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1-β-D-Arabinofuranosylcytosine Conjugates of Corticosteroids as Potential Antitumor Agents*

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Abstract—*The antitumor activity and toxicity of two new 1-β-D-arabinofuranosylcytosine (ara-C) conjugates of cortisol and corticosterone linked through a phosphodiester bond between the 5′ and 21 positions of the respective moieties (cortisol- and corticosterone-p-ara-C) were investigated in L1210 lymphoid leukemia cells in mice. They are highly active against both i.p.- and i.c.-implanted ara-C-sensitive lymphoid leukemia in mice, exceeding the activity produced by the parent drug, ara-C. For example, corticosterone-p-ara-C exhibited the respective ILS values of 306% at 50 mg/kg/day × 9 and 294% at 75 mg/kg/day × 9 on survivals of i.p.- and i.c.-inoculated L1210 leukemic mice. The effectiveness of the conjugates seems to depend on schedules of the treatments. The 9-day continuous treatments showed a better therapeutic effectiveness than those with either a 5-day, a single or a widely spaced (q 4 d., 1, 5, 9) treatment. However, they were found to be marginally effective against i.p.-implanted ara-C-resistant L1210 leukemia in mice. They were also inhibitory against proliferation of human leukemia-lymphoid cells in culture. Their superior antitumor activity and resistance to cytidine deaminase suggests that they serve as a prodrug form of ara-C or ara-CMP.*

INTRODUCTION

RECENTLY, a series of new prodrugs of 1-β-D-arabinofuranosylcytosine (ara-C) were synthesized in our laboratory by conjugating a variety of corticosteroids to ara-C through a naturally occurring phosphodiester bond between the 21 and 5′ positions of the respective moieties [1–4]. In preliminary antitumor evaluation against L1210 lymphoid leukemia in mice at least 4 of the new ara-C conjugates have shown a more than 2-fold greater increase in life span than ara-C alone or in combination with the steroids [1, 4]. The present work is an extension of our ongoing studies to provide further information concerning the efficacy of the conjugates and includes results of variations of the treatment schedules found for 2 of these new conjugates, cortisol-*p*-ara-C and corticosterone-*p*-ara-C, given to mice inoculated with L1210 sensitive and resistant to ara-C, their antiproliferative activity against human leukemia-lymphoma cell lines and other biochemical properties.

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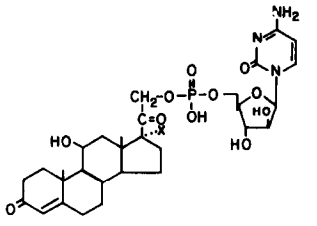
Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; cortisol-*p*-ara-C, 5′-(cortisol-21-phosphoryl)-1-β-D-arabinofuranosylcytosine; corticosterone-*p*-ara-C, 5′-(corticosterone-21-phosphoryl)-1-β-D-arabinofuranosylcytosine; ara-CMP, 1-β-D-arabinofuranosylcytosine 5′-monophosphate; L1210/0, ara-C-sensitive L1210 lymphoid leukemia; L1210/ara-C, ara-C-resistant L1210 lymphoid leukemia; i.p. intraperitoneally; i.c., intracerebrally; ara-U, 1-β-D-arabinofuranosyluracil.

MATERIALS AND METHODS

Chemicals

Cortisol-*p*-ara-C (NSC 321808) and corticosterone-*p*-ara-C (NSC 342432) (Fig. 1) were prepared essentially as described earlier by us [1, 4]. Ara-C was obtained from Sigma Chemical Co., St. Louis, MO and ara-CMP was prepared by phosphorylation of ara-C using the literature procedure [5].



Cortisol-p-ara-C , X=OH
Corticosterone-p-ara-C, X=H

Fig. 1. Structures of ara-C conjugates.

