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Liposomal Sustained-Release Delivery Systems for Intravenous Injection. IV. Antitumor Activity of Newly Synthesized Lipophilic 1-β-D-Arabinofuranosylcytosine Prodrug-Bearing Liposomes

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A lipophilic prodrug of 1-β-D-arabinofuranosylcytosine (Ara-C), namely N^4-[*N*-(cholesteryloxycarbonyl)glycyl]-Ara-C (COCG-Ara-C), was synthesized and its antitumor activity in a liposome-entrapped form was studied. COCG-Ara-C showed an increased lipophilicity and almost complete entrapment in liposomes. COCG-Ara-C was hydrolyzed to the parent drug chemically, but the hydrolysis was accelerated in the presence of mouse, rat and human plasma. The *in vitro* cytotoxicity of the prodrug against P 388 leukemia was approximately one-fifth that of Ara-C and four times that of N^4-behenoyl-Ara-C (BHAC). For *in vivo* antitumor activity tests, unilamellar vesicles composed of egg phosphatidylcholine (PC), egg sphingomyelin (SM) and COCG-Ara-C in a molar ratio of $7:3:X$ ($X=0$—2.0) were prepared by the combination of controlled dialysis and sequential extrusion. The vesicle size ranged from 108 ± 18 nm to 124 ± 18 nm. In all the antitumor activity studies, chemotherapy was performed intravenously. The antitumor activity of COCG-Ara-C-bearing liposomes against intraperitoneally- or intravenously-inoculated mouse L 1210 leukemia was clearly superior to those of Ara-C and BHAC aqueous solutions. The efficacy of COCG-Ara-C against L 1210 leukemia was dependent upon the dosage form: regardless of implantation route, liposomal COCG-Ara-C showed a more potent activity than free COCG-Ara-C (aqueous solution). Prodrug-bearing liposomes also inhibited the growth of a human lung adenocarcinoma A 549 xenograft implanted under the renal capsule more efficiently than did Ara-C and BHAC aqueous solutions. These results suggest the potential usefulness of COCG-Ara-C-bearing liposomes in cancer chemotherapy.

Keywords——1-β-D-arabinofuranosylcytosine; prodrug; hydrolysis; antitumor activity; L 1210 leukemia; human lung adenocarcinoma A549; liposome; sustained release

1-β-D-Arabinofuranosylcytosine (Ara-C) is an important chemotherapeutic agent in the treatment of acute myelogenous leukemia,[1] but has limited efficacy against solid tumors.[2] Because of the S-phase specificity of its antitumor action,[3] continual contact with tumor cells is a requirement for Ara-C to exert its cytotoxic effects. After intravenous (i.v.) injection, Ara-C is rapidly converted to the inactive methabolite 1-β-D-arabinofuranosyluracil (Ara-U) by the catabolic action of cytidine deaminase, which is widely distributed in both normal and neoplastic tissues.[4] As a consequence, Ara-C has a very short plasma half-life,[5] which usually necessitates frequent administration[6] or continuous i.v. infusion[7] to maintain adequate plasma levels for maximum therapeutic efficacy. To circumvent the scheduling problems of Ara-C therapy, numerous attempts have been made to modulate the pharmacokinetics of Ara-C by means of drug delivery systems.[8] Several investigators[9] have shown that the antitumor activity of Ara-C can be enhanced by encapsulation of the drug within liposomes. Improved drug efficacy could be explained by the depot functions of liposomes. However, the encapsulation of Ara-C in the liposomal aqueous phase has some disadvantages, *i.e.*, Ara-C

shows low entrapment and relatively fast leakage out of lipid vesicles.[9c,10]

One of many ways of overcoming these difficulties may be chemical modification of drug molecules to lipophilic prodrugs having adequate physicochemical properties for incorporation in liposomes.[11] In a previous study,[11e] we synthesized several lipophilic derivatives of mitomycin C (MMC) by introduction of the cholesteryl moiety into its 1a-*N*-position through different spacers, and investigated their feasibility as prodrugs for liposomal sustained-release carrier systems to be administered by i.v. injection. Among the derivatives tested, *N*-(cholesteryloxycarbonyl)glycyl MMC (COCG-MMC) proved to have the best properties as regards entrapment efficiency, lability and affinity to liposome carriers in the circulation. Furthermore, COCG-MMC-bearing liposomes successfully maintained the blood levels of the parent drug over a prolonged period of time after i.v. injection.[12]

In the present study, N^4-[*N*-(cholesteryloxycarbonyl)glycyl]-1-β-D-arabinofuranosylcytosine (COCG-Ara-C), which has the same lipophilic moiety and spacer as COCG-MMC, was synthesized and the antitumor activity of the liposome-entrapped derivative against mouse L 1210 leukemia and human lung adenocarcinoma A 549 was investigated to evaluate the usefulness of this delivery system in cancer chemotherapy.

