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STUDIES ON THE MECHANISM OF DRUG-INDUCED LIPIDOSIS

CATIONIC AMPHIPHILIC DRUG INHIBITION OF LYSOSOMAL PHOSPHOLIPASES A AND C

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Abstract—Previously, chloroquine and 4,4′-bis(diethylaminoethoxy)α,β-diethyldiphenylethane, two drugs that have been reported to cause lipidosis in man, had been found to be potent inhibitors of lysosomal phospholipase *in vitro* [Y. Matsuzawa and K. Y. Hostetler, *J. biol. Chem.* **255**, 5190 (1980).] Seven additional cationic amphiphilic agents were examined for their effects on degradation of phosphatidylcholine (the major phospholipid of lysosomes) by lysosomal phospholipases A and C. The drugs were amantadine, 1,7-bis(*p*-aminophenoxy)heptane, chlorophentermine, chlorpromazine, imipramine, propranolol and tripelennamine, agents that have widely varying pharmacologic properties but that share cationic amphiphilic structural features. All of these agents inhibited phospholipases A and C. The results strongly support the hypothesis that drug-induced lipidoses is due to direct inhibition of lysosomal phospholipid catabolism.

More than thirty cationic amphiphilic drugs, including chloroquine, 4,4′-bis(diethylaminoethoxy)α,β-diethyldiphenylethane, chlorphentermine, imipramine and chloropromazine, have been reported to cause phospholipid storage in humans, laboratory animals, and cultured cells, as noted in a recent article by Lüllmann-Rauch [1]. The cellular phospholipid content may increase by as much as 2- to 4-fold in some instances. Morphologically, the polar lipid accumulation appears to be confined to lysosomes which usually have a multilamellar appearance [1]. It has been shown that these multilamellar bodies are rich in phospholipids [2]. Recently, we found that a high percentage of two cationic amphiphilic drugs and all of the excess phospholipid was confined to lysosomes in liver of rats treated with chloroquine or 4,4′-bis(diethylaminoethoxy)α,β-diethyldiphenylethane [3]. The accumulation of phospholipid and drug principally in lysosomes suggested that direct inhibition of lysosomal phospholipases might be a mechanism of major importance in drug-induced lipidosis.

Several investigators have shown that lysosomes exhibit phospholipase A_1 and A_2 activities [4, 5]. In addition, we recently demonstrated phospholipase C activity in rat liver lysosomes using [1-^{14}C] dioleoylphosphatidylcholine as the substrate. Lysosomal phospholipase C has an acid pH optimum, does not require Ca^{2+}, and can also hydrolyze phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine [6] and bis(monoacylglycero)phosphate [7] to diglyceride and the respective phosphorylated constituent. This appears to be the first demonstration of phospholipase C in mammalian tissue, although phosphatidylinositol-specific phospholipase C activity has been reported to be present in mammalian intestinal mucosa, liver, brain, and platelets [8–11]. When the effects of chloroquine and 4,4′bis(diethylaminoethoxy)α,β-diethyldiphenylethane on lysosomal phospholipases were examined, these cationic amphiphilic agents were found to be potent *in vitro* inhibitors of both phospholipases A and C [12].

In this publication, we have examined the effects on lysosomal phospholipases of four agents that have been implicated in the accumulation of polar lipids in animals, cultured cells, and schistosomes: chlorphentermine, imipramine, chlorpromazine and 1,7-bis(*p*-aminophenoxy)heptane [1, 13]. Three other drugs having cationic amphiphilic structures, but not yet reported to cause polar lipid storage, have also been studied: amantadine (an anti-influenzal agent), propranolol (a beta-adrenergic blocking agent) and tripelennamine (an antihistamine). Our results indicate that all seven of these agents inhibit lysosomal phospholipases.