Tumor cells and animals

Ara-C-sensitive L1210 (L1210/0) and ara-C-resistant L1210 (L1210/ara-C) lymphoid leukemia cells were purchased from Arthur D. Little, Inc., Cambridge, MA and routinely transplanted into DBA/2J mice. The *in vivo* studies were carried out in C3H/HEJ × DBA/2J (hereafter called $C_3D_2F_1/J$) mice obtained from the Jackson Laboratory, Bar Harbor, ME and in DBA/2J mice supplied by Roswell Park Memorial Institute. Human leukemia-lymphoma cells (RPMI 8402, MOLT 4F, NALM-6, BALM-1, REH and KM-3) were kindly supplied by Dr. J. Minowada of our Institute.

Evaluation of toxicity

For the preliminary evaluation of the toxicity each group of 3 $C_3D_2F_1/J$ female mice (mean wt 26 g) was given an i.p. injection of varying doses of cortisol-*p*-ara-C as a representative conjugate and the animals after the injection were observed twice daily for lethality for 2 weeks according to the published procedure [6].

Antitumor activity against i.p.-implanted L1210 in mice

Female $C_3D_2F_1/J$ mice (mean wt 26 g) were inoculated i.p. with 1×10^6 L1210/0 lymphoid leukemia or female DBA/2J mice (mean wt 19 g) with 1×10^5 L1210/ara-C and 24 hr (day 1) later i.p. treatment with drugs in 0.9% NaCl was initiated with the following schedules: q.d. 1, q 4 d. 1, 5, 9, q.d. 1–5 and q.d. 1–9, according to the procedures outlined in the NCI Protocols [7] with some modifications [1, 2, 4]. Each drug was tested over a wide range of doses. The results reported in Table 2 are those giving ILS values $\geq 25\%$. Optimum dose is a dose producing the greatest increase in life span on a particular treatment schedule.

Antitumor activity against i.c.-implanted L1210 in mice

Female $C_3D_2F_1/J$ mice (mean wt 26 g) were anesthesized with diethyl ether and the right parietal scalp was washed with 70% ethanol. A 27½ G needle with a guard placed on the outside of the barrel 1.5 mm from the top of the bevel was inserted through the skull and 1×10^5 L1210/0 cells in 0.05 ml suspension were inoculated into the mouse according to the procedures published previously [8, 9]. Drug solution in 0.9% NaCl was administered i.p. once daily starting on day 1 according to the dose range and the treatment schedules as described above.

Antiproliferative activity in vitro

The drugs were tested for *in vitro* growth-inhibitory activity against 6 human leukemia-lymphoma cell lines in culture using the methodology described previously [10, 11]. All cultures were run in duplicate and experiments were repeated twice. The culture tubes were incubated at 37°C for a total of 7 days. The drug concentrations that produced 50% inhibition of cell growth (ED_{50}) were determined by plotting the number of viable cells on day 4 (a half point of the logarithmic cell growth), as a percentage of the control, against drug concentrations.

The results were evaluated statistically by Student's *t* test and differences between means were regarded as significant if $P < 0.05$.

Determination of resistance to cytidine deaminase

Human liver cytidine deaminase (EC 3.5.4.5) was prepared from tissue which had been removed at autopsy according to the published procedure [12]. The specific activity of the cytidine deaminase used in this study was 4.27×10^{-5} mU/mg protein and protein concentration was 19.95 mg/ml. For assay of deamination of the conjugates a mixture of compound (10 μmol), 0.2 ml of the enzyme preparation and 0.8 ml of 0.1 M Tris–HCl (pH 8.0) was incubated at 25°C for 24 hr. During the incubation aliquots (0.1 ml) were streaked on a TLC plate (0.1 × 20 × 20 cm) followed by developing with isopropanol–NH_4OH–H_2O (7:1:2). Each band was extracted with 50% ethanol and quantitated by u.v. The band matching with the conjugate was further incubated with 5′-nucleotidase (EC 3.1.3.5) from *Crotalus adamanteus* (Sigma Chemical Co.) in 0.1 M Tris–HCl (pH 9.0) and 0.005 M $MgSO_4$ at 37°C for 24 hr, and the products were separated by paper chromatography and characterized and quantitated by u.v. as described previously [4].