Experimental

General Procedures——Melting points were determined using a Thomas Hoover capillary melting point apparatus and are uncorrected. Ultraviolet (UV) absorption spectra were recorded on a Hitachi U-3200 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AM-200 spectrometer. Mass spectra (MS) were obtained using a VG Analytical ZAB-SE mass spectrometer. Infrared (IR) spectra were recorded on a Hitachi 260-10 spectrophotometer.

Chemicals——Ara-C and Ara-U were purchased from Yamasa Shoyu Co. N^4-Behenoyl-Ara-C (BHAC, Sunrabin®) was from Asahi Chemical Industry Co. Egg phosphatidylcholine (PC) and egg sphingomyelin (SM) were obtained from Sigma Chemical Co. *N*-(Cholesteryloxycarbonyl)glycine was prepared according to the method previously described.[11e] All other chemicals were of reagent grade or better.

Synthesis of COCG-Ara-C——Ethylchlorocarbonate (0.61 g) was added dropwise to a solution of *N*-(cholesteryloxycarbonyl)glycine (2.74 g) and triethylamine (0.78 ml) in tetrahydrofuran (40 ml) with stirring at -15 °C, and the mixture was stirred for 20 min at -15 °C. Then a solution of Ara-C (1.37 g) in dimethylformamide (30 ml) was added dropwise with stirring at -15 °C. The mixture was stirred for 1 h at 5 °C and then for 30 min at room temperature, and the organic solvents were evaporated off *in vacuo*. The residue was washed with methanol and dried to afford a crude reaction product, which was purified by silica gel column chromatography to yield N^4-[*N*-(cholesteryloxycarbonyl)glycyl]-Ara-C (1.80 g, 45%) as a white powder; mp 159—161 °C (dec.). IR (Nujol): 3330, 2850, 1690, 1640, 1570 cm^{-1}. SIMS *m/z*: 713 (M+H), 751 (M+K). ^{1}H-NMR ($CDCl_3+CD_3OD$) δ: 8.08 (1H, d, J=7.6 Hz, 6-H), 7.14 (1H, d, J=7.6 Hz, 5-H), 6.06 (1H, d, J=4.0 Hz, 1′-H), 5.34 (1H, br s, cholesteryl 6-H), 4.10—3.70 (5H, m, 2′, 3′, 4′-H, 5′-CH_2), 3.62 (2H, m, $COCH_2NHCO$), 0.66 (3H, s, cholesteryl 18-CH_3).

Lipophilic Index——The relative lipophilic indices ($\log k'$) were determined by high performance liquid chromatography (HPLC) employing the equation $\log k' = \log[(t_R - t_0)/t_0]$, where t_R is the retention time of the solute and t_0 is the elution time of the solvent. The $\log k'$ values were extrapolated to 0% organic solvent concentration to obtain the lipophilic indices ($\log k'_0$).[13] The stationary phase used was a Develosil ODS-5 packed column (4.6×200 mm, Nomura Chemical Co.). A mixture of isopropanol and distilled water was used as the mobile phase at a flow rate of 1.0 ml/min.

Preparation of Liposomes——Liposomes used in this study were composed of PC, SM and COCG-Ara-C in a molar ratio of 7:3:X (X=0—2.0). All liposome preparations were routinely prepared by the combination of controlled dialysis[14] and sequential extrusion.[15] Lipids and an appropriate amount of COCG-Ara-C were dissolved in chloroform–methanol (2:1, v/v) and the organic solvents were evaporated off. The dried lipid film was suspended in 6 ml of pH 7.4 phosphate-buffered saline (PBS), sodium cholate was added in a molar ratio of total phospholipids to detergent of 0.8, and the mixture was agitated by hand, yielding a lipid concentration of 50 or 100 mM. The formed mixed micellar solution was dialyzed against PBS at 35 °C using a Lipoprep dialyzer (Diachema) for 22 or 44 h depending on the lipid concentration. After completion of dialysis, the liposome suspension was subjected to sequential extrusion through 0.4, 0.2, 0.1 and 0.08 μm pore-sized polycarbonate membranes (Nuclepore) and subsequently sterilized by filtration through a Millex GV (0.22 μm, Millipore). The concentration of liposome-entrapped prodrug was determined by HPLC. The liposome preparations were diluted with sterile PBS to obtain the desired drug concentration and kept under nitrogen at 4 °C until use.

Vesicle Size Measurement——Liposome size was determined by the use of a dynamic laser light scattering instrument (Coulter, model N4).