MATERIALS AND METHODS

Preparation of delipidated lysosomal soluble protein. Secondary lysosomes were isolated from liver of male Sprague–Dawley rats injected intravenously with Triton WR-1339 (850 mg/kg) in 0.9% sodium chloride, according to the method of Trouet [14]. Lysosomal protein was solubilized by repeated freezing and thawing and was delipidated twice with *n*-butyl alcohol as described previously [15]. The final delipidated soluble protein fraction was desalted by gel filtration using a column of Sephadex G-25, as

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noted previously. The final preparation contained less than 5 nmoles lipid phosphorus/mg protein. Protein was determined by the method of Lowry *et al.* [16]. Aliquots of the lysosomal protein were stored frozen at −60° until use.

Hydrolysis of [1-^{14}C] *dioleoylphosphatidylcholine.* The incubation medium contained 50 mM sodium acetate (pH 4.4), 50 μg doubly-delipidated lysosomal soluble protein, and 4.5×10^{-5} M [1-^{14}C] dioleoylphosphatidylcholine (sp. act. 30 mCi/mmole) and the drugs as noted, in a total volume of 0.200 ml. The mixture was incubated for 20 min at 37°, the reaction was stopped by the addition of 20 vol. of chloroform/methanol (2:1, v/v), and total lipids were extracted by the method of Folch *et al.* [17]. The lipid extract was concentrated to a small volume using a nitrogen stream, applied to a 0.25 mm layer of silica gel H prepared with 0.01 M magnesium acetate and developed to 7 cm with chloroform/methanol/water (65:35:5, by vol.), dried in nitrogen for 20 min, and developed to the top of the plate with heptane/diethyl ether/conc. formic acid (90:60:4, by vol.) as described previously [12]. The areas corresponding to phosphatidylcholine, lysophosphatidylcholine, monoglyceride, diglyceride, and fatty acid were scraped into vials and counted by liquid scintillation spectrometry with 15 ml of 0.5% 2,5-diphenyloxazole and 0.02% *p*-bis[2-(5-phenyloxazole)]benzene in toluene/Triton X-100/water (2:1:0.2, by vol.). In making the calculations, a specific activity of 15 mCi/mmole was used for fatty acid, monoglyceride, and lysophosphatidylcholine, and 30 mCi/mmole was used for diacylglycerol. Corrections for quenching were made with an external standard method.

Chemicals. Triton X-100 was obtained from Supelco (Bellefonte, PA) and silica gel H from EM Laboratories (Elmsford, NY). Amantadine was purchased from the Aldrich Chemical Co. (Milwaukee, WI). 1,7-bis(*p*-aminophenoxy)heptane (BW 153C51) was provided by Dr. S. D. M. Watts of the Wellcome Research Laboratories (Beckenham, Kent, England), chlorphentermine by the Warner–Lambert Research Institute (Morris Plains, NJ), chlorpromazine by Smith Kline & French Laboratories (Philadelphia, PA), imipramine and tripelannamine by the Ciba-Geigy Corp. (Summit, NJ) and propranolol by Ayerst Laboratories (New York, NY). [1-^{14}C]Dioleoylphosphatidylcholine was purchased from Applied Science Laboratories (College Park, PA). Other chemicals were of analytical reagent grade. Chloroform and methanol were redistilled before use.

RESULTS

The release of ^{14}C-labeled oleic acid from [1-^{14}C]dioleoylphosphatidylcholine occurs through the action of phospholipase A and lysophospholipase or alternatively through the action of phospholipase C followed by diglyceride and monoglyceride lipase [6, 12] and, therefore, represents the overall catabolism by the two pathways. The effect of the amphiphilic agents on lysosomal catabolism of [1-^{14}C]dioleoylphosphatidylcholine is shown in Fig. 1. [^{14}C]Oleic acid release was inhibited most strongly

