Vol. 85, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 29, 1978 Pages 714-723

THE SYNTHESIS, CHARACTERIZATION, AND PRELIMINARY BIOLOGICAL EVALUATION OF 1-β-D-ARABINOFURANOSYLCYTOSINE-5'-DIPHOSPHATE-L-1,2-DIPALMITIN

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Received October 6, 1978

SUMMARY: Recently, 1-β-D-arabinofuranosylcytosine-5'-diphosphate-DL-1,2-dipalmitin (VIa) was reported to inhibit the growth of L51784 cells in mice and of human colon carcinoma HCT-15 cells, also in mice. This paper describes the synthesis of a single diastereomer by conversion of 1-β-D-arabinofuranosylcytosine 5'-monophosphate (II) to the nucleoside 5'-phosphomorpholidate (III), followed by reaction with L-α-dipalmitoylphosphatidic acid (IV) to give 1-β-D-arabinofuranosylcytosine-5'-diphosphate-L-1,2-dipalmitin (V) in good yield. The separation of the product is described and its characterization by chromatography, elemental analysis, and spectroscopic methods. The lipophilic nature of V renders it insoluble in aqueous media and a method of sample preparation utilizing sonication techniques is described which provides a clear solution suitable for biological evaluation. In addition, the ability of V to inhibit the *in vitro* growth of L1210 cells and of mouse myeloma MPC 11 cells is described and compared with 1-β-D-arabinofuranosylcytosine (I) and other lipophilic prodrugs of I.

The efficacy of araC* (I) as a chemotherapeutic agent has been improved by the use of lipophilic 5'-carboxylic acid esters [e.g., 5'-O-ado-araC (VII) and 5'-O-palm-araC (VIII)] as prodrugs (1,2) which protect the arabinonucleoside from catabolism by deoxycytidine deaminase to the biologically ineffective araU (3). Subsequently, the parent drug (I) is released upon enzymatic or chemical hydrolysis (1,2).

The use of phospholipids instead of carboxylic acid esters as "carriers" for araC is a particularly attractive alternative since ara-CDP-L-dipalmitin (V)

*Abbreviations: araC, 1-β-D-arabinofuranosylcytosine; araU, 1-β-D-arabinofuranosyluracil; ara-CMP, 1-β-D-arabinofuranosylcytosine 5'-monophosphate; ara-CDP-L-dipalmitin, 1-β-D-arabinofuranosylcytosine 5'-diphosphate-L-1,2-dipalmitin; ribo-CDP-DL-dipalmitin, 1-β-D-ribofuranosylcytosine 5'-diphosphate-DL-1,2-dipalmitin; 5'-O-ado-araC, 5'-O-adamantoyl-1-β-D-arabinofuranosylcytosine; 5'-O-palm-araC, 5'-O-palmitoyl-1-β-D-arabinofuranosylcytosine.

0006-291X/78/0852-0714$01.00/0

is the 2'-epimer of ribo-CDP-L-dipalmitin, a natural cellular component. While this work was in progress, ara-CDP-DL-dipalmitin (VIa) was shown to be a substrate for several enzymes which normally utilize ribo-CDP-L-dipalmitin to produce 5'-CMP as one of the products (4), and preliminary data (4,5) have shown ara-CDP-DL-dipalmitin (VIa) to be extremely effective in mice bearing leukemia L51784 and against human colon carcinoma (HCT-15) in immunosuppressed mice.

We report herein the detailed synthesis, separation, and characterization of the single diastereomer ara-CDP-L-dipalmitin (V). In addition, we describe a method for solubilizing this type of liponucleotide for biological testing and the results of preliminary inhibition studies against L1210 and mouse myeloma MPC 11 cells grown in culture.

