Biochemical Pharmacology, Vol. 20, pp. 3295–3308. Pergamon Press, 1971. Printed in Great Britain

BIOCHEMICAL AND PHARMACOLOGIC STUDIES WITH 1-β-D-ARABINOFURANOSYLCYTOSINE 5′-ADAMANTOATE (NSC-117614), A DEPOT FORM OF CYTARABINE

G. L. NEIL, H. H. BUSKIRK, T. E. MOXLEY, R. C. MANAK, S. L. KUENTZEL and B. K. BHUYAN

Research Laboratories, The Upjohn Company, Kalamazoo, Mich. 49001, U.S.A.

(*Received* 14 *January* 1971; *accepted* 12 *May* 1971)

Abstract—In the treatment of L1210 leukemic mice, the antitumor activity observed with 1-β-D-arabinofuranosylcytosine 5′-adamantoate (AdO-*ara*-C) suggested that the agent represents a molecular depot form or sustained action form of 1-β-D-arabinofuranosylcytosine (*ara*-C, cytarabine). The results of the present study provide strong support for this hypothesis. Many biochemical similarities between *ara*-C and AdO-*ara*-C were observed, suggesting similar or identical modes of action. Cytotoxicity of both agents in cell culture could be prevented with deoxycytidine. Like *ara*-C, AdO-*ara*-C markedly inhibited DNA synthesis with little effect on RNA or protein synthesis. Cross-resistance (L1210 cells in culture) was also observed. The differences observed *in vitro* (e.g. lower intrinsic cytotoxicity, lower extent of uptake by L1210 cells, slower kinetics of inhibition of DNA synthesis with the derivative) were also consistent with the hypothesis that hydrolysis of AdO-*ara*-C to *ara*-C is required for cytotoxic activity. Direct evidence for hydrolysis was obtained in studies of the metabolism *in vitro* of AdO-*ara*-C in mammalian plasma and in plasma level studies in mice. Inhibition of enzymatic hydrolysis with an esterase inhibitor (eserine sulfate) markedly reduced the cytotoxicity of AdO-*ara*-C towards L1210 cells in culture. Plasma level and excretion studies indicated that i.p. administration of AdO-*ara*-C to mice yielded cytotoxic *ara*-C levels which persisted for much longer than is possible with a single dose of the parent compound itself. These data, when considered with those concerning the effects of low levels of *ara*-C in contact with cells in culture for long periods help to explain the unusual therapeutic effects of AdO-*ara*-C.

1-β-D-ARABINOFURANOSYLCYTOSINE 5′-adamantoate (NSC-117614; AdO-*ara*-C*), a novel derivative of 1-β-D-arabinofuranosylcytosine (NSC-63878; *ara*-C), has been shown to exert a potent antitumor effect in the treatment of L1210 leukemic mice.[1,2] The agent is an immunosuppressant in mice[3,4] and rats,[4,5] and is active against experimental allergic encephalomyelitis in rats.[6] In the treatment of L1210 leukemic mice,[2] AdO-*ara*-C is more active than the parent compound when administered as a single dose or in a short course of daily doses, and single-dose treatment with AdO-*ara*-C is almost as effective as treatment with *ara*-C on its optimum schedule (three or four courses of multiple, closely spaced doses with appropriate intervals for host recovery).

* Abbreviations (and synonyms) used: AdO-*ara*-C, 1-β-D-arabinofuranosylcytosine 5′-(1-adamantanecarboxylate) (NSC-117614, *ara*-C adamantoate, adamantoyl cytarabine); *ara*-C, 1-β-D-arabinofuranosylcytosine [NSC-63878, cytosine arabinoside, cytarabine, Cytosar (trademark of The Upjohn Company)]; THU, 1-(β-D-ribofuranosyl)-4-hydroxy-3,4,5,6-tetrahydropyrimidin-2-[1H]-one (NSC-112907, tetrahydrouridine); AdO-*ara*-U, 1-β-D-arabinofuranosyluracil 5′-(1-adamantanecarboxylate); TCA, trichloroacetic acid; Hyamine hydroxide, *p*-(diisobutyl-cresoxyethoxyethyl) dimethylbenzammonium hydroxide (trademark of Packard Instrument Company); CdR, 2′-deoxycytidine; UR, uridine.