RESULTS

Preliminary mouse toxicity

Table 1 shows some preliminary data of toxicity of cortisol-*p*-ara-C in $C_3D_2F_1/J$ mice. The results indicated that 3 divided i.p. injections of over 2000 mg/kg were found to be lethal in all 3

Table 1. *Preliminary estimation of toxicity of cortisol-p-ara-C**

	Dose (mg/kg)					
	3000	2000	1600	1400	1200	1000
Dead mice/group of 3	3	3	2	1	0	0
Weight change on day 14 (g/mouse)†	-	-	+4.43	+1.43	+0.70	+3.00

*Each group of 3 $C_3D_2F_1/J$ (female, mean wt 26 g) was given 3 divided i.p. injections (at 2-hr intervals) of the various dose levels of cortisol-*p*-ara-C in a total of 0.5 ml of 0.9% NaCl containing 0.05 ml of 95% ethanol. Mice were observed twice daily for lethality after drug injection for 2 weeks.

†Weight change for the control mice averaged +1.82 g/mouse.

mice. However, dose levels at and below 1200 mg/kg were not lethal and far exceeded the optimum dose (50 mg/kg/day) previously found for this conjugate [1]. At no time during the 14 days of observation did the animals lose weight.

Antitumor activity against i.p.-implanted L1210/0 in mice

Table 2 summarizes the results obtained by i.p. treatments with ara-C, cortisol-*p*-ara-C and corticosterone-*p*-ara-C at the optimal doses on the various treatment schedules. The untreated mice died on days 8–11 after tumor cell implantation. Under a treatment regimen of daily injection of ara-C for 5 days the maximum activity was obtained at 20.0 mg (82.2 μmol)/kg/day and the ILS value was 65%. In the case of the extended treatments for 9 days the most effective dose found for ara-C was 10.0 mg/kg/day and the ILS value was 138%. With treatment by cortisol- and corticosterone-*p*-ara-C at a dose of 50.0 mg (75.0 and 76.7 μmol)/kg/day for 5 days the maximal ILS values found were 128 and 130% respectively. When the same daily dose was given for 9 days cortisol- and corticosterone-*p*-ara-C exhibited more impressive ILS values, of 244 and 306% respectively. Aside from the highly significant activity demonstrated for the conjugates against L1210/0 leukemia in mice, average loss of weight of the animals provided preliminary indications that toxicity was less than that of ara-C. Furthermore, the total dose administered (250 or 450 mg/kg) was only one- or two-fifths of the maximum tolerated dose (1200 mg/kg).

Table 2 also shows the results obtained by treatments with cortisol- and corticosterone-*p*-ara-C at single and widely spaced (q. 4 d. 1, 5, 9) doses. A single i.p. dose of the conjugates showed no significant therapeutic effect (ILS, 18%) and the widely spaced i.p. doses exhibited a marginal effect (ILS 40–55%), significantly less than those found for the 5 consecutive daily administrations of the conjugates of the same total dose. Thus the effectiveness of the conjugate appears to be sensitive to the treatment schedule used.

Antitumor activity against i.c.-implanted L1210/0 in mice

Table 3 summarizes the results obtained by i.p. treatments with ara-C, cortisol-*p*-ara-C and corticosterone-*p*-ara-C at optimal doses on the various treatment schedules. The control mice lost about 7 g of weight and died by day 9. All of these animals developed severe exophthalmos. The most effective dose of ara-C for the 5-day treatment was 27.5 mg (113 μmol)/kg/day and the ILS was 72%, while that for the 9-day treatment was 18.2 mg (75.0 μmol)/kg/day, with the ILS value of 78%. With the treatment of cortisol- and corticosterone-*p*-ara-C at the most effective dose of 75.0 mg (112 and 115 μmol)/kg/day for 5 days the ILS values found were 100 and 172% respectively. When cortisol-*p*-ara-C (50.0 mg/kg/day) and corticosterone-*p*-ara-C (75.0 mg/kg/day) were given for 9 days the conjugates exhibited more impressive ILS values, of 144 and 294% respectively. The average weight losses of the treated animals with the conjugates were less than those of the control and the treated animals with ara-C. A single or the widely spaced i.p. doses showed no significant therapeutic effect (ILS 22–33%).