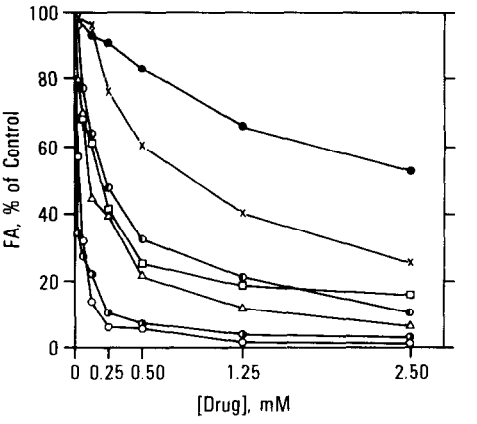


Fig. 1. Inhibition of [1-^{14}C]oleic acid release from [1-^{14}C]dioleoylphosphatidylcholine by cationic amphiphilic drugs. Incubations were carried out as described in Materials and Methods, and the drugs were added in the concentrations shown. Each data point is the average of two separate experiments for each drug tested. The control rate of oleic acid release in the absence of drugs was 490 ± 72 nmoles·mg^{-1}·hr^{-1} (mean ± S.D.; N = 5). Key: (●) amantadine; (◑) 1,7-bis(*p*-aminophenoxy)heptane; (◐) chlorphentermine; (○) chlorpromazine; (□) imipramine; (△) propranolol; and (X) tripelennamine.

by 1,7-bis(*p*-aminophenoxy)heptane and chlorpromazine. At 25 μM, the lowest concentration studied, these agents caused a 64 and 43 per cent decrease in activity respectively. At 2.5 mM, the release of [1-^{14}C]oleic acid was reduced by these two drugs to less than 3 per cent of the control. Chlorphentermine, imipramine, and propranolol also inhibited the release of [1-^{14}C]oleic acid from [1-^{14}C]phosphatidylcholine. At 2.5 mM, fatty acid release was decreased to 10.4, 5.8 and 15.8 per cent of control by chlorphentermine, propranolol and imipramine respectively. Less marked inhibition was noted with amantadine and tripelennamine at 2.5 mM; the release of fatty acids from phosphatidylcholine was reduced to 52 and 26 per cent of control, respectively, as shown in Fig. 1.

The effects of the cationic amphiphilic drugs on the phospholipase A and C pathways were estimated by measuring [^{14}C]lysophosphatidylcholine (a product of phospholipase A) and [^{14}C]diglyceride and [^{14}C]monoglyceride (products of phospholipase C action). This requires the rate of phospholipase A to be greater than the rate of lysophospholipase; similarly, the rate of phospholipase C must be faster than that of diglyceride and monoglyceride lipases. Because the products of the phospholipase A and C activity can be removed by further metabolism, as noted above, the rates for phospholipases A and C, based on the generation of radioactive lysophosphatidylcholine, diglyceride and monoglyceride, should be regarded as minimum estimates. For the purposes of studying drug inhibition, however, the assumptions appear to be valid with the time and protein concentration studied [6, 12].

Figure 2 shows the effects of the cationic amphiphilic drugs on release of [1-^{14}C]monooleoylglycerophosphocholine from [1-^{14}C]dioleoylphos-

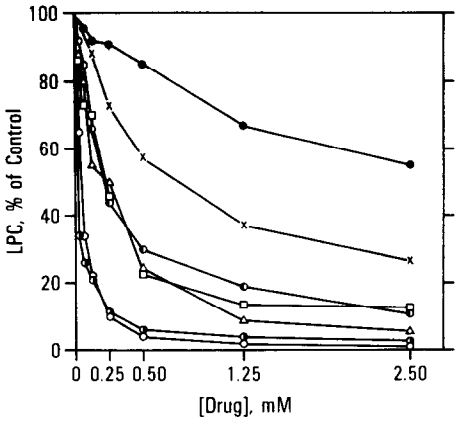


Fig. 2. Inhibition of lysosomal phospholipase A by cationic amphiphilic drugs. The uninhibited rate of formation of lysophosphatidylcholine was 330 ± 45 nmoles·mg^{-1}·hr^{-1} (mean ± S.D.; N = 5. Symbols are defined in the legend of Fig. 1. Each result is the average of two experiments.