Indeed, in contrast to *ara*-C, effects with AdO-*ara*-C are quite insensitive to dose scheduling. Significant activity was observed even when AdO-*ara*-C was administered up to 48 hr prior to tumor inoculation. This sustained action effect and other data were interpreted[2] in terms of the hypothesis that AdO-*ara*-C represents a depot form of the parent compound, releasing *ara*-C (by hydrolysis) *in vivo*. The results of the present studies provide biochemical and pharmacologic support for this hypothesis.

MATERIALS AND METHODS

^{3}H-AdO-*ara*-C was prepared by New England Nuclear Corp. (Boston, Mass.) by a procedure devised in this laboratory. AdO-*ara*-C (100 mg) was dissolved in 1·0 ml glacial acetic acid containing 10 c of tritiated water. The solution was heated at 95° for 20 min, then frozen, and solvent was removed by lyophilization. Labile tritium was removed by washing the lyophilized powder three times with 5 ml water. The material obtained had a specific activity of 1·28 mc/mg (520 mc/m-mole). By analogy with deuterium-labeling experiments (monitored with proton magnetic resonance techniques), the tritium label should be exclusively at the 5-position of the pyrimidine ring. Thin-layer chromatography (solvents I and II, see below) indicated a radiochemical purity of >95 per cent. The tritium label was relatively stable to exchange under physiological conditions. Less than 1 per cent exchange was observed after 4 hr of incubation (37°) in various tissue culture media. For disposition studies, material with a specific activity of 3·25 μc/mg was prepared by recrystallization (with nonradioactive material) from methanol.

^{3}H-*ara*-C (1·4 c/m-mole) was obtained from Schwarz Bioresearch (Orangeburg, N.Y.). Eserine sulfate (physostigmine H_2SO_4) was obtained from Calbiochem (Los Angeles, Calif.). Tetrahydrouridine (THU) and 1-β-D-arabinofuranosyluracil 5′-(1-adamantanecarboxylate) (AdO-*ara*-U) were obtained from Drs. C. Y. Peery and P. F. Wiley (The Upjohn Company) respectively.

Cytotoxicity studies. KB (human epidermoid carcinoma) cells were maintained in culture in Eagle's basal medium (Grand Island Biological Company) supplemented with fetal calf serum (10%), bactopeptone (0·5 mg/ml), bacitracin (0·1 mg/ml) and streptomycin (0·15 mg/ml). L1210 (mouse leukemia) cells were maintained in RPMI-1634 medium (Grand Island Biological Company) supplemented with sodium bicarbonate (0·075%, w/v), penicillin (0·1 mg/ml) and streptomycin (0·05 mg/ml). Cytotoxicities toward KB and L1210 cells growing in culture were determined by measuring the inhibition of growth (over a 3-day period) with the protein determination method of Smith *et al.*[7] and the direct cell-count method of Buskirk,[8] respectively. Except in cross-resistance studies (see below), stock solutions of AdO-*ara*-C were prepared in dimethyl sulfoxide. Cell incubation mixtures contained no more than 0·05% dimethyl sulfoxide (v/v), a concentration which had no significant effect on cell growth.

Ara-C-resistant L1210 cells,[9] obtained by subculture in the presence of 0·10 μM *ara*-C, were employed in cross-resistance studies (see Table 1). These cells were provided by Dr. M. K. Bach, The Upjohn Company. Dimethyl sulfoxide was not employed in these studies, as the concentration required would have been toxic to the cells. Instead, AdO-*ara*-C was added to the culture tubes in ethanol solution and the ethanol was evaporated prior to addition of culture medium and cells.

Inhibition of macromolecular synthesis. The method of Bhuyan and Smith[10] as modified by Li *et al.*[11] was employed to determine the inhibition of radioactive precursor incorporation into DNA, RNA and protein of intact L1210 cells in culture. DNA was determined by Burton's modification of the diphenylamine method[12] and RNA by the orcinol method.[13] Cell protein was determined by the method of Oyama and Eagle.[14] Radioactivity was determined by liquid scintillation techniques.