Antitumor activity against i.p.-implanted L1210/ara-C in mice

The activities of ara-C, cortisol-*p*-ara-C and corticosterone-*p*-ara-C against L1210/ara-C leukemia in mice were also examined. The 5-day i.p. treatments with ara-C at a dose of 50.0 mg/kg/day produced ILS values of 77%. When cortisol-*p*-ara-C (75.0 mg/kg/day) and corticosterone-*p*-ara-C (100 mg/kg/day) were administered i.p. for 5 days the ILS determined were 53% each. The data indicates that ara-C and the conjugates possessed some antitumor activity against the L1210/ara-C. However, the activity was marginal.

Antiproliferative activity against human lymphocytes in culture

The antiproliferative activities of ara-C, cortisol-*p*-ara-C and corticosterone-*p*-ara-C

*Table 2. Antitumor activity of ara-C and the conjugates against i.p.-inoculated L1210/0 leukemic mice**

Compound	Treatment schedule (day)	Active dose range† mg(μmol)/kg/day	Optimal dose‡ mg(μmol)/kg/day	Weight change§ (g/mouse) on day 8	Survival days‖ Range	Median (T/C)	% ILS¶	45-Day survivors
Ara-C	1		100(411)	+1.42	8–10	9.00/8.00	13	0/6
	1, 5, 9	25 (103)–100(411)	50(206)	+2.80	13–14	13.0/8.00	63	0/6
	1–5	5(20.6)–80(329)	20(82.2)	+0.50	14–15	14.0/8.50	65	0/6
	1–9	5(20.6)–20(82.2)	10(41.1)	−4.58	18–30	25.0/10.5	138	0/6
Cortisol-*p*-ara-C	1		250(375)	+0.23	10–13	13.0/11.0	18	0/6
	1, 5, 9	85 (127)–125(187)	85(127)	+0.93	10–16	14.0/10.0	40	0/6
	1–5	25(37.4)–100(150)	50(75.0)	−0.30	17–29	20.5/9.00	128	0/8
	1–9	25(37.4)–100(150)	50(75.0)	−1.30	19–39	27.5/8.00	244	0/8
Corticosterone-*p*-ara-C	1		250(384)	−3.69	11–13	13.0/11.0	18	0/6
	1, 5, 9	85 (130)–125(192)	125(192)	+2.91	9–20	15.5/10.0	55	0/6
	1–5	25(38.4)–75(115)	50(76.7)	−0.18	14–35	23.0/10.0	130	0/6
	1–9	25(38.4)–100(153)	50(76.7)	−0.63	22–>45	36.5/9.00	306	1/8

*Each group of 6–8 $C_3D_2F_1$/J mice (mean wt 26 g) received i.p. inoculation of 1×10^6 cells on day 0. Treatment (i.p.) was initiated 24 hr after tumor inoculation. Animals were observed daily until death or 45 days.

†Dose producing an increase in life span ≥25% over the controls.

‡Dose producing greatest increase in life span.

§Mean weight change for the control studies (44 mice) was +2.70 ± 1.13 (S.D.) g/mouse.

‖Median survival days of 44 mice used for the control studies were 9.16 ± 1.17 (S.D.) days.

¶Percentage increase in life span: (T/C − 1) × 100.

*Table 3. Antitumor activity of ara-C and the conjugates against i.c.-inoculated L1210/0 leukemic mice**

