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PHOSPHOLIPID-NUCLEOSIDE CONJUGATES. 5.[1] THE INTERACTION OF SELECTED 1-β-D-ARABINOFURANOSYLCYTOSINE-5'-DIPHOSPHATE-L-1,2-DIACYLGLYCEROLS WITH SERUM LIPOPROTEINS

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SUMMARY: The phospholipid-nucleoside conjugates 1-β-D-arabinofuranosylcytosine-5'-diphosphate-L-1,2-dipalmitin (**1**), -distearin (**2**), and -diolein (**3**) have been shown to interact rapidly with canine high density lipoprotein and with both high density and low density lipoproteins isolated from human serum. The extent of interaction with the high density lipoproteins appears to be dependent upon the characteristic gel-liquid crystalline phase transition of the conjugate's phospholipid. Since the phospholipid-nucleoside conjugates under study represent sustained release forms of the antileukemic agent 1-β-D-arabinofuranosylcytosine, the therapeutic efficacy of these conjugates should now be considered in light of these interactions.

Recent work from this laboratory (4-9) and from others (10-12) has indicated an improved efficacy against various leukemias in mice (5-7,9,10,12) of phospholipid conjugates of 1-β-D-arabinofuranosylcytosine (araC) such as araCDP-L-dipalmitin (**1**), araCDP-L-distearin (**2**), and araCDP-L-diolein (**3**) (4-9), as well as mixed multispecies (araCDP-L-diacylglycerols) and diastereomeric mixtures such as araCDP-D,L-dipalmitin (10-12) relative to the parent drug. Because of their amphipathic nature, these prodrugs form large supramolecular assemblies (e.g., bilayer sheets, interlocking networks of bilayers, or micellar structures) depending on the ionic strength, temperature, the nature of their fatty acid side chain, and the mode of preparation (12,13). Recent work has shown that high density lipoproteins (HDLs) readily incorporate phospholipids from unilamellar (14-17) or multilamellar (18) liposomes and also from discoidal complexes of phospholipid and apolipoprotein A-1 (19). In addition, human plasma low density lipoprotein (LDL) has been shown to aggregate with unilamellar liposomes (20). These

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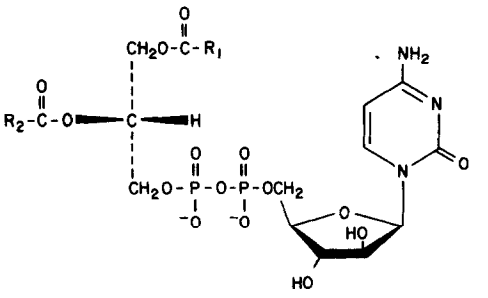

1, $R_1 = R_2 = -(CH_2)_{14}CH_3$
2, $R_1 = R_2 = -(CH_2)_{16}CH_3$
3, $R_1 = R_2 = -(CH_2)_7-CH=CH-(CH_2)_7CH_3$

Figure 1. Structures of the araCDP-L-1,2-diacylglycerols used in this study.

findings, along with current efforts to utilize lipoproteins as site-specific delivery systems for chemotherapeutic agents (21), prompted our current investigation into interactions of lipoproteins with compounds 1-3 (see Figure 1). We utilized canine HDL and human HDL and LDL to evaluate the potential of lipoproteins as carriers for phospholipid-drug conjugates.

MATERIALS AND METHODS

Compounds 1-3 as their disodium salts were prepared as described previously (4,6). Samples for investigation were all prepared by sonication of a suspension of the material in 0.9% NaCl, 0.1 mM Tris, pH 7.1. Sonication was carried out using a Kontes Microultrasonic cell disrupter (with microprobe) at power setting 8. All sonications were performed in a Forma Scientific water bath at a preset temperature (± 0.5°C) and used a routine of alternating 20 sec on, then 20 sec off (6 times - i.e., 2 min of total sonication) in all cases in order to minimize local heating effects. The aggregational states of compounds 1-3, when prepared in this fashion, have been well defined (13). The extinction coefficient at 280 nm of the conjugate at this pH is ca. 10,000 (6).

Isolation of lipoproteins. Canine HDL was isolated from the plasma of purebred beagles by preparative ultracentrifugation as described by Edelstein et al. (22). Lipoprotein concentrations were calculated from the protein, quantitated by the method of Lowry (23), using the chemical composition of canine HDL (22). Human HDL and LDL were similarly prepared by ultracentrifugation of plasma from normal donors (24). The lipoproteins were dialyzed against 0.9% NaCl, 0.1 mM Tris, pH 7.1 before use.

Incubation of phospholipid-nucleoside conjugates with lipoproteins. Samples of conjugate (approx. 1 mL of 1 mM) were prepared by sonication at 37°C as described above, and their optical density was determined spectrophotometrically. Aliquots of lipoprotein solutions of known concentration and optical density were then mixed with the conjugate samples and incubated at 37°C for 1 h. The optical density of this solution was then measured and a known volume (usually 0.8-1.0 mL) was applied to the gel filtration column or the density gradient.