Materials and Methods

Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Ultraviolet spectra were recorded on a Beckman Model 25 spectrophotometer. ^1H NMR spectra ($CDCl_3$-CD_3OD-D_2O, TMS internal or D_2O, TSP internal) were determined on a Varian HR-220 instrument operating in the FT mode. Thin layer chromatography was carried out on Eastman cellulose sheets (No. 13254 with fluorescent indicator in solvent A: 2-propanol-conc. NH_4OH-H_2O (7:1:2) or on Merck silica gel 60, F-254 in solvent B: $CHCl_3$-MeOH-H_2O-HOAc (25:15:4:2), and solvent C: isobutylketone-HOAc-H_2O (40:30:7). Uv-absorbing compounds were detected by visualization under a uv-lamp and phosphate-containing materials by spraying with a modified Dittmer-Lester reagent (6) as described elsewhere (7). Ion-exchange chromatography was performed on Whatman DEAE-52 cellulose (Ac^- form) or Cellex-CM (Na^+ form) presoaked and packed in $CHCl_3$-MeOH-H_2O (2:3:1 by volume). Monitoring of column eluant was at 254 nm using an LKB Uvicord II uv analyzer. Sonication was carried out using a Kontes Microultrasonic cell disrupter. Sedimentation coefficients were determined using a Beckman Model L-2 ultrachentrifuge following procedures described by Anderson (8). Ara-CMP was synthesized by known procedures (9) or was purchased from Sigma Chemical Co., St. Louis, Missouri. L-α-Dipalmitoylphosphatidic acid disodium salt was purchased from Sigma; ribo-CDP-DL-dipalmitin was purchased from P-L. Biochemicals and purified by ion-exchange chromatography before use; 5'-O-adamantoyl-1-β-D-arabinofuranosylcytosine and 5'-O-palmitoyl-1-β-D-arabinofuranosylcytosine were gifts from the Upjohn Company, Kalamazoo, Michigan. Elemental analyses were carried out by Galbraith Laboratories, Inc., Knoxville, Tennessee. Pyridine was stored, refluxed over, and then distilled from CaH_2 and used immediately. Acetone was distilled before use and all other solvents were reagent grade.

Preparation of samples for biological evaluation. The compound to be tested (IV, V, or VIb, 0.005 mmole) was suspended in H_2O (1 ml) and sonicated for 60 mins while jacketed in a water bath (for IV, 22°; for V, 0°; for VIb, 10 min at 22° then 50 min at 0°). The clear solution was then filtered through a 0.45 μm millipore filter, its concentration checked by uv and used directly for testing or sedimentation studies.

Culture techniques. Subclone 66.2 of mouse myeloma MPC 11 cell line (10) was grown in suspension at 37°C in 5% CO_2, 95% air that was water saturated. Culture medium was Dulbecco's modified Eagle's medium, containing heat inactivated horse serum (20%), NCTC-109 medium (10%), non-essential amino acid solution (100X) (1%), penicillin (80 units/ml), streptomycin (80 µg/ml), and L-glutamine (3.3 mM) (10).

Growth curve analyses. Growth curves of mouse myeloma cells were determined by electronic counting. A modified Coulter transducer with a multichannel analyzer (127 channels) was used with a 100 µm diameter aperture. An aliquot (0.1 ml) of cell suspension was placed in a plastic dilution vial and electrolyte (10 ml) was added. Electrolyte was formalin (0.04%) in Dulbecco's phosphate buffered saline (11). After mixing by inversion, readings were taken immediately. Electrical size signals from the Coulter transducer were sampled for 0.1 min and subjected to pulse-height analyses as well as visual electrical volume determination. The electrically larger peak in the bimodal distribution of cells was used as the viable cell number in the growth curves, according to the method of Matsushita et al. (12).