Entrapment Measurement——The extent of entrapment of the prodrug in liposomes was determined by gel filtration as described previously.[11]

Hydrolysis Test——Hydrolysis experiments were carried out on the liposome-entrapped COCG-Ara-C in PBS and three kinds of plasma. Female ICR mice weighing about 23 g and Sprague–Dawley rats weighing about 240 g were used to obtain plasma. Human plasma was obtained from a healthy volunteer. All hydrolysis experiments were performed at 37 ± 0.2 °C and initiated by adding a liposome suspension (25 μl) to preheated media (0.975 ml) to give a final drug concentration of 1×10^{-4} M. At 22 h after initiation, aliquots of the solution were withdrawn. An aliquot (0.2 ml) of the sample was deproteinized by addition of isopropanol (0.5 ml), and the supernatant after centrifugation was analyzed immediately for the derivative remaining. Another aliquot (0.2 ml) was diluted with PBS (0.5 ml) and immediately assayed for regenerated Ara-C.

HPLC Assay——Ara-C, Ara-U and COCG-Ara-C were determined by using a Waters HPLC system equipped with a model 510 pump, a WISP 710 B automatic sample injector and a Lamda-Max model 481 absorbance detector. Detection of Ara-C and Ara-U was performed at 274 nm and the stationary phase used was a NOVA-PAK C_{18} cartridge (8.0 × 100 mm, Waters) packed in an RCM 100 radial compression module (Waters). Phosphate buffer (0.05 M, pH 7.0) was used as the mobile phase at a flow rate of 2.5 ml/min. COCG-Ara-C was detected at 300 nm and the stationary phase used was a Develosil ODS-5 packed column (4.6 × 200 mm, Nomura Chemical Co.) Acetonitrile–isopropanol–phosphoric acid (650 : 350 : 1, v/v) was used as the mobile phase at a flow rate of 2.0 ml/min. Standard solutions were chromatographed and calibration lines were constructed on the basis of peak-area measurements.

***In Vitro* Cytotoxicity Assay**——*In vitro* cytotoxicity was evaluated using primary cultured P 388 cells as described previously.[12,16]

Preparations for Antitumor Activity Evaluation——Ara-C was dissolved in PBS. BHAC solution was prepared by adding distilled water to the commercial vials (Sunrabin®). COCG-Ara-C was solubilized in PBS using HCO-60 (final detergent concentration: 25%, w/v). COCG-Ara-C-bearing liposomes were prepared as described above. All the preparations were subjected to sterilization by filtration through a Millex GV.

Antitumor Activity against Mouse L 1210 Leukemia——L 1210 leukemia cells were maintained by weekly intraperitoneal passage through female DBA/2 mice. The evaluation of antitumor activity was performed essentially according to the National Cancer Institute protocols for screening[17] with some minor modifications. L 1210 leukemia (1×10^5 cells in 0.1 ml) was inoculated intraperitoneally or intravenously into female BDF_1 mice weighing 17.0—21.1 g. Mice were treated intravenously with drug preparations in a volume of 0.01 or 0.015 ml/g body weight, according to the following schedules. 1) Chemotherapy was performed on day 1 after intraperitoneal (i.p.) implantation (Fig. 3) or i.v. implantation (Table IV). 2) Treatment was carried out on days 2 and 6 after i.p. inoculation (Fig. 4). Control groups received PBS, plain liposomes or 25% (w/v) HCO-60 solution as a vehicle. Antitumor activity was expressed as T/C values calculated on the basis of the mean survival time of treated relative to control groups. The observation period was 60 d.

Antitumor Activity against Human Lung Adenocarcinoma A 549——Human lung adenocarcinoma A 549 was maintained by serial subcutaneous passage of tumor fragments (1 × 1 × 1 mm) through female BALB/c-nu/nu mice. The chemotherapy study was performed as follows. Female BALB/c-nu/nu mice were anesthetized with an intraperitoneal injection of 0.25 ml of 10% (w/v) pentobarbital solution. The skin was prepared with alcohol (70%, v/v) and an incision was made over the kidney. The kidney was gently externalized, and a tumor fragment (1 × 1 × 1 mm) was implanted under the capsule using a trocar (1 × 120 mm). Following implantation, the tumor-bearing kidney was returned to the peritoneal cavity and the abdominal incision was closed with silk and wound clips. Animals were treated intravenously with drug preparations in a volume of 0.01 or 0.015 ml/g body weight on days 1, 5 and 9 after implantation. Mice were weighed on the first treatment day and day 9. Control groups received either PBS or plain liposomes as a vehicle. On day 12 animals were killed by cervical dislocation, and the tumor-bearing kidney taken out for caliper measurements of xenograft size. The tumor volume (V) was calculated from the formula $V = a \times b^2/2$,[17] where a and b are the largest and smallest diameters of the tumor mass, respectively. Antitumor activity was expressed as tumor mass growth inhibition (G.I.), calculated by means of the formula G. I. (%) = $(\bar{V}_c - \bar{V}_t)/\bar{V}_c \times 100$, where $\bar{V}_c$ and $\bar{V}_t$ represent the mean tumor volumes of control (treated with PBS or plain liposomes) and treated groups, respectively.