Esterase assay. Fresh plasma (2 ml) was incubated with ^{3}H-AdO-*ara*-C (3·8–5·1 μc/3–4 μg/ml) at 37°. A parallel plasma sample was preincubated with THU (10 μg/ml) for 30 min prior to addition of substrate. THU was employed to inhibit deamination of the substrate and/or hydrolysis product (*ara*-C) to the corresponding uracil derivative.[15] Aliquots (0·2 ml) were removed at designated time intervals and added to an equal volume of absolute ethanol to precipitate protein. After centrifugation, 50 μl of the supernatant was applied to Silica gel thin-layer plates (Brinkmann Instruments, Inc., Westbury, N.Y.). Appropriate nonradioactive standards (*ara*-C, *ara*-U, AdO-*ara*-C and AdO-*ara*-U) were applied. The chromatograms were developed in solvent I (methyl ethyl ketone–acetone–water, 7:2:1). Standards were located under ultraviolet light and had the following R_f values: *ara*-C, 0·25; *ara*-U, 0·50; AdO-*ara*-C, 0·43; AdO-*ara*-U, 0·66. Radioactivity associated with each of the standards was determined by integration of scans obtained with a radiochromatogram scanner (model 7201, Packard Instrument Company, Downers Grove, Ill.).

Since the hydrolysis reaction followed first-order kinetics, half-lives and rate constants were determined from semilogarithmic plots of substrate concentration as a function of time. Because deamination of *ara*-C to *ara*-U proved significant in some cases (see below) and because *ara*-U and AdO-*ara*-C were poorly separated chromatographically, hydrolysis kinetic parameters were determined from data where THU was employed. THU had no effect on AdO-*ara*-C hydrolysis itself. Where eserine sulfate was employed, it was added to the plasma immediately prior to the addition of ^{3}H-AdO-*ara*-C.

Deaminase assay. Plasma (1–2 ml) was incubated with ^{3}H-*ara*-C (20 μc/3·5 μg/ml) at 37°. Aliquots were removed and treated as described for the esterase assay, using the same chromatography system. Conversion of *ara*-C to *ara*-U was determined by integration of peak areas. Deamination was first-order for much of the reaction, but deviated at high conversion, presumably due to product inhibition by *ara*-U.[16] Rates and half-lives were, however, determined from initial portions of semilogarithmic plots. For inhibition studies, THU was included in the reaction mixture. When direct deamination of ^{3}H-AdO-*ara*-C was studied, eserine sulfate (10^{-4} M) was included to inhibit the hydrolysis reaction. In this experiment (see Table 5), another solvent system (solvent II) consisting of isopropanol–concentrated NH_4OH–H_2O (7:1:2) capable of separating *ara*-U and AdO-*ara*-C (R_f values 0·5 and 0·65 respectively) was also employed.

Uptake studies. L1210 cells (4×10^6/ml) were incubated with ^{3}H-*ara*-C (1 μg/0·8 μc/ml) or ^{3}H-AdO-*ara*-C (1 μg/1·28 μc/ml) at 37°. At various times, aliquots were removed and centrifuged. Radioactivity of the supernatant medium was determined by scintillation techniques. The cell pellet was washed with 5·0 ml each of the following: cold 0·85% NaCl (three times); cold 10% trichloroacetic acid (TCA) (three times); ethanol–ether (3:1, v/v); and ether. Radioactivity of 0·1-ml aliquots of all washes was determined. The pellet (TCA-insoluble material) was dissolved in Hyamine

hydroxide (1 M in methanol) and radioactivity was determined. Uptake into the acid-soluble (TCA washes) and acid-insoluble fractions was calculated as per cent of radioactivity added.

Plasma level studies. ^{3}H-AdO-*ara*-C (200 mg/650 μc/kg) was administered i.p. to female BDF_1 mice (19–21 g; Jackson Memorial Laboratory, Bar Harbor, Me.) as a suspension in 0·25% aqueous methyl cellulose. At various times after administration, animals were anesthetized and heparinized blood was obtained by puncture of the vena cava. Blood from six animals per time was pooled. Plasma was obtained by centrifugation (3000 *g*, 4°, 20 min) and stored (<48 hr) frozen. Plasma samples were assayed for *ara*-C (or equivalent) using two independent assays.

Microbiological assay. The microbiological disc-plate assay for *ara*-C of Hanka *et al.*[17] (a modification of the procedure of Pittillo and Hunt[18]) was employed. Neither *ara*-U nor AdO-*ara*-C was cytotoxic in this assay system. A minimum of ca. 0·1 μg *ara*-C (or cytotoxic equivalent) per ml could be detected in mouse plasma.

Radioactivity assay. The method of Neil *et al.*[19] was employed. Radioactivity was determined by direct scintillation counting of an aliquot of plasma. Correction for tritium exchange was made after microdistillation. Deproteinized plasma (ethanol precipitation) was chromatographed (Silica gel thin-layer plates, solvents I and II). Nonradioactive standards (*ara*-C, *ara*-U and AdO-*ara*-C) were located under ultraviolet light. Radioactivity of scrapings from the plates was determined by scintillation counting techniques. All radioactivity was found associated with *ara*-C or *ara*-U.