1-β-D-Arabinofuranosylcytosine-5'-monophosphomorpholidate, 4-morpholine-N,N'-dicyclohexylcarboxamidinium salt (III). This was prepared by condensation of ara-CMP (II) (0.323 g, 1 mmole) with morpholine (0.34 ml) in the presence of N,N'-dicyclohexylcarbodiimide using published procedures for related compounds (13,14). The reaction was monitored by tlc (cellulose) in solvent A and the product III was obtained in two crops (0.585 g; 85.3%) after drying over P_2O_5 in vacuo for 5 hr at 60°. Mp 155-165° (slowly collapsed to a gum); uv (H_2O), λmax 272 nm (ε 9,100), λmin 252 nm (ε 6,000); (0.01 N HCl, λmax 281 nm (ε 13,100), λmin 246 nm (ε 2,300); (0.01 N NaOH), λmax 272 nm (ε 9,400), λmin 253 nm (ε 6,200). NMR (D_2O; δ from TSP) 7.86 (d, J_{6-5} = 7.5 Hz, H_6); 6.24 (d, $J_{1'2'}$ = 5.4 Hz, $H_{1'}$); 6.08 (d, J_{5-6} = 7.5 Hz, H_5); 4.86 (s, HDO); 4.45 (t, $J_{2'3'} \simeq J_{2'1'}$ = 5.3 Hz, $H_{2'}$); 4.27-4.02 (m, $H_{3'}$, $H_{4'}$, $H_{5'}$, $H_{5''}$); 4.00-2.98 (m's, morpholine-CH_2-'s); 2.09-1.02 (m's, cyclohexyl-CH_2-'s). Rf's: (A) 0.47, (B) 0.16, (C) 0.04. Anal. Calcd. for $C_{30}H_{52}N_7O_9P$ (M.Wt. 685.77): C, 52.54; H, 7.64; N, 14.30; P, 4.52. Found: C, 52.70; H, 7.73; N, 14.40; P, 4.37.

L-α-Dipalmitoylphosphatidic acid, pyridinium salt (IV). This was prepared from the commercially available disodium salt [Rf's: (B) 0.70; (C) 0.53] by passage through a Dowex 50W x 8 (pyridinium form) column pre-equilibrated and eluted with $CHCl_3$-MeOH-pyridine-H_2O (3:3:1:1). The eluate was evaporated to small volume and the gel-like residue was lyophilized. A further lyophilization from benzene gave a white solid.

1-β-D-Arabinofuranosylcytosine-5'-diphosphate-L-1,2-dipalmitin (V). This was prepared by modification of published procedures for related compounds (14). To 150 mg (0.22 mmole) of III as the 4-morpholine-N,N'-dicyclohexylcarboxamidinium salt, was added dry pyridine (10 ml). The material was rendered anhydrous by repeated evaporation from pyridine (4 x 3 ml) in vacuo. To a solution of the residue in pyridine (5 ml) was added IV (0.29 mmole) as the pyridinium salt. The solution was evaporated to dryness from dry pyridine (5 x 5 ml; admitting only dry N_2 into the evaporator) and finally concentrated to ca. 3 ml. This mixture was stoppered and stored in a desiccator for 5 days at room temperature, the progress of the reaction being monitored by tlc (silica gel, solvent B). The reaction mixture was evaporated to dryness in vacuo and then co-evaporated with toluene (5 x 3 ml) to remove traces of pyridine. The residue so obtained was dissolved in $CHCl_3$-MeOH-H_2O (2:3:1, 5 ml), cooled to 0° and adjusted to pH 3.5 by addition of 0.01 N HCl. Centrifugation gave two layers which were separated. The upper layer was extracted with $CHCl_3$ (2 x 5 ml), using centrifugation to aid the separation of the phases. The combined $CHCl_3$ layers were washed once with water (1/5 by volume) and concentrated to a small volume (∿ 3 ml). To the resi-