Results

Characterization of the Lipophilic Derivative

The structures of Ara-C and its lipophilic derivative are shown in Fig. 1 and their physicochemical data are listed in Table I. The derivative was identified as N^4-[*N*-(cholesteryloxycarbonyl)glycyl]-Ara-C (COCG-Ara-C) on the basis of mass, UV, IR and

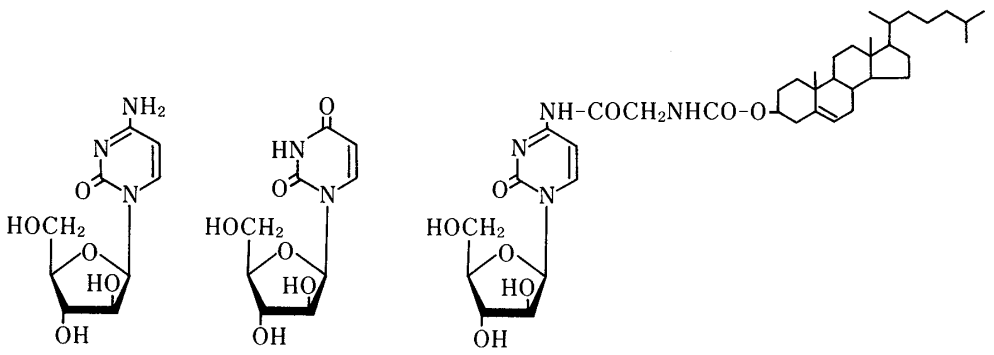

Fig. 1. Structures of Ara-C, Ara-U and a Lipophilic Ara-C Derivative (COCG-Ara-C)

TABLE I. Physicochemical Data for Ara-C and COCG-Ara-C

Compound	mp (°C)	Molecular weight	UV_{max} (nm) ($\varepsilon \times 10^{-3}$)	$\log k_0'$ [a]	Entrap percent
Ara-C	212—214	243.2	272 (9.79)[b]	0.445	<0.1
COCG-Ara-C	159—161 (dec.)	712.9	246 (14.86)[c] 301 (7.73)[c]	3.454	99.8

a) Lipophilic index in HPLC (see the text). *b*) Values in distilled water. *c*) Values in ethanol.

TABLE II. Size of Liposomes

Preparation[a]	Lipid conc. (mM)	COCG-Ara-C conc. (mM)	Diameter[b] (nm)
A	50	0	114±22
A′	50	0	108±18
B	50	1.0	120±23
C[c]	50	3.0	123±23
D	50	10.0	118±23
D′	50	10.0	120±22
E′	100	18.0	120±23
F	100	20.0	124±18
F′	100	20.0	116±22

a) Preparations A, B, C, D and F were used for chemotherapy of L 1210 leukemia. Preparations A′, D′, E′ and F′ were for human lung adenocarcinoma A 549 xenograft. *b*) Liposome size was determined by dynamic laser light scattering and is expressed as the mean±S.D. *c*) Preparation C was also used in the hydrolysis test.

NMR spectra. The marked difference in UV maximum between Ara-C and the derivative suggests that the *N*-(cholesteryloxycarbonyl)glycyl group is introduced into the cytosine moiety. As expected, COCG-Ara-C showed an increased lipophilic index ($\log k_0'$), being almost completely entrapped in liposomes. In contrast, liposomes incorporating Ara-C proved impossible to prepare by the controlled dialysis method because of the high diffusibility of Ara-C through the dialysis membranes during detergent removal.

Characterization of Liposomes

The mean diameters of the liposomes used in this study are summarized in Table II.

Regardless of the amount of COCG-Ara-C incorporated, derivative-bearing liposomes showed a fairly narrow size distribution and their mean diameters were almost equal to those of plain liposomes.

Hydrolysis Test

In order to evaluate the susceptibility of the derivative to chemical and enzymatic hydrolysis, a hydrolysis test was performed in PBS, mouse, rat and human plasma. Table III shows percentages of COCG-Ara-C remaining and percentages of Ara-C regenerated after incubation of the liposome-entrapped derivative with various media for 22 h. COCG-Ara-C was hydrolyzed to Ara-C to some extent in PBS, but its hydrolysis was accelerated in the presence of each plasma. No significant differences were observed in these bioactivation phenomena. The production of Ara-U, the inactive metabolite of Ara-C, was observed in human plasma.

***In Vitro* Cytotoxicity Test**

In vitro cytotoxicity assay was performed using primary cultured P 388 leukemia cells, and the results are shown in Fig. 2. The concentrations of drugs required to inhibit cell growth to 50% of the control values (IC_{50}'s) were determined to be 49 nM for Ara-C, 256 nM for COCG-Ara-C and 1120 nM for BHAC. In this culture system, the activities of COCG-Ara-C and BHAC were approximately one-fifth and one-twenty-third of that of the parent drug, respectively.