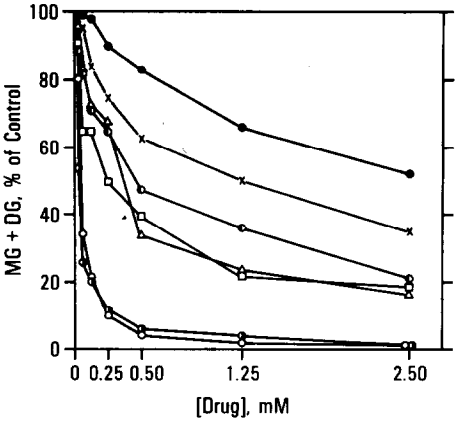


Fig. 3. Inhibition of lysosomal phospholipase C by cationic amphiphilic drugs. The uninhibited rate of monoglyceride + diglyceride release was 125 ± 20 nmoles·mg^{-1}·hr^{-1} (mean ± S.D.; N = 5). Each result is the average of two experiments. Symbols are defined in the legend of Fig. 1.

phatidylcholine. 1,7-bis(*p*-Aminophenoxy)heptane and chlorpromazine were extremely potent inhibitors of phospholipase A; chlorphentermine, imipramine and propranolol were of intermediate potency as inhibitors, and tripelennamine and amantadine were the least effective.

Figure 3 shows the inhibitory effects of these agents on phospholipase C; the results were generally similar to those found with phospholipase A. The order of effectivenss in inhibiting phospholipase C was: 1,7-bis(*p*-aminophenoxy)heptane and chlorpromazine >> imipramine, chlorphentermine, propranolol > tripelennamine > amantadine.

Table 1 shows the millimolar concentrations of these agents required to produce 50 per cent inhibition of lysosomal phospholipases A and C or of total [^{14}C]oleic acid release under standard incubation conditions (calculated from the data shown in Figs. 1–3). In the cases of amantadine and tripelennamine, these values were calculated from data obtained at 5 and 10 mM concentrations of these agents (data not shown). As noted above, all of these cationic amphiphilic agents inhibited lysosomal phospholipases A and C. The effects of these agents on the two phospholipases were generally similar, although the inhibitory effects on phospholipase A were slightly greater than on phospholipase C. As shown in Table 1, the order of effectiveness of these compounds in inhibiting phospholipases A and C was nearly identical: 1,7-bis(*p*-aminophenoxy) heptane > chlorpromazine >> chlorphentermine, imipramine, propranolol > tripelennamine > amantadine. Fifty percent inhibition of phospholipases A and C under these experimental conditions required 1,7-bis(*p*-aminophenoxy)heptane and chlorpromazine concentrations of only 0.01 to 0.03 mM and 0.03 to 0.07 mM, respectively, whereas chlorphentermine, imipramine and propranolol produced 50 per cent inhibition of phospholipases A and C at 0.21 to 0.25 mM and 0.25 to 0.45 mM, respectively. Higher concentrations of tripelennamine (0.8 and 1.25 mM) were required to inhibit phospholipases A and C by 50 per cent, and amantadine inhibition of lysosomal phospholipases A and C was least pronounced, with 3.25 and 6.75 mM amantadine required to give 50 per cent inhibition.

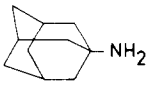
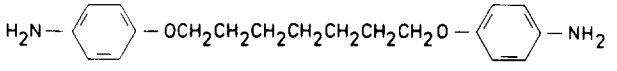
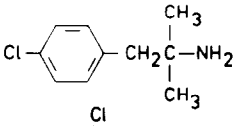
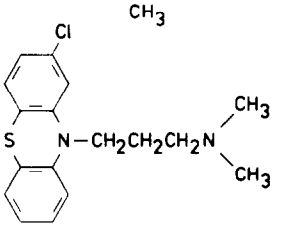
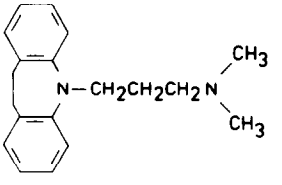
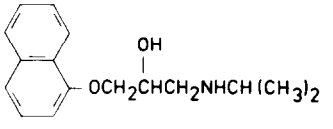
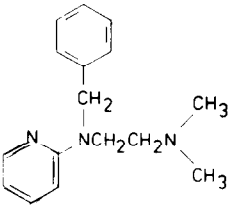
DISCUSSION