due was added $CHCl_3$-MeOH-H_2O (2:3:1; 20 ml) and, if necessary, additional MeOH was added to give a clear solution. This was applied to a cooled (4°), jacketed DEAE-52 (Ac^-) column (2.5 x 45.0 cm) and washed with $CHCl_3$-MeOH-H_2O (2:3:1; 200 ml). A linear gradient of the same solvent (1 L) to 0.15 M NH_4Ac (1 L, also made up in the same solvent) was then commenced. The appropriate fractions containing V (0.17 mmole, 76%) were pooled, concentrated to yield an opalescent gel-like product, and then lyophilized. The residue was separated between $CHCl_3$ - H_2O (4:1, 50 ml) and evaporation of the organic phase gave V as the diammonium salt. Conversion to the disodium salt was carried out by passage down a Cellex-CM (Na^+ form) column (1.5 x 15.0 cm) in $CHCl_3$-MeOH-H_2O (2:3:1) as solvent. The eluate was evaporated to dryness and the product was precipitated from a minimum volume of $CHCl_3$ by addition of acetone. The white solid was filtered, washed with acetone, and dried in vacuo over P_2O_5 at room temperature for 17 hr. Mp 185-189° (decomp.); uv ($CHCl_3$-MeOH-H_2O, 2:3:1), λmax 274 nm (ε 7,200), λmin 251 nm (ε 4,500); ($CHCl_3$-MeOH-0.1 N HCl, 2:3:1), λmax 284 nm (ε 15,900), λmin 252 nm (ε 1,600); ($CHCl_3$-MeOH-0.01 N NaOH, 2:3:1), λmax 274 nm (ε 7,400), λmin 252 nm (4,800). NMR ($CDCl_3$-CD_3OD-D_2O; δ from TMS) 7.89 (d, J_{6-5} = 7.6 Hz, H_6); 6.20 (d, $J_{1'-2'}$ = 5.0 Hz, $H_{1'}$); 6.02 (d, J_{5-6} = 7.6 Hz, H_5); 4.32 (t, $J_{2'3'} \simeq J_{2'-1'}$ = 4.9 Hz, $H_{2'}$); 4.28-3.86 ($H_{3'}$, $H_{4'}$, $H_{5'}$, $H_{5''}$); 2.33 (q, $-CH_2-CO-$); 1.68-1.07 (m's, palmitoyl-CH_2-'s); 0.98-0.80 (m, $CH_3(CH_2)_{14}-$). Rf's: (B) 0.50, (C) 0.46. Anal. Calcd. for $C_{44}H_{79}N_3O_{15}P_2Na_2 \cdot 1.5H_2O$ (M.Wt. 1025.05): C, 51.55; H, 8.06; N, 4.10; P, 6.04. Found: C, 51.36; H, 8.19; N, 4.24; P, 6.34.

Results and Discussion

Reaction of ara-CMP with morpholine by the procedure of Moffat and Khorana (13) (see Figure 1) gave the 5'-monophosphomorpholidate (III) in good yield. The reaction of III with the pyridinium salt of L-α-dipalmitoylphosphatidic acid (IV) to produce ara-CDP-L-dipalmitin (V) occurred readily in anhydrous pyridine (14). Detection of non-uv-absorbing material (e.g., IV) on Merck silica gel tlc plates (see Experimental) proved inconclusive when the familiar phosphorus-detecting sprays (6,15) were used and a simple modification of the Dittmer-Lester spray was required for observation of these phosphorus-containing compounds at low concentrations (7). Separation of V from unwanted by-products proved to be difficult largely due to the solubility properties of the product. Solubility was obtained in $CHCl_3$-MeOH-H_2O (2:3:1) and complete separation was achieved on DEAE-cellulose (Ac^- form), using a cold-jacketed column and a linear gradient of NH_4Ac in the same solvent mixture (16). After evaporation of volatiles, the precipitated ammonium salt was converted to the disodium salt using CM-cellulose (Na^+ form) in the same $CHCl_3$:MeOH:H_2O solvent mixture. The product was fully characterized by nmr and uv spectroscopy, elemental analysis, and chromatography (see Materials and Methods).