Antitumor Activity against Mouse L 1210 Leukemia

The antitumor activity of intravenously-injected COCG-Ara-C-bearing liposomes were investigated in intraperitoneally- and intravenously-implanted L 1210 leukemia systems, and compared with those of Ara-C and BHAC aqueous solutions. The results of single (day 1) and double (days 2 and 6) dose treatments of intraperitoneally-inoculated L 1210 leukemia are depicted in Figs. 3 and 4, respectively. As shown in Fig. 3, a single treatment with prodrug-

TABLE III. Conversion of Liposome-Entrapped COCG-Ara-C into Ara-C and Ara-U in Various Media[a]

Medium	% COCG-Ara-C remaining	% Ara-C regenerated	% Ara-U regenerated	Total (%)
PBS	95.8	2.7	n.d.[b]	98.5
Mouse plasma	79.1	15.2	n.d.	94.3
Rat plasma	85.9	9.2	n.d.	95.1
Human plasma	86.6	4.2	7.5	98.3

a) Liposome-entrapped COCG-Ara-C was incubated at 37 °C for 22 h. *b*) Undetectable (<0.1%).

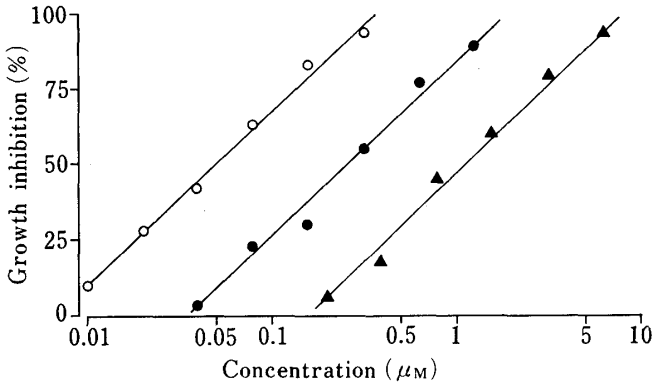

Fig. 2. Growth-Inhibitory Effect of Ara-C and Its Derivatives in a Primary Cultured P 388 Leukemia System

○, Ara-C; ●, COCG-Ara-C; ▲, BHAC. Each point represents the mean of three tests.

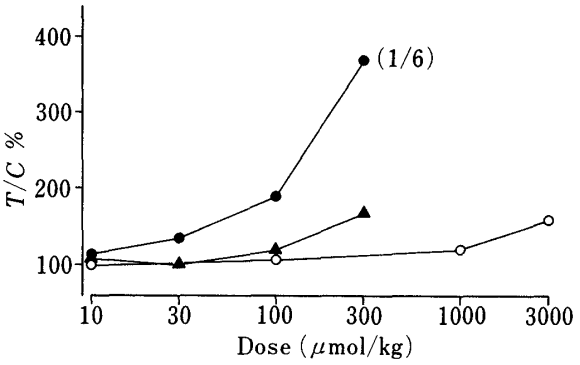

Fig. 3. Antitumor Activity of Ara-C Aqueous Solution, BHAC Aqueous Solution and COCG-Ara-C-Bearing Liposomes against Intraperitoneally-Implanted L 1210 Leukemia (Single Dose Treatment)

○, Ara-C aqueous solution; ▲, BHAC aqueous solution; ●, COCG-Ara-C-bearing liposomes. L 1210 cells (1×10^5) were implanted intraperitoneally into female BDF_1 mice (6 mice/group). Treatment was carried out intravenously 24 h later. The number in parenthesis refers to 60-day survivors.

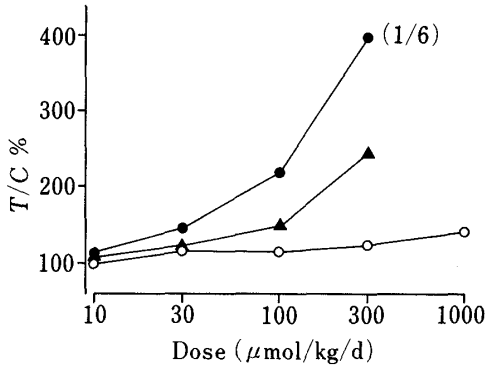

Fig. 4. Antitumor Activity of Ara-C Aqueous Solution, BHAC Aqueous Solution and COCG-Ara-C-Bearing Liposomes against Intraperitoneally-Implanted L 1210 Leukemia (Double Dose Treatment)

○, Ara-C aqueous solution; ▲, BHAC aqueous solution; ●, COCG-Ara-C-bearing liposomes. L 1210 cells (1×10^5) were implanted intraperitoneally into female BDF_1 mice (6 mice/group) on day 0. Treatment was performed intravenously on days 2 and 6. The number in parenthesis refers to 60-day survivors.