Compound	Treatment schedule (day)	Active dose range† mg(μmol)/kg/day	Optimal dose‡ mg(μmol)/kg/day	Weight change§ (g/mouse) on day 8	Range	Survival days‖ Median (T/C)	% ILS¶	45-Day survivors
Ara-C	1	200.0(822)–400.0(1645)	350.0(1439)	-3.73	10–13	11.5/8.00	44	0/6
	1, 5, 9	33.3(137)–150.0(617)	150.0(617)	-5.93	12–16	13.0/9.00	44	0/6
	1–5	25.0(103)–80.0(329)	27.5(113)	-5.01	11–17	15.5/9.00	72	0/8
	1–9	10.0(41.1)–40.0(165)	18.2(75.0)	-4.41	15–17	16.0/9.00	78	0/6
Cortisol-*p*-ara-C	1		375.0(561)	-5.92	11–22	11.0/9.00	22	0/6
	1, 5, 9	150.0(225)–225.0(337)	150.0(225)	-8.05	10–13	12.0/9.00	33	0/6
	1–5	75.0(112)–100.0(150)	75.0(112)	-2.53	13–28	18.0/9.00	100	0/8
	1–9	25.0(37.4)–125(187)	50.0(74.9)	-2.83	14–25	18.0/8.00	144	0/8
Corticosterone-*p*-ara-C	1		250.0(384)	-2.95	9–11	11.0/9.00	22	0/6
	1, 5, 9	125.0(192)–225.0(384)	225.0(345)	-6.87	10–13	12.0/9.00	33	0/6
	1–5	50.0(76.6)–100.0(153)	75.0(115)	-2.06	18–>45	24.5/9.00	172	1/8
	1–9	75.0(115)–200.0(307)	75.0(115)	-1.65	17–>45	31.5/8.00	294	2/6

*Each group of 6–8 $C_3D_2F_1/J$ mice (mean wt 26 g) received i.c. inoculation of 1×10^5 cells on day 0. Treatment (i.p.) was initiated 24 hr after tumor inoculation. Animals were observed daily until death or 45 days.

†Dose producing an increase in life span ≥25% over the controls.

‡Dose producing greatest increase in life span.

§Mean weight change for the control studies (44 mice) was -7.44 ± 1.10 (S.D.) g/mouse.

‖Median survival days of 44 mice used for the control studies were 8.64 ± 0.47 (S.D.) days.

¶Percentage increase in life span: (T/C - 1) × 100.

Table 4. Inhibitory effects of ara-C and the conjugates on human lymphocytes in culture

Cell type	Cell line	Concentration (μM) for 50% loss of viability on day 4 (ED_{50}) Ara-C	Cortisol-*p*-ara-C	Corticosterone-*p*-ara-C
T lymphocyte	RPMI 8402	0.19 ± 0.08*	-	1.03 ± 0.20†
	MOLT 4F	-	5.03 ± 1.50‡	0.86 ± 0.44
Pre-B lymphocyte	NALM-6	-	0.65 ± 0.62	0.40 ± 0.08
B lymphocyte	BALM-1	3.17 ± 1.26§	5.00 ± 2.00	1.10 ± 0.78
Non-T, non-B lymphocyte	REH	0.17 ± 0.02‖	0.70 ± 0.10	0.50 ± 0.15
	KM-3	0.11 ± 0.04¶	1.63 ± 0.55	0.49 ± 0.17

*Mean ± S.D.

†$P < 0.01$ compared to the ED_{50} of ara-C in RPMI 8402.

‡$P < 0.05$ compared to the value in REH and $P < 0.01$ compared to the ED_{50} of corticosterone-*p*-ara-C in MOLT 4F.

§$P < 0.05$ compared to the values in other cell lines.

‖$P < 0.01$ compared to the ED_{50} of cortisol-*p*-ara-C in REH.

¶$P < 0.01$ and $P < 0.05$ compared to the ED_{50} of cortisol-*p*-ara-C and corticosterone-*p*-ara-C in KM-3 respectively.

against T, B and non-T non-B lymphocytes are shown in Table 4. The data show that the conjugates are inhibitory against the human lymphocytes *in vitro*. However, their observed ED_{50} in T and non-T, non-B cells were significantly greater than those of ara-C ($P < 0.01$). This is in accord with previous observations in which the conjugates have shown ED_{50} values similar to or higher than ara-C in L1210/0 lymphoid leukemia cell culture [1, 2, 4]. ED_{50} values of ara-C in T and non-T, non-B cells were 0.11–0.19 μM, whereas the ED_{50} in B cells was 3.17 μM. Thus B cells were significantly less sensitive to ara-C than T and non-T, non-B cells ($P < 0.05$). Corticosterone-*p*-ara-C showed ED_{50} values of 0.49–1.10 μM in all the cells tested. However, cortisol-*p*-ara-C was less inhibitory against B and even T cells than non-T, non-B cells ($P < 0.05$) and less active than corticosterone-*p*-ara-C against B and T cells ($P < 0.01$).