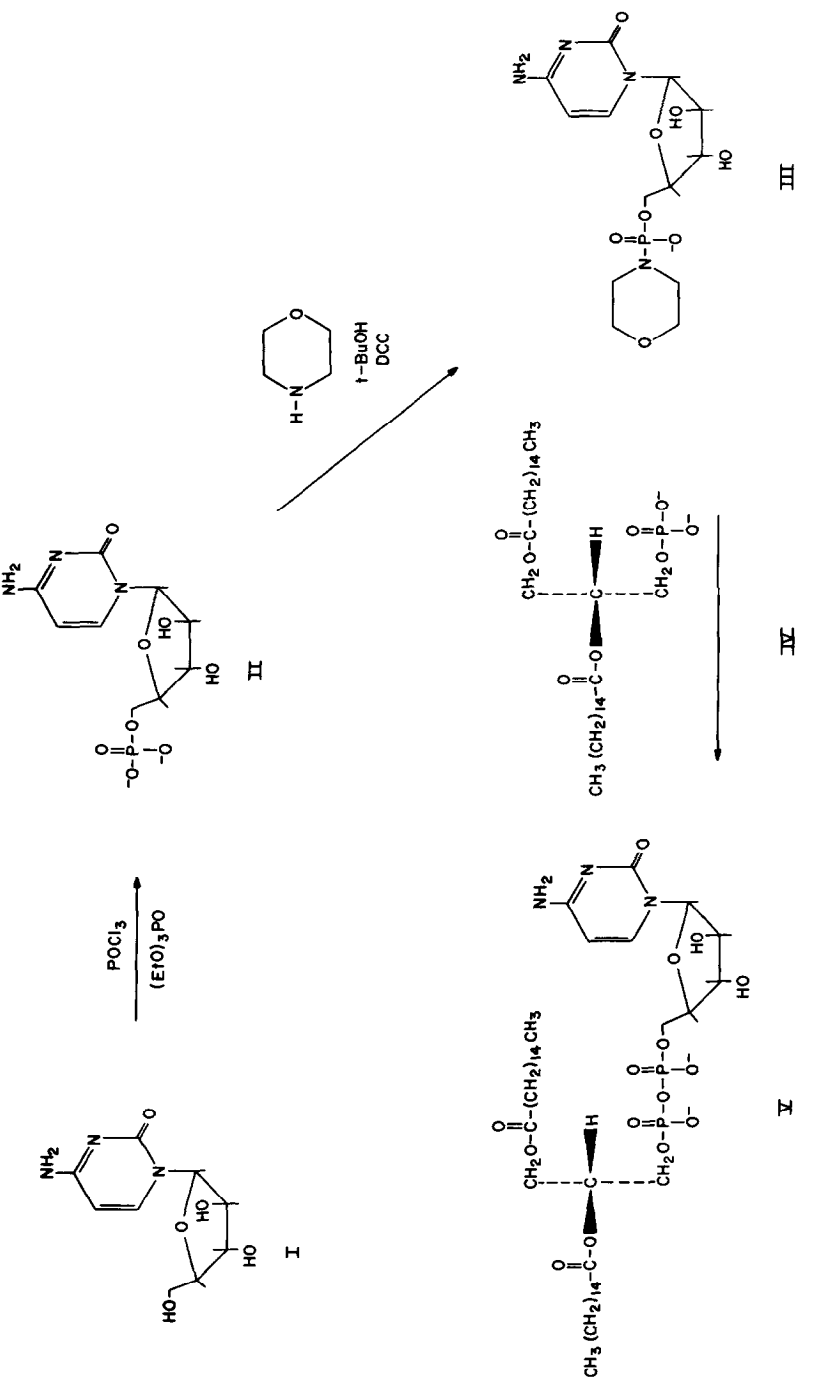

Figure 1. Synthesis of ara-CDP-L-dipalmitin (V).

The ara-CDP-L-dipalmitin (disodium salt) produced was insoluble in DMSO and in H_2O, and our initial testing (against L1210 cells) utilized a suspension of V in DMSO [see ref (17) for details]. Using appropriate dilutions of this suspension, there was a small inhibition of growth (68% after 48 hr; 80% after 72 hr) at 10^{-4} M (based upon original weight of V in the suspension). [The parent araC (I) is totally soluble at these concentrations and is much more effective (95% inhibition after 48 hr at 10^{-6} M).] Suspensions of V in water did not inhibit the mouse myeloma cell line at any of the concentrations tested. We therefore sought a new method for preparing these liponucleotides for biological evaluation in in vitro systems.

Since sonication of phospholipid-containing compounds has been shown to lead to the formation of micelles or vesicles (18), it seemed appropriate to examine the effect of sonication on a suspension of V in H_2O. Sonication of such a suspension lead to a clearing of the sample after only $\sim$ 10 mins. Thereafter, samples of IV, V, and VIb were routinely sonicated for 1 hr and then filtered through a 0.45 μm millipore filter before being tested in in vitro systems. Examination of the solution by tlc after sonication showed no degradation of the material; in addition, examination by uv spectroscopy before and after filtration showed no retention of uv-absorbing material by the millipore filter.