TABLE IV. Antitumor Activity of Ara-C Aqueous Solution, BHAC Aqueous Solution and COCG-Ara-C-Bearing Liposomes against Intravenously-Inoculated L 1210 Leukemia[a)]

Preparation	Dose (μmol/kg)	MST[b)] ± S.E. (d)	T/C (%)	60-day survivors
PBS	0	7.2 ± 0.1	100	0/12
Ara-C aqueous solution	3000	9.8 ± 0.3[c)]	136	0/6
	6000	10.8 ± 0.2[c)]	150	0/6
BHAC aqueous solution	30	8.0 ± 0.0[c)]	111	0/6
	300	13.0 ± 0.0[c)]	181	0/6
Plain liposomes	0	7.0 ± 0.0	100	0/6
COCG-Ara-C-bearing liposomes	30	9.3 ± 0.2[d,e)]	133	0/6
	300	24.9 ± 2.8[d,e)]	356	0/7

a) L 1210 cells (1×10^5) were implanted intravenously into female BDF_1 mice. i.v. treatment was carried out 24 h later. *b*) Mean survival time. *c*) Significantly different from the group treated with PBS ($p < 0.001$). *d*) Significantly different from the group treated with plain liposomes ($p < 0.001$). *e*) Significantly different from the group treated with BHAC aqueous solution at an equimolar dose ($p < 0.001$).

bearing liposomes exhibited a moderate activity (*T/C*: 135%) at 30 μmol/kg and a maximum effect (*T/C*: 370%) at 300 μmol/kg. At the highest dose, one of six mice survived more than 60 days. In contrast, the same treatments with Ara-C and BHAC aqueous solutions gave only low *T/C* values (less than 200%) even at doses of 3000 and 300 μmol/kg, respectively. Sixty-day survivors were not observed. The superiority of COCG-Ara-C-bearing liposomes over Ara-C and BHAC aqueous solutions was also demonstrated in the double dose treatment schedule (Fig. 4). At a daily dose of 300 μmol/kg, prodrug-bearing liposomes showed maximum antitumor activity with a *T/C* value of 391%, and one of six mice survived more than 60 days. In the same treatment schedule, however, Ara-C (1000 μmol/kg/d) and BHAC (300 μmol/kg/d) aqueous solutions yielded *T/C* values of 141% and 245%, respectively.

Table IV shows the antitumor effect of COCG-Ara-C-bearing liposomes against intravenously-implanted L 1210 leukemia when the i.v. therapy was carried out according to the single dose (day 1) treatment schedule. Prodrug-bearing liposomes exhibited a moderate antitumor activity (*T/C*: 133%) even at a dose of 30 μmol/kg and showed maximum

therapeutic effect (T/C: 356%) at 300 μmol/kg. On the other hand, an equimolar dose of BHAC aqueous solution showed a less potent antitumor activity than COCG-Ara-C-bearing liposomes. The parent drug Ara-C was also less effective with a T/C value of 150% even at a dose of 6000 μmol/kg.

The results (Figs. 3 and 4 and Table IV) indicate that COCG-Ara-C-bearing liposomes yield an equal or greater chemotherapeutic effect at molar doses which are three and thirty times lower than those of BHAC and Ara-C aqueous solutions, respectively.

Effect of Liposome Entrapment on the Antitumor Activity of COCG-Ara-C

In order to assess the effect of liposome entrapment on the antitumor activity of COCG-Ara-C, the therapeutic effect of the prodrug when administered in an aqueous solution was also evaluated and compared with that of the liposome-entrapped form. Table V summarizes the antitumor effect of COCG-Ara-C against intraperitoneally- or intravenously-implanted L 1210 leukemia when administered intravenously in the free (an aqueous solution) or liposome-entrapped form. Regardless of the route of tumor inoculation, liposome-entrapped COCG-Ara-C was significantly more effective than the free derivative, indicating that liposome

TABLE V. Effect of Liposome Entrapment on the Antitumor Activity of COCG-Ara-C against L 1210 Leukemia

Preparation	Dose (μmol/kg)	i.p.–i.v. system[a]		i.v.–i.v. system[b]	
		MST[c] ± S.E. (d)	T/C (%)	MST[c] ± S.E. (d)	T/C (%)
Plain liposomes	0	10.0 ± 0.0	100	7.2 ± 0.1	100
COCG-Ara-C liposomes	100	17.0 ± 0.3[d,e]	170	13.8 ± 0.4[d,f]	189
25% HCO-60 solution	0	10.2 ± 0.2	100	7.3 ± 0.2	100
COCG-Ara-C solution	100	14.7 ± 0.4[g]	144	11.6 ± 0.5[g]	159

a) The study was performed as described in Fig. 3. *b*) The test was carried out as in Table IV. *c*) Mean survival time. *d*) Significantly different from the group treated with plain liposomes ($p<0.001$). *e*) Significantly different from the group treated with COCG-Ara-C aqueous solution ($p<0.001$). *f*) Significantly different from the group treated with COCG-Ara-C aqueous solution ($p<0.01$). *g*) Significantly different from the group treated with 25% HCO-60 solution ($p<0.001$).