The ratio of counts in the *ara*-C and *ara*-U sections was employed with the nondistillable counts and the specific activity to determine the amounts of *ara*-C and *ara*-U present in each plasma sample.

Excretion studies. ^{3}H-AdO-*ara*-C (200 mg/650 μc/kg) was administered i.p. to five BDF_1 mice. Animals were housed in metabolism cages (Acme Metal Products, Chicago, Ill.) and urine and feces were collected at various intervals over a period of 1 week. Radioactivity in the urine was determined by direct scintillation counting. Feces were extracted with 0·5 N perchloric acid (70°, 30 min) and radioactivity was determined on the supernatant after centrifugation. Urine was chromatographed (solvents I and II).

Cell-kill studies. The per cent survival of L1210 cells treated with *ara*-C was determined by a modification of the cloning method of Himmelfarb *et al.*[20] After various periods of incubation with *ara*-C, cell suspensions were centrifuged and resuspended in RPMI-1634 medium. The cells were then diluted with medium to the required concentration of cells. The last dilution was made by adding 1 ml of the diluted cells to 39 ml of RPMI-1634 medium containing 20% serum and 0·2% agar. To prepare the agar medium, 1·5% Noble agar (Difco Laboratories, Detroit, Mich.) was autoclaved in 0·9% NaCl. The agar was then diluted with RPMI-1634 medium containing 20% serum to an agar concentration of 0·2%. Five ml cells were planted in tubes, and colonies were counted visually after 10–12 days of incubation at 37° in an atmosphere of 5% CO_2–95% N_2. We attempted to obtain about 5–30 colonies per tube. The cloning efficiency was about 50 per cent.

RESULTS

*Cytotoxicity of AdO-*ara*-C and* ara*-C toward L1210 and KB cells.* AdO-*ara*-C (on a mole-equivalent basis) inhibited growth of L1210 and KB cells less than *ara*-C. ID_{50}

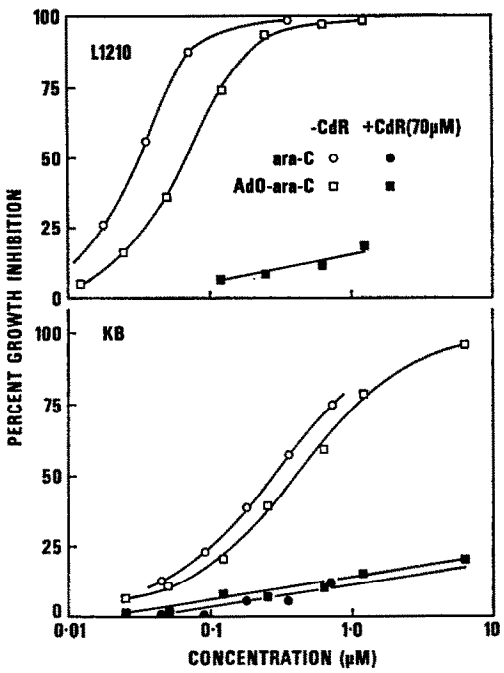

FIG. 1. Effect of AdO-*ara*-C and *ara*-C on the growth of KB and L1210 cells in culture and prevention of cytotoxicity by CdR. KB or L1210 cells growing in culture were incubated with *ara*-C or AdO-*ara*-C with or without simultaneous addition of CdR (70 μM). Inhibition of cell growth was determined by comparison with control cultures (with or without CdR).

values of 0·36 (KB) and 0·07 μM (L1210) were obtained for AdO-*ara*-C as compared to values of 0·27 (KB) and 0·03 μM (L1210) for *ara*-C (Fig. 1). The shapes of the dose–response curves for the two agents are very similar.

Figure 1 shows that the cytotoxicity of both *ara*-C and AdO-*ara*-C was prevented by CdR. For example, 1·23 μM AdO-*ara*-C with or without CdR (70 μM) inhibited KB cell growth[16] and 80 per cent, respectively.

TABLE 1. CROSS-RESISTANCE OF *ara*-C AND AdO-*ara*-C

Agent	Cytotoxicity ID_{50} (μM)*	
	Ara-C-sensitive L1210 cells	*Ara*-C-resistant L1210 cells†
Ara-C	0·037	0·45
AdO-*ara*-C‡	0·24	2·2

* ID_{50} values are levels required to give 50 per cent growth inhibition.