Examination by analytical ultracentrifugation (8) of an aqeuous solution of V, after sonication and filtration, indicated that the bulk of the material had a sedimentation coefficient of $\sim$ 4 $S_{25,w}$ indicating that vesicles or micelles had indeed been formed, the exact nature of which is currently being investigated.

In contrast to a suspension of V in water, a sample prepared as described above showed good inhibition (Figure 3), with 50% inhibition after two generations ($\sim$ 50 hrs) at 6.2 M x 10^{-5} while controls of ribo-CDP-DL-dipalmitin (VIb) and L-α-dipalmitoylphosphatidic acid (IV) (samples similarly prepared by sonication and filtration) showed little or no inhibition at any of the concentrations

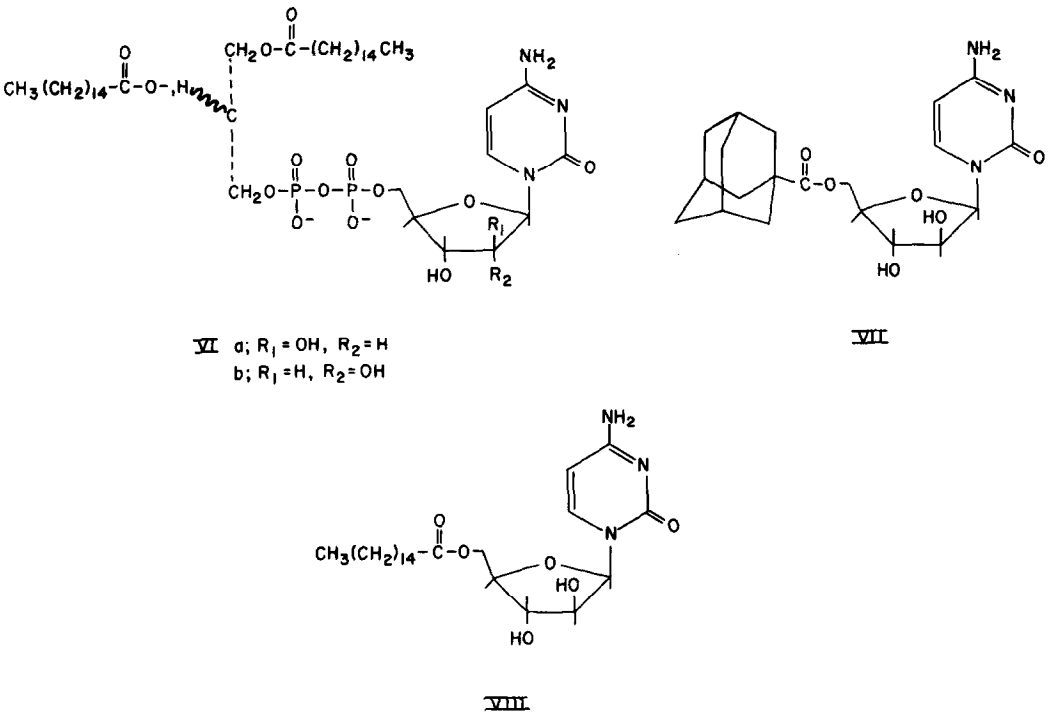

Figure 2. Structures of ara-CDP-DL-dipalmitin (VIa), ribo-CDP-DL-dipalmitin (VIb), 5'-O-ado-araC (VII), and 5'-O-palm-araC (VIII).