Although the site of action of the cationic amphiphilic agents on drug-induced lipodosis has not been identified with certainty, as noted by Lüllmann-

Table 1. Inhibition of lysosomal phospholipases by cationic amphiphilic drugs: millimolar concentration required to produce 50 per cent inhibition

	IC_{50} (mM)		
	Phospholipase		Release of [1-^{14}C]oleic acid by all pathways
Drug	A	C	
1,7-bis(*p*-Aminophenoxy)heptane	0.01	0.03	0.02
Chlorpromazine	0.03	0.07	0.03
Chlorphentermine	0.21	0.45	0.22
Imipramine	0.23	0.25	0.20
Propanolol	0.25	0.38	0.11
Tripelennamine	0.50	1.25	0.88
Amantadine	3.25	6.75	2.50

Table 2. Structures and pharmacological actions of some inhibitors of lysosomal phospholipases A and C

Drug	Structure	Pharmacological effect
Amantadine		Anti-influenzal agent
1,7-bis(*p*-Aminophenoxy)-heptane		Anti-schistosomal agent
Chlorphentermine		Appetite suppressant
Chlorpromazine		Neuroleptic
Imipramine		Antidepressant
Propranolol		Beta-adrenergic blocker
Tripelennamine		Antihistamine

Rauch [1], the present studies have extended our previous findings with chloroquine and 4.4'-bis(diethylaminoethoxy)α,β-diethyldiphenylethane [12] and have demonstrated that seven additional cationic amphiphilic drugs inhibit lysosomal phospholipases A and C directly at pH 4.4. These findings, together with our previous results showing phospholipid and drug storage in lysosomes [3], support the concept that the lysosome is the principal site at which these drugs act. Although the drugs have markedly different pharmacologic actions, they all have the property of inhibiting lysosomal phospholipases. Their structures and actions are summarized in Table 2. Their inhibitor potencies varied widely—from 10 μM 1,7-bis(*p*-aminophenoxy) heptane required to produce 50 per cent inhibition of phospholipase A to 6.75 mM amantadine required to inhibit phospholipase C by 50 per cent (Table 1). Of the nine agents studied to date, all but three have been reported to cause cellular phospholipid storage [1, 13].

One requirement for the production of phospholipidosis may be the ability of a given agent to concentrate in lysosomes. Since all of these agents are weak bases, it seems likely that they might tend to accumulate in lysosomes by the mechanisms described by de Duve *et al.* [18]. In fact, several of these agents have already been found to exhibit lysosomotropism. Wibo and Poole [19] found that chloroquine concentrates in the lysosomal fraction of cultured skin fibroblasts. In rat liver, we showed that after 7 days of oral administration of chloroquine or 4,4'-bis(diethylaminoethoxy)α,β-diethyldiphenylethane, the content of the drug in the lysosomes is ten to thirteen times greater than the content in the liver homogenate [3]. Okhuma and Poole [20] provided evidence that amantadine and chloroquine raise the intracellular pH of cultured macrophages, suggesting that they concentrate in lysosomes.

Cationic amphiphilic drugs that are lysosomotropic may cause phospholipidosis if they also inhibit lysosomal phospholipase as do the seven agents

reported in this study and the two agents reported previously [12]. All of the inhibitors reported to date are cationic, with an amino group at one pole. The typical inhibitor molecule also has a hydrophobic portion lacking a polar functional group. It is interesting that two of the most potent inhibitors of lysosomal phospholipases, 1,7-bis(*p*-aminoethoxy)-heptane and 4,4′-bis(diethylaminoethoxy)α,β-diethyldiphenylethane, are bipolar, having a hydrophobic central region in the molecule, with amino groups on either end.