Gel filtration. All gel filtration column chromatography was performed on a Sepharose 4B (Pharmacia) column having dimensions 3.0 x 43.0 cm, and

using 0.9% NaCl, 0.1 mM Tris, pH 7.1 as the eluant in a water-jacketed column. The temperatures in the column and the reservoir were monitored using a thermocouple and a mercury thermometer and, for any one experiment, they were kept constant, ± 1°C. The flow rate was approximately 1 mL/min. All columns were monitored at 280 nm using an LKB Uvicord II, and the absorbance of individual fractions was read using a Beckman A-25 spectrophotometer. The column had an excluded volume (as measured with tobacco mosaic virus, TMV) of 50 mL and a fully included volume (as measured with araC) of 172 mL. Ferritin was eluted at 110 mL. The larger bilayer sheets of araCDP-L-distearin formed by sonication at 37°C are excluded on the column (elutes at 50 mL). Canine HDL elutes after 122 mL and is quantitatively recovered.

Density gradient ultracentrifugation. Fractionation was carried out by isopycnic density gradient ultracentrifugation using a discontinuous gradient consisting of 14%, 16%, 18%, and 20% NaBr. Centrifugation was carried out with a SW41 rotor at 38,000 rpm, 15°C, for 48 h.

Turbidity profiles. Turbidity profiles were measured at 320 nm in tightly stoppered 1 mL cuvettes using a heating-cooling water bath. Temperatures in the UV cuvette were maintained at ± 0.1°C and were measured accurately using a small thermocouple.

RESULTS AND DISCUSSION

Initially, the interaction between the nucleoside-phospholipid conjugates 1-3 and canine HDL was examined because canine HDL is a well-characterized, homogeneous system that is structurally similar to human HDL but lacks the distinct subclasses (HDL_2 and HDL_3) of the latter (22). The araCDP-L-distearin (conjugate 2) prepared by sonication at 24°C or 37°C forms large bilayer sheets, which scatter incident light (13). When these turbid solutions are mixed with canine HDL, the solution clears in a temperature-dependent fashion, faster at 37°C than at 24°C (see Materials and Methods). Control solutions of conjugate 2 alone showed little or no turbidity change over the same time period. These observations prompted a more detailed examination by density gradient ultracentrifugation and column chromatography of the reaction products resulting from the interaction of HDL with conjugates 1-3. Canine HDL is quantitatively recovered in one symmetrical peak, banding in fractions 4-6 (as shown in Figure 2A) when fractionated by density gradient ultracentrifugation. In contrast, the macromolecular aggregates of araCDP-L-dipalmitin (conjugate 1) formed by sonication at 37°C band in fractions 12-15. In some instances excess conjugate precipitated during the centrifugation and pelleted to the bottom of the centrifuge tube. When the araCDP-L-dipalmitin and canine HDL are incubated in varying ratios for 1 h at 37°C and then fractionated by

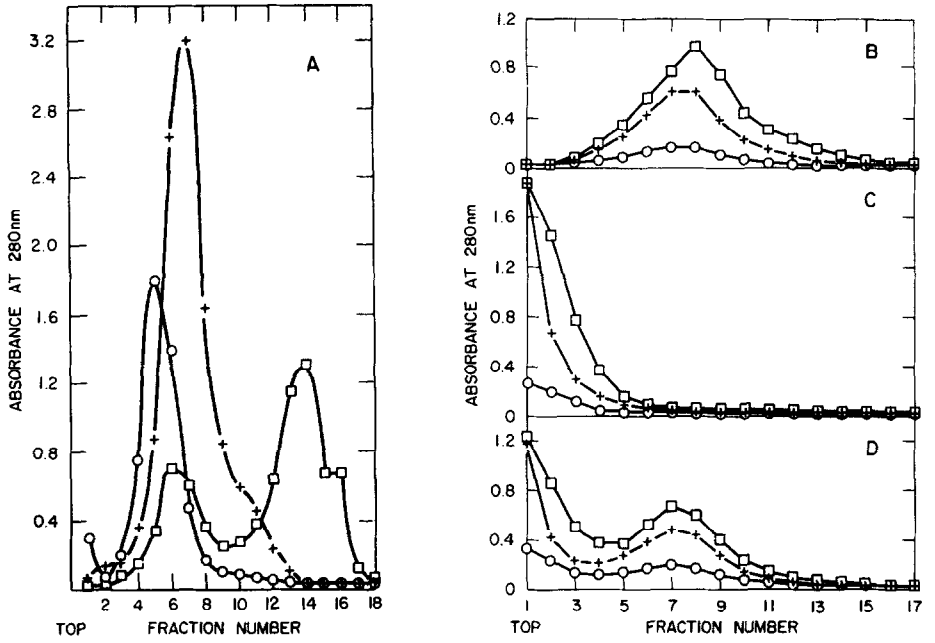

Figure 2. Interaction of araCDP-L-dipalmitin with canine HDL (panel A) and human HDL and LDL (panel B-D). All incubations were carried out at 37°C and the incubation products were fractionated by density gradient ultracentrifugation (see Materials and Methods). The data shown are representative of many similar runs.