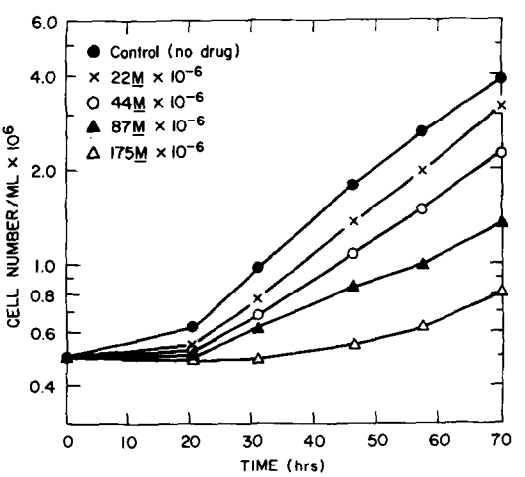

Figure 3. Ara-CDP-L-dipalmitin (V) Inhibition of Mouse Myeloma Cell Growth. Sonicated (V) was added to 5 ml suspension cultures at the concentrations indicated. All cultures contained 4% H_2O V/V and were grown as described in text. Cell number was determined by electronic Coulter analyses.

Table 1. Inhibition of Mouse Myeloma Cell Growth.

	Inhibiting Dose (M x 10^{-6})[a]	
	in DMSO	Sonicated
araC (I)	4.6	1.6
L-α-dipalmitoylphosphatidic acid (IV)	-	No inhibition
ara-CDP-L-dipalmitin (V)	-	62.2
ribo-CDP-DL-dipalmitin (VIb)	-	Slight inhibition[b]
5'-O-ado-araC (VII)	9.2	-
5'-O-palm-araC (VIII)	5.0	-

[a] Inhibiting dose is drug concentration causing 50% inhibition of cell growth, relative to control (non-drug containing) cultures after two generations (ca. 50 hrs, mid-exponential) growth. All DMSO cultures and controls contained 0.6% V/V DMSO. Sonicated drug cultures and controls contained 4% H_2O V/V

[b] 11% inhibition at 69 M x 10^{-6}.

tested. AraC in this system showed 50% inhibition after two generations (∿ 50 hrs) at 1.6 M x 10^{-6}. The other lipophilic drugs tested, 5'-O-ado-araC (VII) and 5'-O-palm-araC (VIII) were examined as solutions in DMSO, since sonication of aqueous suspensions did not lead to dissolution in water. Tested in this fashion VII and VIII showed 50% inhibition after two generations (∿ 50 hrs) at 9.2 and 5.0 M x 10^{-6}, respectively (see Table 1). A solution of araC in DMSO showed 50% inhibition at 4.6 M x 10^{-6} after two generations (∿ 50 hrs).

The lower activity of V relative to araC against the mouse myeloma cell line can be explained by slow release of the active parent drug I from V. After initial uptake by cells of the micelles/vesicles of V, breakdown of these micelles/vesicles is required for release of the prodrug V which, in turn, must release the ara-CMP (or araC) following enzymatic action. The somewhat higher activity of the other lipophilic analogues tested, 5'-O-ado-araC (VII) and 5'-O-palm-araC (VIII), is not inconsistent, because their uptake by cells is presumably by a different mechanism and the enzymatic (or chemical) hydrolysis of the prodrug to the parent araC also follows different pathways.

The inhibition of the mouse myeloma cell line by V (Table 1) is comparable to that described for the ara-CDP-DL-dipalmitin (VIa) against L5178Y cells grown in culture (1.4 M x 10^{-5}, 50% inhibition, 72 hrs] (19). This latter study describes a slight inhibition by ribo-CDP-DL-dipalmitin (VIb) but indicates a limiting solubility of this compound. Sonication of VI at room temperature caused dissolution in ∿ 10 mins and sonication was then continued at 0° for an additional 50 mins. A slight inhibition [11% at 6.9 M x 10^{-5}, after two generations (∿ 50 hrs)] could be detected with this sample.

The present study describes the first detailed procedures for the chemical synthesis of ara-CDP-L-dipalmitin (V), and tabulates identifying spectral and chromatographic data. A new method of sample preparation of phospholipid analogues for biological evaluation has allowed in vitro testing of V against a mouse myeloma cell line. These encouraging results along with other data presented by Turcotte and coworkers (4,5,20) serve to further emphasize the need for additional investigation of this new type of prodrug.

Acknowledgments: We thank Drs. S.S. Danyluk, N.G. Anderson, and S.P. Spragg for helpful discussions, and Dr. H.C. Hong for the test against cell line L1210. Supported in part by the American Cancer Society, Illinois Division Grant 77-57, and the U.S. Department of Energy.

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