Although we have shown that chloroquine and 4,4′-bis(diethylaminoethoxy)α,β-diethyldiphenylethane are greatly increased in lysosomes after 7 days [3], their concentrations have not been determined due to lack of a volume term. It will be of great importance to determine the lysosomal concentration of the various cationic amphiphilic agents at an early stage in the development of phospholipidosis, because our results at 7 days might have been a consequence of passive binding to accumulated phospholipids by the mechanism suggested by Lüllmann *et al.* [21]. For these agents to be the cause of phospholipidosis, it will be necessary to show that they are present in lysosomes early in the course of the disorder in concentrations sufficient to account for a substantial inhibition of lysosomal phospholipases.

The mechanism of drug inhibition of lysosomal phospholipases is unknown. Our previous attempts to determine the type of inhibition, using chloroquine and 4,4′-bis(diethylaminoethoxy)α,β-diethyldiphenylethane, did not give definitive results; this is probably due to the complexity of the lysosomal soluble protein fraction used [12]. Studies with purified lysosomal phospholipases A and C may be more revealing. Lüllman *et al.* [21] proposed that cationic amphiphilic drugs inhibit phospholipid catabolism by forming a complex with phospholipids; it is hypothesized that this drug–phospholipid complex resists hydrolysis [21]. Seydel and Wassermann [22], using n.m.r., have provided evidence of drug–phospholipid binding. It should be noted that our studies do not indicate whether the observed inhibition is due to drug–enzyme or drug–phospholipid interactions. Nevertheless, our studies reported here and previously [3, 12] provide strong support for the hypothesis that the lysosome is the principal site of action of the cationic amphiphilic agents and that the molecular basis for phospholipid storage is inhibition of lysosomal lipid catabolism.

Chien *et al.* [23] demonstrated that pretreatment of rats with chlorpromazine blocked the degradation of phosphatidylcholine and phosphatidylethanolamine that follows hepatic ischemia. They proposed that chlorpromazine exerts its action, in part, by reducing cellular Ca^{2+} content. This would not be likely to affect lysosomal phospholipases since they do not require Ca^{2+} [6]. A direct inhibitory effect of chlorpromazine seems more likely, based on our results, but the role of lysosomal phospholipases in ischemic liver is not thought to be great [23]. Nevertheless, if the intracellular pH falls during ischemia and lysosomal phospholipases are released, chlorpromazine would be expected to inhibit their activities as shown here. Evidence for decreased latency of liver lysosomal hydrolases during ischemia has been provided recently by Wattiaux and Wattiaux-de Conick [24] who also showed that this decreased latency of lysosomal acid hydrolases could be partially prevented by chlorpromazine pretreatment.

Ruth *et al.* [25] recently found that Triton-containing lysosomes from rat liver were stabilized by chlorpromazine. They presented evidence that the stabilization was not due to mechanical actions and suggested that inhibition of phospholipases might be involved. They showed *in vitro* inhibition of endogenous lysosomal phospholipase A activity as manifested by the release of lysophosphatidylcholine and lysophosphatidylethanolamine from lysosomes incubated in the presence or absence of 500 μM chlorpromazine. It should be noted that the chlorpromazine concentration required to produce half-maximum stabilization of lysosomes was three to seven times that required for half-maximum inhibition of soluble lysosomal phospholipases A and C (Table 1). Nevertheless, our results are in general agreement with Ruth *et al.* [25]. Interestingly, Watts and Atkins [26] observed that 1,7-bis(*p*-aminophenoxy)heptane also stabilizes rat liver lysosomes against osmotic lysis [26]. Both chlorpromazine (Ref. 9, Table 1) and 1,7-bis(*p*-aminophenoxy)heptane are potent inhibitors of lysosomal phospholipases, suggesting that stabilization may be due, in part, to blockage of a lytic action of these enzymes on the lipid bilayer membrane. This appears to be the first report of inhibition of lysosomal phospholipases by propranolol, imipramine, amantadine, 1,7-bis(*p*-aminophenoxy)heptane, chlorphentermine and tripelennamine.

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