carbamates and of a newly synthesized diethyl phosphate compound in inhibiting the cholesteryl ester hydrolases of Fu5AH rat hepatoma cells. These compound were chosen because they, or related compounds, have been previously demonstrated to be active site-directed, irreversible inhibitors of purified lipid ester hydrolases with neutral pH optima (14, 15). The results demonstrate that all compounds tested were effective in markedly and specifically inhibiting neutral but not acid cholesteryl ester hydrolase activity as assessed in enzyme assays of cell homogenates. When added to cultured cells, the N-alkyl carbamates partially inhibited the hydrolysis of cholesteryl esters in cytoplasmic lipid droplets, and the diethyl phosphate completely inhibited this hydrolysis. Moreover, the latter compound caused only minor inhibition of the lysosomal hydrolysis of LDL-cholesteryl esters.

Abbreviations: PBS, phosphate-buffered saline; ACAT, acyl-CoA: cholesterol acyltransferase; LDL, low density lipoprotein; TLC, thin-layer chromatography.

EXPERIMENTAL PROCEDURES

Chemicals, incubation media and lipoproteins

Chemicals, physiological saline (PBS), and the media, sera, and antibiotics for tissue culture have been described previously (5). All incubation media consisted of minimal essential medium (MEM) containing 50 μg/ml gentamicin and buffered with 25 mM $NaHCO_3$. Stock cultures of Fu5AH hepatoma cells (16, 17) were grown in MEM supplemented with 5% calf serum. Cell culture incubations were carried out at 37°C in an atmosphere of 5% CO_2 in air. Sonicated lipid dispersions having a free cholesterol: phosphatidylcholine molar ratio > 2 were prepared as previously described (18). Reconstituted human low density lipoprotein (LDL, d 1.006–1.063) was prepared following the procedure of Krieger et al. (19). [1,2-^{3}H]Cholesteryl oleate (0.25 mCi/μmol) was prepared as described (20) from [1,2-^{3}H]cholesterol (Amersham) and oleic anhydride (Sigma) and was purified prior to use by thin-layer chromatography (TLC) (21). At the time of use, more than 98% of the radiolabel was present in the reconstituted LDL as cholesteryl ester.

Synthesis of inhibitors

The N-butyl and N-octyl carbamate derivatives of *p*-nitrophenol were synthesized, purified, and characterized as described (14). Methods used for the synthesis and characterization of the N-dodecylcarbamate were the same as those used for the N-octyl derivative. The diethyl phosphate compound was synthesized by coupling diethylchlorophosphate with the appropriate organic alcohol in dry pyridine. Details of the synthesis and characterization of this compound will be reported elsewhere.

Preparation of cell homogenates

Fu5AH cells were cultured as described above on 100-mm plastic Petri dishes. Generally, 16 dishes of confluent cells were used for each homogenate preparation. The medium was aspirated and the monolayers were rinsed two times with 5 ml of PBS. Two ml of PBS was added, the cells were scraped with a rubber spatula, and the cell suspension was transferred to a centrifuge tube. This procedure was repeated and the combined cell suspensions were centrifuged at 1500 rpm for 15 min at 10°C in an IEC Centra-7R centrifuge. The supernatants were removed and discarded, and the cell pellets were resuspended in PBS and combined into a tared centrifuge tube. The tube was centrifuged at 1000 rpm for 10 min, the supernatant was removed, and the packed cell pellet was weighed. Nine volumes of 0.25 M sucrose were added, the cells were resuspended, and then homogenized with a Tekmar homogenizer for 20–60 sec. The homogenates were divided into small portions and stored at −70°C prior to use in the enzyme assays that were generally conducted within 2 weeks of homogenate preparation. The protein concentration of the homogenates ranged from 4 to 5 mg/ml.