Resistance to cytidine deaminase

Under the experimental condition using a crude preparation of cytidine deaminase from a human liver [12], the conjugates remained intact during a 24-hr incubation period at 37°C.

DISCUSSION

Results obtained in this study and the previous works [1–4] demonstrate that the majority of the ara-C conjugates of corticosteroids are highly active against both i.p.- and i.c.-implanted L1210/0 lymphoid leukemia in mice, exceeding the activities produced by ara-C. Moreover, the most effective total doses are 450–675 mg/kg, which are two- to three-fifths of the maximum non-lethal dose (1200 mg/kg). However, the effectiveness of the conjugates seems to depend on schedules of treatments, since 5-day continuous treatments showed better therapeutic effectiveness than those with a single or widely spaced (q. 4 d., 1, 5, 9) treatments of the same total dose (Table 2). Furthermore, the extended treatments for 9 days with all the conjugates tested against i.p.-implanted L1210 in mice were found to be more effective than those for 5 days. A similar result was observed when the i.c.-inoculated leukemic mice were treated with the conjugates. The 9-day treatments with the optimum daily dose were more effective than the 5-day treatments (Table 3).

When the conjugates were incubated in human and mouse plasma at 37°C for 24 hr they remained 40–80% intact and the products were the conjugate, ara-CMP, ara-U and the steroid [1–4]. Also, the conjugates were found to be resistant to hydrolysis by cytidine deaminase. However, single dose treatments on day 1 were found to be essentially ineffective (Table 2). Thus they do not appear to be a depot form of ara-C such as 5′-palmitoyl-, 5′-adamantoyl- and N^4-acyl-ara-C [6, 13, 14]. Like the simple 5′-*O*-alkylphosphorylation of ara-C [15], conjugation of the steroid to ara-C through a phosphodiester linkage has not thus far provided a means to overcome ara-C resistance based on kinase deficiency.

It remains to be established whether the conjugates are active *per se* or whether hydrolysis to ara-CMP and the steroids are required for the cytotoxic activity. However, it seems likely that the hydrolysis may well be required for the activity since the conjugates start to show their *in vitro* antiproliferative activity against both L1210/0 and the human lymphocytes after 48 hr of incubation, while ara-C has already started to kill the cells at 24 hr of incubation. Thus the hydrolysis may occur mainly at the cellular level [4]. It is of interest to note that the lower antiproliferative activity of cortisol-*p*-ara-C against T cells than that of non-T, non-B cells (Table 4) might be related to the lower

glucocorticoid receptor levels in T cells than those in non-T, non-B cells [16].

Finally, it is not certain whether the conjugates cross the cell membrane intact and the hydrolysis occurs at the cellular level followed by releasing the parent drug and the steroid. Although we have not yet studied this mechanism, this possibility might be more plausible since previous reports [1, 4] indicated that: (1) the conjugates remained intact when incubated in the cell culture medium; (2) their enzymatic degradation in plasma was quite slow; and (3) they were more effective than the combinations, possibly by their resistance to the ara-C-inactivating enzyme cytidine deaminase in plasma before cellular uptake. Further studies will be needed to verify this point. However, their superior antitumor activity and the above-mentioned biochemical properties suggest that they serve as a prodrug of ara-C or ara-CMP, which might be useful as a target-specific delivery system. Unlike the other lipophilic derivatives of ara-C [6, 13, 14], our new conjugates are quite water-soluble (5–8 mg/ml of saline) because of the phosphodiester linkage, which can be advantageous over the previously mentioned lipophilic derivatives in clinical utilization. These preliminary results demonstrate the potential interest of the conjugates in cancer chemotherapy and especially in brain cancer chemotherapy.

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