A) Elution profiles of canine HDL and araCDP-L-dipalmitin mixtures. O, HDL alone (6 mg); +, incubation mixture contained 10 OD drug and 1.18 mg HDL; □, incubation mixture contained 10 OD drug and 0.27 mg HDL.

B-D) Elution profiles of human HDL and LDL and araCDP-L-dipalmitin mixtures. Aliquots of a stock solution of 1 (1.3 mg/mL) were incubated with 1.5 mg aliquots of lipoprotein and made up to 1 mL with buffer. (B) O, HDL alone; +, HDL plus drug (160 µL); □, HDL plus drug (330 µL). (C) O, LDL alone; +, LDL plus drug (160 µL); □, LDL plus drug 330 µL). (D) O, HDL plus LDL; +, HDL plus LDL plus drug (160 µL); □, HDL plus LDL plus drug (330 µL).

density gradient ultracentrifugation, the amount of araCDP-L-dipalmitin banding in the HDL peak increases with increasing amounts of HDL until quantitative recovery of the conjugate (total optical density) is obtained in the HDL peak (25). Because the recovery of the HDL can be considered quantitative (vide supra), the amounts of conjugate and HDL in the lipoprotein peak (fractions 4-8) can be estimated (Figure 3). Similar results were obtained with araCDP-L-distearin when the incubation mixtures were fractionated by gel filtration column chromatography on a Sepharose 4B column (Figure 3). In addition, the elution position of the HDL peak did not vary,

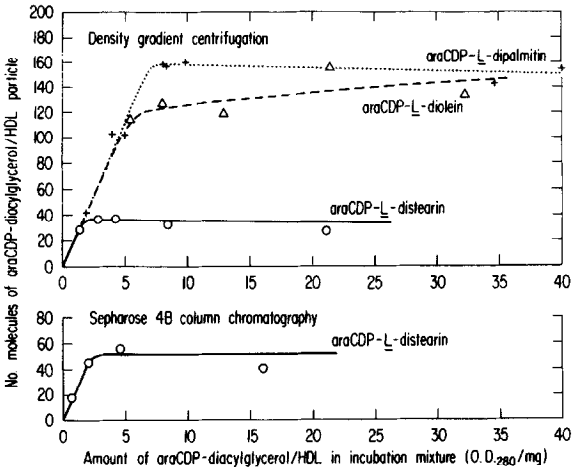

Figure 3. Plot of number of molecules of selected araCDP-L-diacylglycerols per canine HDL particle (obtained from HDL-drug complex peak) against the amount of araCDP-L-diacylglycerol per HDL in the original incubation mixture (OD_{280}/mg). Upper plot shows data obtained from density gradient centrifugation; lower plot shows data obtained from gel filtration. +, araCDP-L-dipalmitin; O, araCDP-L-distearin; Δ, araCDP-L-diolein.

even when large amounts of conjugate were present, indicating that the conjugate is incorporated into the HDL with little or no change in overall size of the lipoprotein. The amounts of araCDP-diacylglycerol incorporated into the canine HDL particles were calculated and plotted as a function of the ratio of drug/HDL in the original incubation mixture (Figure 3). The number of molecules incorporated per HDL particle are approximately 160, 40, and 130 for conjugates 1, 2, and 3, respectively. The values obtained for araCDP-L-distearin by the ultracentrifugation method and the gel filtration method are in good agreement. Also, the results are consistent with the work of others, who have shown an increased exchange of phosphatidylcholine from liposomes at or above their phase transition with serum lipoproteins (26,27).

The incorporation of araCDP-L-dipalmitin into human lipoproteins is shown in Figure 2(B-D). Ready uptake into both human HDL (HDL_2 plus HDL_3) and human LDL is apparent [Figure 2(B,C)]. Furthermore, when the araCDP-L-dipalmitin was incubated with human HDL and LDL, present in the same proportions found in serum, uptake into both classes of lipoprotein was clearly seen (Figure 2D).

In conclusion, the ready uptake of the prodrugs 1-3 into both HDL and LDL lipoproteins has been demonstrated under physiological conditions. Whether the uptake and transport by the lipoproteins is responsible, to some extent, for the improved efficacy of this type of prodrug is presently unclear, but the results described herein provide further evidence for a possible role of lipoproteins in the transport of lipophilic drugs and offer encouragement for the use of lipoproteins as a site-specific drug delivery system (21). Such possibilities and a deeper understanding of the structural nature of the lipoprotein-conjugate complex, as has been derived for HDL-phosphatidylcholine complexes (14), are to be investigated.

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