Assay of cholesteryl ester hydrolase activity

A radiometric assay for cholesterol ester hydrolase activity that has previously been described in detail (22) was modified for these studies. Reaction mixtures were prepared in a final volume of 0.2 ml by adding in order: 0.1 ml buffer (see below), 0.085 ml of diluted Fu5AH cell homogenate (100–200 μg protein), and either 0.005 ml of inhibitor in DMSO or DMSO alone. Assays were initiated within 10 min by the addition of 2 nmol of cholesterol [1-^{14}C]oleate (0.025 μCi/nmol) (Amersham) in 0.01 ml of ethanol and were carried out at 37°C for 1 h. Reactions at pH 4.0 used 0.1 M sodium acetate buffer and those at pH 7.0 used 0.1 M Tris-maleate buffer. Reactions were terminated by the addition of organic solvents and extraction of the product, [1-^{14}C]oleic acid, into an alkaline aqueous phase (23). All enzyme assays were carried out in duplicate, and results are presented as the mean of the two determinations. Duplicates varied by less than 10%.

Assay of cholesteryl ester hydrolysis in intact cells

For studies involving the quantitation of the hydrolysis of cholesteryl esters in cytoplasmic lipid droplets, Fu5AH cells were plated into 35-mm dishes at a density of 1×10^5 cells per ml, 2 ml per dish, and grown for 2 days in MEM supplemented with 5% calf serum. Cells were loaded with cholesteryl esters, stored as cytoplasmic inclusions, by exposure for 48 h to medium containing 5% calf serum, 5% fetal bovine serum, and free cholesterol-rich phospholipid dispersions at a final concentration of 250 μg dispersion free cholesterol per ml (24). [1,2-^{3}H]Cholesterol (Amersham) was included in all loading media at a concentration of 0.5 μCi/ml. The labeled cholesterol was added in ethanol (final concentration 0.5%), and the media were preincubated overnight at 37°C prior to incubation with cells. After the 48-h loading period, during which time the radiolabeled cholesterol was incorporated and esterified, the cell monolayers were washed and incubated an additional 24 h in medium containing 2.5 mg/ml bovine serum albumin (BSA) to allow all of the intracellular pools of labeled cholesterol to equilibrate to the same specific activity (5). To quantitate cholesteryl ester hydrolysis, the loaded cells were incubated for up to 12 h in MEM containing 0.1% BSA and Sandoz compound 58035 which inhibits ACAT (6). The inhibition of ACAT prevents the reesterification of any free cholesterol generated by cholesteryl ester hydrolysis and thus allows assessment of the activity of the hydrolase. To examine the effect of the various hydrolase inhibitors, parallel cultures of Fu5AH cells were incubated in the clearance media described above to which were added the inhibitors as described in the text. Sandoz 58035 and the hydrolase in-

hibitors were added to the culture media in DMSO; the final concentration of DMSO was 0.2%. The inhibitory effects of the compounds that were tested are expressed relative to the hydrolysis of cholesteryl esters obtained in the presence of Sandoz 58035, which was set equal to 100%.

The mass of cellular free and esterified cholesterol present in the cells at the beginning of the clearance phase of the experiment was determined by GLC analysis using cholesteryl methyl ether as an internal standard after isopropanol extraction of washed monolayers (18). After lipid extraction, protein determinations were performed on the remaining monolayers (25) with BSA as a standard. In the experiments presented here, the cellular free cholesterol content was approximately 25 μg per mg cell protein while the esterified cholesterol content ranged from 60 to 104 μg per mg cell protein. The hydrolysis of the cholesteryl esters was quantitated by determining the reduction of radiolabeled cholesteryl esters and the appearance of labeled free cholesterol in the cultures. These values were obtained by first solubilizing the cell monolayer into the incubation media with SDS as previously described (21) and extracting the lipids using the procedure of Bligh and Dyer (26). The distribution of radiolabel between free cholesterol and cholesteryl esters was determined after separation of the lipid classes by thin-layer chromatography on ITLC-SA plates developed in petroleum ether–ethyl ether–acetic acid 85:15:1 (v/v/v). Radioactive bands were cut from the plates and counted in a Beckmann LS7500 counter.