TABLE VI. Effect of Ara-C Aqueous Solution, BHAC Aqueous Solution and COCG-Ara-C-Bearing Liposomes on the Growth of Human Lung Adenocarcinoma A 549 Xenograft Implanted under the Renal Capsules[a]

Preparation	Dose (μmol/kg/d)	MBWC[b] (g)	MTV[c] ± S.E. (mm^3)	G.I.[d] (%)
PBS	0	0.7	17.6 ± 4.0	0
Ara-C aqueous solution	1000	0.1	13.7 ± 1.2	22.5
	1800	−0.2	10.6 ± 2.1	39.8
	3200	0.5	15.0 ± 4.0	14.9
BHAC aqueous solution	100	0.5	11.5 ± 2.0	34.6
	180	1.0	10.9 ± 1.4	38.2
	320	0.4	12.3 ± 2.4	30.5
Plain liposomes	0	0.6	16.9 ± 2.6	0
COCG-Ara-C liposomes	100	1.0	10.8 ± 1.1	35.7
	180	0.6	8.7 ± 1.6[e]	48.3
	320	0	7.6 ± 0.8[f]	54.9

a) A fragment of A 549 (1 × 1 × 1 mm) was implanted under the kidney capsules of female BALB/c-nu/nu mice (6 mice/group) on day 0. Chemotherapy was carried out intravenously on days 1, 5 and 9. *b*) Mean body weight change (day 9 − 1). *c*) Mean tumor volume. *d*) Growth inhibition. *e*) Significantly different from the group treated with plain liposomes ($p<0.05$). *f*) Significantly different from the group treated with plain liposomes ($p<0.01$).

entrapment enhances the *in vivo* antitumor effect of the prodrug.

Antitumor Activity against Human Lung Adenocarcinoma A 549

The antitumor activity of COCG-Ara-C-bearing liposomes against human lung adenocarcinoma A 549 was investigated using the subrenal capsule assay, and the results are summarized in Table VI. The therapeutic effect of Ara-C and BHAC aqueous solutions are included for comparison. Prodrug-bearing liposomes inhibited the growth of the xenograft dose-dependently and 54.9% growth inhibition was obtained at a dose of 300 μmol/kg/d. On the other hand, an equimolar dose of BHAC aqueous solution caused 30.5% inhibition of the growth of the xenograft and only 39.8% growth inhibition occurred with Ara-C aqueous solution at 1800 μmol/kg/d. Apparently, in this tumor system, COCG-Ara-C-bearing liposomes exhibited an antitumor activity superior to those of Ara-C and BHAC aqueous solutions.

Discussion

In an effort to circumvent the scheduling problems of Ara-C therapy, a variety of Ara-C analogs have been synthesized. 5′-Esters,[18] 3′-esters,[19] N^4-acyl derivatives[20] and 2,2′-anhydro-Ara-C (cyclocytidine)[21] have been prepared as prodrugs resistant to the action of cytidine deaminase, the Ara-C-inactivating enzyme. Steroid-[22] and phospholipid-[23] linked Ara-C 5′-monophosphate (Ara-CMP) derivatives have also been synthesized in attempts to improve cell penetration properties and/or tumor specificity. Some derivatives have already been incorporated in liposomes and tested in various tumor models.[9d,11c] However, little has been synthesized for the purpose of designing sustained-release delivery systems by combination with liposome carriers.

In the present study, a novel lipophilic Ara-C derivative, COCG-Ara-C, was synthesized as a prodrug applicable to intravenously injectable liposomal sustained-release carrier systems, and the antitumor activity of prodrug-bearing liposomes was investigated using two tumor models.

Generally, lipid–drug interactions are closely related to the lipophilicity of the drugs.[24] COCG-Ara-C has a sufficiently high lipophilicity to be more efficiently entrapped in liposomal phospholipid bilayers compared with Ara-C itself (Table I). This property would be advantageous in not only large-scale production but also lyophilization of drug-bearing liposomes.[25]

Vesicle size and size distribution have been proved to affect the pharmacokinetics of liposomes,[26] which may be closely related to the antitumor effect of liposome-entrapped cytotoxic agents. As a result, it is essential to control these parameters for a successful design of drug-bearing liposomes. As to the quality of the liposomes used in this study, prodrug-bearing liposomes showed a fairly narrow size distribution and their mean diameters were almost equal to those of plain liposomes (Table II).