To determine the effect of the hydrolase inhibitors and the lysosomotropic agent chloroquine on hydrolysis of LDL-derived cholesteryl esters, Fu5AH cells were plated into 12-well plates at a density of 5×10^4 cells per well in MEM with 5% calf serum and grown for 3 days. The cells were then rinsed 3 times with PBS and incubated overnight in MEM containing 10 mg/ml lipoprotein-deficient serum to up-regulate LDL receptors (9). The cells were again rinsed 3 times with PBS and incubated for 3 h with 2 ml/dish of media containing the inhibitors, as described in the text. After this 3-h preincubation period, LDL that had been reconstituted with [1,2-^{3}H]cholesteryl oleate (0.2 mCi/ml cholesteryl ester) was added to each dish for a final concentration of 10 μg LDL protein/dish (2 μCi/dish). The cellular uptake and hydrolysis of the LDL-derived [1,2-^{3}H]cholesteryl oleate was determined after 4 h incubation at 37°C. The medium was removed from the dishes, centrifuged to sediment any detached cells, and the lipids were extracted from the cell-free media by the Bligh and Dyer method (26). After rinsing the monolayer with PBS, lipids were extracted with isopropanol as described above, and the distribution of labeled free and esterified cholesterol in both the media and cells was quantitated by TLC as described above.

RESULTS

Studies with cell homogenates

The effects of the *p*-nitrophenyl N-alkyl carbamates on the cholesteryl ester hydrolase activities of Fu5AH cell homogenates are shown in **Fig. 1**. As shown in panel A, the butyl derivative at a concentration of 125 μM, was effective in inhibiting the neutral cholesteryl ester hydrolase activity by 80% but had little effect on the acid cholesteryl ester hydrolase activity. Panels B and C show that the same selectivity for the neutral hydrolase was exhibited by the octyl and dodecyl derivatives. However, in contrast to the butyl derivative, the longer chain carbamates were effective inhibitors at much lower concentrations. Thus, concentrations in the 2–5 μM range led to marked inhibition (80–85%) of the neutral cholesteryl es-

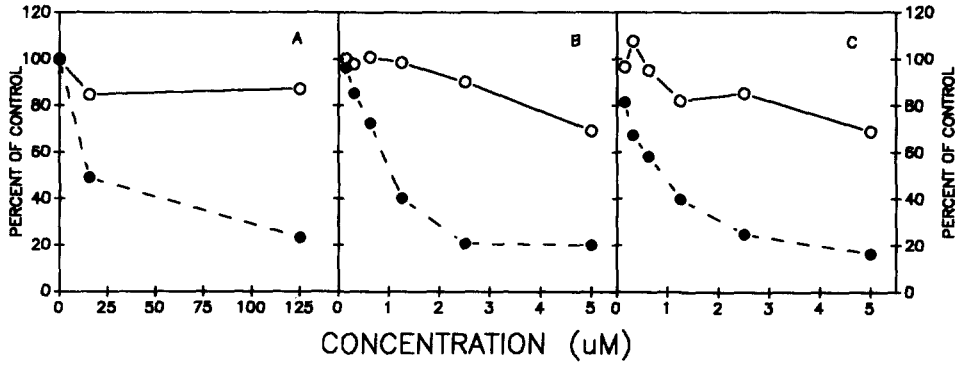

Fig. 1. Inhibition of cholesteryl ester hydrolase activities in homogenates of Fu5AH hepatoma cells by *p*-nitrophenyl N-alkyl carbamates. Whole homogenates were assayed for cholesteryl ester hydrolase activity at acid (pH 4, open symbols) or neutral (pH 7, filled symbols) pH in the presence of the indicated concentrations of *p*-nitrophenyl N-butyl carbamate (panel A), *p*-nitrophenyl N-octyl carbamate (panel B), or *p*-nitrophenyl N-dodecyl carbamate (panel C). Activities are expressed as percent of control as determined in assays conducted in the absence of inhibitors.

ter hydrolase activity with little effect on the acid cholesteryl ester hydrolase activity.

The effects of the diethyl phosphate inhibitor on the hepatoma cholesteryl ester hydrolase activities are presented in **Fig. 2** and **Fig. 3**. As shown in Fig. 2, this compound was also effective in selectively inhibiting the neutral cholesteryl ester hydrolase activity in the cell homogenates. However, concentrations of 300 μM or greater were required for maximal inhibition, which was at most 70%. The data presented in Fig. 3 demonstrate the bimodal nature of the complete pH profile for the uninhibited reaction and clearly show the selective inhibition of the neutral activity by the diethyl phosphate compound. As a potential active site-directed, irreversible enzyme inhibitor it would be predicted that the extent of inhibition would be independent of substrate concentration. Indeed, another experiment (data not shown) demonstrated that the inhibitor concentration dependence was nearly identical for reactions conducted with 10 μM and with 120 μM cholesteryl oleate.

In the experiments described above, the diethyl phosphate compound was added to the enzyme reaction mixtures at the pH of the assay. We considered the possibility that the apparent specificity for inhibition of the neutral activity might be due to the lack of ability of the inhibitor to react with the enzyme at acid pH (pH 4). In order to address this, the following experiment was carried out. Homogenates were preincubated with a maximally effective inhibitor concentration (approximately 750 μM) for 30 min at room temperature and at neutral pH, prior to dilution and assay at both pH 4 and pH 7. The results obtained with these preincubation conditions were identical to those of controls in which the inhibitor was added in the usual way at the time of assay (data not shown). Thus,

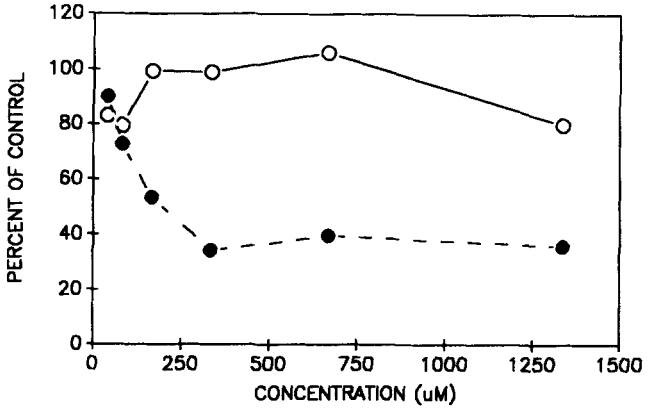

Fig. 2. Inhibition of cholesteryl ester hydrolase activities in homogenates of Fu5AH hepatoma cells by the diethyl phosphate compound. Whole homogenates were assayed for cholesteryl ester hydrolase activity at acid (pH 4, open symbols) or neutral (pH 7, filled symbols) pH in the presence of the indicated concentration of inhibitor. Activity is expressed as percent of control determined in assays conducted in the absence of inhibitor.

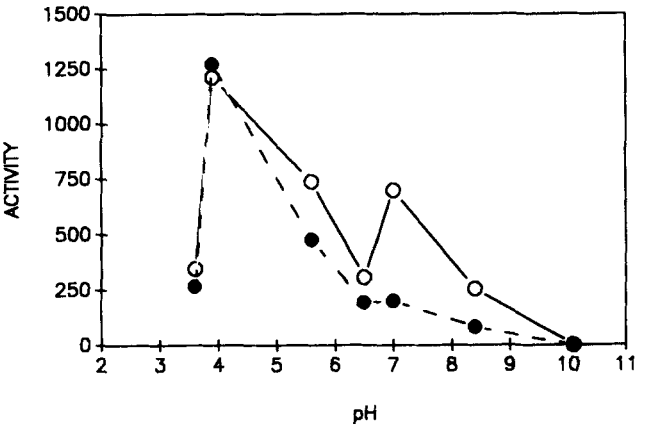

Fig. 3. pH profile of cholesteryl ester hydrolase activity in homogenates of Fu5AH hepatoma cells. Whole homogenates were assayed for cholesteryl ester hydrolase activity in the absence (open symbols) or presence (filled symbols) of the diethyl phosphate inhibitor (669 μM). Activity is expressed as cpm of the product ([^{14}C]oleate) extracted in a 1-ml aliquot of the upper phase using the standard assay described in Methods. Buffers, used at a final concentration of 50 mM, were sodium acetate (for pH 3.6, 4.0, and 5.6), Tris-maleate (for pH 6.5 and 7.0), and glycine (for pH 8.4 and 10.0).

in both cases there was little effect on the acid cholesteryl ester hydrolase activity and marked inhibition of the neutral activity.