In the *in vitro* hydrolysis test, COCG-Ara-C was hydrolyzed to Ara-C to some extent in PBS, but its hydrolysis was accelerated in the presence of various plasma samples (Table III). This finding indicates that the cleavage of the derivative occurs by not only chemical but also enzyme-mediated hydrolysis. Similar hydrolysis mechanisms have been reported for COCG-MMC,[11e] which has the same lipophilic moiety and spacer structure as COCG-Ara-C. The appearance of Ara-U after incubation with human plasma may be due to the species-specific high deaminase activity in the plasma, as demonstrated by Mulligan and Mellett.[27]

In the *in vitro* cytotoxicity test, COCG-Ara-C and BHAC exhibited reduced antitumor activities against primary cultured P 388 leukemia relative to the parent drug (Fig. 2). The difference in cytotoxic activity between the derivatives could be explained by the different regeneration rates of active Ara-C from the inactive derivatives. Extremely low *in vitro*

cytotoxicity of BHAC against L 1210 leukemia has also been reported by Aoshima *et al.*[28]

A number of studies on the antitumor effect of Ara-C derivatives[18-23] and liposome-encapsulated Ara-C[9] have been reported. In some cases, however, both tumor cells and drugs were administered by the i.p. route. The i.p.–i.p. system, where direct contact between drugs and target cells may occur, is convenient for screening active compounds, but it seems to be impractical from the viewpoint of clinical application. In the present study, the chemotherapeutic effect of COCG-Ara-C-bearing liposomes was examined using not only L 1210 leukemia but also human lung adenocarcinoma A 549 xenograft systems, treatment being performed by i.v. injection, considered as a more practical administration route.

As shown in Figs. 3 and 4 and Table IV, Ara-C was virtually ineffective in the treatment schedules used in this study. Because of rapid conversion of Ara-C to an inactive metabolite Ara-U, Ara-C has been proved to require complicated treatment schedules to attain its maximum therapeutic effect.[6] BHAC is one of the N^4-acyl derivatives of Ara-C that has been shown to exhibit a marked antitumor activity against L 1210 leukemia, irrespective of treatment schedule.[28] In fact, BHAC aqueous solution gave a significant antitumor effect ($T/C \geqq 150\%$)[17] at 300 μmol/kg, which was about a 10 times lower dose than that of Ara-C aqueous solution (Fig. 3). The superior antitumor effect of BHAC has been explained by the findings that BHAC acts as a depot of Ara-C *in vivo*.[28,29] Compared with Ara-C and BHAC aqueous solutions, COCG-Ara-C-bearing liposomes exhibited a markedly increased antileukemic activity, regardless of treatment schedule and tumor implantation route (Figs. 3 and 4 and Table IV). The extremely potent antitumor activity of COCG-Ara-C-bearing liposomes might also be explained by the hypothesis that prodrug-bearing liposomes act as slow release reservoirs of the parent drug and provide a therapeutic level of Ara-C at a more optimal rate than BHAC over a prolonged period of time. This hypothesis is supported in part by the slow cleavage of the derivative to Ara-C observed in the *in vitro* hydrolysis test (Table III).

The extremely high T/C values obtained in the single dose treatment with COCG-Ara-C-bearing liposomes (Fig. 3 and Table IV) imply the schedule-independency of its efficacy. Although we have not yet examined the sensitivity of COCG-Ara-C to cytidine deaminase, the derivative might be resistant to the enzyme to a considerable extent, because chemical modification of the N^4-position of Ara-C has been proved to lead to an increase in resistance to the catabolic action of the Ara-C-inactivating enzyme.[20,28]

Liposome-entrapped COCG-Ara-C showed a significantly more potent antitumor effect relative to the free derivative (Table V). This finding indicates the potential utility of the liposomes as a dosage form of COCG-Ara-C. The difference in efficacy between the two preparations might be attributable to the different biological disposition of COCG-Ara-C.

The superiority of COCG-Ara-C-bearing liposomes to Ara-C and BHAC aqueous solutions was also demonstrated in the subrenal capsule assay using human lung adenocarcinoma A 549 (Table VI). These results suggest the potential usefulness of COCG-Ara-C-bearing liposomes for the treatment of some kinds of solid tumors.

Although *in vivo* kinetic studies are necessary to characterize the antitumor action of COCG-Ara-C-bearing liposomes in more detail, the present results suggest that derivative-bearing liposomes might be effective against not only leukemias but also solid tumors. Further studies are under way to investigate the biological disposition of liposome-entrapped COCG-Ara-C for clarifying the *in vivo* sustained-release characteristics of this delivery system. The results will be published in a subsequent paper.

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