Sensitivity to inhibitors of neutral cholesteryl ester hydrolase in intact cells

Cholesteryl ester-loaded Fu5AH cells were used as a model to test the effectiveness of potential inhibitors of the neutral cholesteryl ester hydrolase in living cells. **Table 1** shows the effects of the three nitrophenyl carbamates, the diethyl phosphate compound, and the lysosomotropic agent, chloroquine, on the hydrolysis of cholesteryl esters stored in Fu5AH cells in cytoplasmic inclusions. The concentrations of the carbamates and of the diethyl phosphate compound added to the culture media were the highest concentrations that could be present without producing cellular toxicity after 24 h incubation, as determined microscopically in preliminary experiments. The inhibition observed with the nitrophenyl carbamates ranged from 50% obtained with nitrophenyl-N-butyl carbamate to 83% with nitrophenyl-N-dodecyl carbamate. The most effective inhibitor was the diethyl phosphate compound, which inhibited the hydrolysis of the cellular cholesteryl esters by more than 90% when present in the medium at a concentration of 100 μM. Chloroquine, at a concentration that causes nearly complete inhibition of the lysosomal hydrolysis of cholesterol esters (see below), had no effect in these studies, confirming that the observed hydrolysis is mediated by an extra-lysosomal, neutral cholesteryl ester hydrolase.

Because the diethyl phosphate compound appeared to be the most effective of the tested compounds, complete dose response curves were constructed using Fu5AH cells

TABLE 1. Effect of inhibitors of cholesteryl ester hydrolase on the hydrolysis of endogenous cholesteryl esters in Fu5AH cells

Compound	Concentration	% Inhibition
	μM	
P-Nitrophenyl-N-butyl carbamate	250	49.7 ± 2.1
P-Nitrophenyl-N-octyl carbamate	100	48.2 ± 7.0
P-Nitrophenyl-N-dodecyl carbamate	250	83.0 ± 1.8
Diethyl phosphate compound	100	100.0 ± 1.2
Chloroquine	50	0 ± 1.9

Fu5AH cells, loaded with [^{3}H]cholesteryl esters in cytoplasmic inclusions, were incubated for 12 h in the presence of the indicated inhibitors together with Sandoz compound 58035 (1 μg/ml). The reduction of labeled esterified cholesterol obtained in cultures exposed to Sandoz 58035 alone averaged 19.1 ± 5.0% and represented maximum hydrolysis. Percent inhibition of hydrolysis = [(hydrolysis with 58035 − hydrolysis with 58035 and inhibitor)/hydrolysis with 58035] × 100. Values are the mean ± SD; n = 4.

loaded with cholesteryl esters. The data presented in **Fig. 4** demonstrate that incubation of the cells for 12 h in the diethyl phosphate compound inhibits the hydrolysis of cholesteryl esters in the range of 1 to 100 μM.

Sensitivity to inhibitors of acid cholesteryl ester hydrolase in intact cells

To determine whether the diethyl phosphate inhibited the acid cholesteryl hydrolase activity in Fu5AH cells, the extent of hydrolysis of labeled esterified cholesterol, supplied by LDL, was determined in cultures incubated in the presence of reconstituted LDL containing [1,2-^{3}H]cholesteryl oleate. Because the cells were exposed to LDL in the presence of the various compounds, different amounts of LDL were taken up in each case. The results presented in **Table 2** demonstrate that the lysosomotropic agent chloroquine inhibited hydrolysis by 94%, confirming that the labeled cholesteryl esters were being hydrolyzed by the lysosomal acid cholesteryl ester hydrolase. Exposure of cell monolayers to 10 and 100 μM of the diethyl phosphate compound resulted in a small reduction

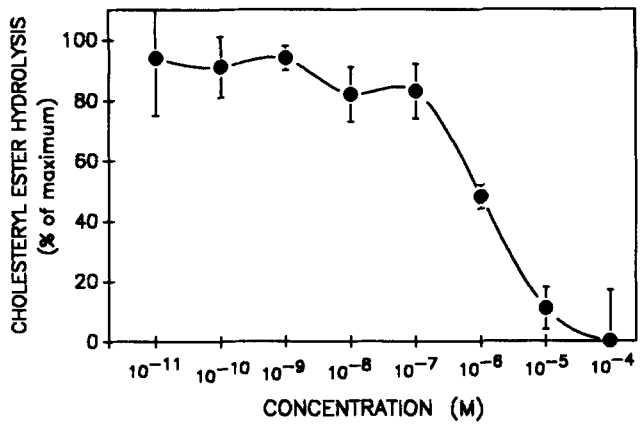

Fig. 4. Inhibition of hydrolysis of cellular cholesteryl esters in Fu5AH cells by the diethyl phosphate compound. Cells were loaded with cholesteryl esters in cytoplasmic inclusions as described in the text. The cultures were then exposed to media containing 0.1% BSA, 1 μg/ml Sandoz 58035, and the diethyl phosphate compound at the indicated concentrations. Maximum hydrolysis is defined as that observed in the absence of the diethyl phosphate inhibitor. Values are mean ± SD; n = 4.

in the fractional hydrolysis of cholesteryl esters, but there was no significant impact on the mass of cholesteryl ester that was hydrolyzed.

DISCUSSION

In this report we have demonstrated the efficacy of *p*-nitrophenyl-N-alkyl carbamates and of a newly synthesized diethyl phosphate compound in inhibiting neutral cholesteryl ester hydrolase in the rat hepatoma cell, Fu5AH. Previous work with the N-alkyl carbamate and diethyl phosphate derivatives of *p*-nitrophenol had shown that they were effective, active site-directed, irreversible inhibitors of porcine pancreatic cholesteryl esterase and of bovine lipoprotein lipase, respectively (14, 15), both being enzymes that function extracellularly. The mechanism of inhibition involves the enzyme-catalyzed formation of the N-alkyl carbamyl- or phosphoryl-enzyme at an active site

TABLE 2. Effect of the diethyl phosphate compound on the hydrolysis of LDL-derived cholesteryl esters in Fu5AH cells

Inhibitor	μg CE Uptake per mg Cell Protein per 4 h	μg CE Hydrolysed per mg Cell Protein per 4 h
Sandoz 58035 (1 μg/ml)	0.19 ± 0.02	0.13 ± 0.01
Sandoz 58035 + diethyl phosphate compound (100 μM)	0.23 ± 0.01	0.12 ± 0.01
Sandoz 58035 + diethyl phosphate compound (10 μM)	0.20 ± 0.03	0.12 ± 0.02
Sandoz 58035 + chloroquine (50 μM)	0.17 ± 0.01	0.01 ± 0.00

Fu5AH cells were preincubated 3 h with the indicated inhibitors followed by incubation for 4 h with the inhibitors plus LDL reconstituted with [1,2-^{3}H]cholesteryl oleate. Hydrolysis of the labeled cholesteryl oleate was determined as described in the Methods. Masses are calculated based on the specific activity of the LDL cholesteryl ester. Values are mean ± SD; n = 4.

serine. Since evidence was available indicating that hepatic neutral cholesteryl esterases were inhibited by reagents such as diisopropylfluorophosphate that react with serine residues (11), we reasoned that lipophilic N-alkyl carbamates and diethyl phosphate derivatives might also inhibit this (these) enzyme(s). Moreover, since the lysosomal, acid cholesteryl esterase (acid lipase) is not inhibited by diisopropylfluorophosphate or diethyl-*p*-nitrophenyl phosphate (11, 13), we predicted that inhibition by the compounds studied might be specific for the neutral cholesteryl esterase activity. Indeed, the results of our experiments using cell homogenates are consistent with these expectations, and, of particular interest, is the fact that these inhibitors are effective in living cells.

Assays of whole homogenates of Fu5AH hepatoma cells for cholesteryl ester hydrolase activities demonstrated that the *p*-nitrophenyl-N-alkyl carbamates were effective in markedly inhibiting the neutral hydrolase without affecting acid hydrolase activity. As previously observed for the inhibition of purified pancreatic cholesteryl esterase (14), the longer chain carbamates were more effective inhibitors than the butyl derivative. The same specificity for inhibition of the neutral as opposed to the acid cholesteryl ester hydrolase activity was observed with the diethyl phosphate. Thus, these results demonstrate that the enzyme(s) responsible for the major amount of this activity in the hepatoma cell homogenate is (are) likely to be a serine esterase(s). The fact that, for all the inhibitors, maximal inhibition of neutral cholesteryl ester hydrolase was at most 70–80% suggests that other enzymes present in the homogenate can also catalyze the hydrolysis of cholesteryl esters to some extent.

Perhaps the most significant aspect of the present work is the demonstration that the compounds studied also inhibit the hydrolysis of cholesteryl esters stored in cytoplasmic inclusions in the intact hepatoma cells in culture. Interestingly, there was little relationship between the effectiveness of a given compound in inhibiting cholesteryl ester hydrolase activity in homogenates and in cell culture systems. In the studies with cell homogenates, the N-alkyl carbamates were effective in inhibiting hydrolysis at much lower concentrations than the diethyl phosphate. In contrast, the latter compound was most effective in inhibiting the neutral cholesteryl ester hydrolase in intact cells. Concentrations of the diethyl phosphate compound of greater than 300 μM were necessary for maximal inhibition in homogenates, while concentrations of 10–100 μM were maximally effective in inhibiting cholesteryl ester hydrolysis in the intact hepatoma cells. In the absence of any information on the cellular uptake or accumulation of the inhibitors, or even on their effective concentration in the heterogeneous homogenate assay mixtures, the reasons for the apparently different activities in the two systems remain unknown.

The use of the diethyl phosphate inhibitor has allowed us to fully dissect two distinct pathways of cholesteryl ester hydrolysis in intact Fu5AH cells. Hydrolysis of endogenous cholesteryl esters in cytoplasmic lipid droplets is completely abolished by inhibition of neutral cholesteryl ester hydrolase activity whereas the hydrolysis of cholesteryl esters delivered to the cell via receptor-mediated endocytosis of LDL is unaffected. Conversely, the lysosomotropic agent, chloroquine, leads to the complete inhibition of hydrolysis of LDL-derived cholesteryl esters without affecting the hydrolysis of endogenous cholesteryl esters. The combined use of the two inhibitors should now allow further investigations on the relative roles of these two pathways in the hydrolysis of cholesterol esters delivered to the cell in other forms. For example, the subcellular sites and details of the enzymes responsible for the hydrolysis of cholesteryl esters delivered to hepatic cells in association with chylomicron remnants, other lipoproteins (eg., HDL), or in exogenous lipid droplets (21) have not been fully defined. The availability of specific inhibitors of neutral cholesteryl ester hydrolase should now make it possible to assess quantitatively the relative roles of acid cholesteryl ester hydrolase and neutral cholesteryl ester hydrolase activity in the hydrolysis of these forms of cholesteryl esters.

As a potent inhibitor of the hydrolytic arm of the "cholesteryl ester cycle," another possible use of the diethyl phosphate inhibitor would be in studies of the absolute rates and regulation of cholesterol esterification by ACAT. Just as the ACAT inhibitor, Sandoz 58035, has allowed better experimental assessment of the rates of cellular cholesteryl ester hydrolysis (24, 27), so too, inhibition of neutral cholesteryl ester hydrolase activity should allow more detailed studies of cellular ACAT activity.

The ultimate utility of the diethyl phosphate or related compounds for studies of cellular cholesteryl ester metabolism will obviously depend on how widely the results obtained here with Fu5AH cells can be generalized to other cell types. Although this determination will require further studies, we have preliminary evidence that the compound is effective in inhibiting neutral cholesteryl ester hydrolase activity in rat liver homogenates and the neutral hydrolase activity of both intact J-774 macrophages and homogenates of J-774 cells. It thus appears that further study of its action may enhance our understanding of cholesteryl ester metabolism in a number of systems.

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