† L1210 cells obtained from subculture in the presence of 0·10 μM *ara*-C.

‡ AdO-*ara*-C was added to culture tubes in ethanol solution and the ethanol was evaporated prior to addition of cells.

Cross-resistance of ara-*C and AdO*-ara-*C.* Cross-resistance of *ara*-C and AdO-*ara*-C is shown in Table 1. L1210 cells resistant to *ara*-C and requiring approximately 12 times as much *ara*-C (ID_{50} = 0·45 μM) to inhibit growth as a sensitive line (*ara*-C ID_{50} = 0·037 μM) were also much (nine times) less sensitive to AdO-*ara*-C. The AdO-*ara*-C ID_{50} value obtained with the sensitive line (0·24 μM) was higher than that observed in Fig. 1 (0·07 μM). This may be due to the fact that in the cross-resistance studies, dimethyl sulfoxide was not employed to dissolve the AdO-*ara*-C, necessitating the use of a suspension.

*Inhibition of L*1210 *cell macromolecular synthesis by AdO*-ara-*C and* ara-*C.* In Fig. 2, the inhibition of incorporation of tritiated thymidine (^{3}H-TdR) into DNA of L1210 cells is shown as a function of contact time with *ara*-C and AdO-*ara*-C.

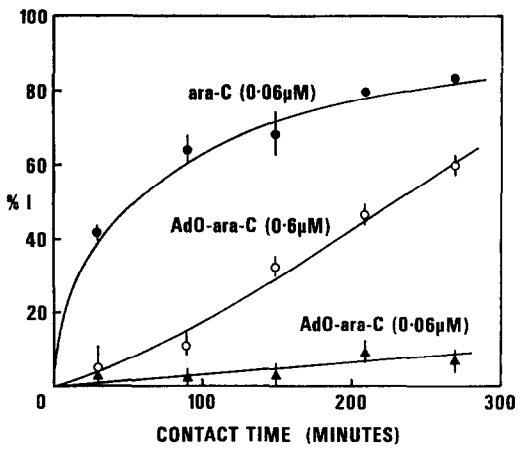

FIG. 2. Inhibition of L1210 cell DNA synthesis by AdO-*ara*-C and *ara*-C as a function of cell-agent contact time. L1210 cells (7·5 × 10^5/ml of medium) were preincubated with saline (control), AdO-*ara*-C (0·06 or 0·6 μM) or *ara*-C (0·06 μM) for 0, 1, 2, 3 or 4 hr prior to addition of ^{3}H-TdR (0·45 μc/1·5 μg/ml medium). Cell samples were removed at 15-min intervals thereafter (for 1 hr) and incorporation of ^{3}H-TdR into DNA was determined. Per cent inhibition was determined from specific incorporation rates (counts/min/μg DNA/min). Contact time includes one-half the labeling time (30 min), as well as the preincubation time.

L1210 DNA synthesis, as measured by precursor incorporation, was much less sensitive (over the 5-hr period of study) to AdO-*ara*-C than it was to *ara*-C. With 0·06 μM *ara*-C, inhibition approached a plateau value of ca. 80 per cent after 2–3 hr. With the same concentration of AdO-*ara*-C, however, there was less than 10 cer cent inhibition in this time. At a 10-fold higher concentration (0·6 μM), AdO-*ara*-C was still less effective than 0·06 μM *ara*-C for the contact times studied. The inhibition curve with 0·6 μM AdO-*ara*-C, however, showed no "plateau" effect and inhibition was still increasing at 5 hr.

The effects of AdO-*ara*-C (1·1 μM) and *ara*-C (0·05 μM) on precursor incorporation into L1210 cell DNA, RNA and protein are shown in Table 2. AdO-*ara*-C, like *ara*-C, inhibited DNA synthesis significantly with little or no inhibition of RNA and protein synthesis. After 240 min of contact with 1·1 μM AdO-*ara*-C, incorporation of precursors into DNA, RNA and protein were inhibited 61, 12 and 3 per cent, respectively, compared to 67, 9 and 0 per cent, respectively, for 0·05 μM *ara*-C.

TABLE 2. EFFECTS OF AdO-*ara*-C AND *ara*-C ON THE INCORPORATION OF PRECURSORS INTO L1210 CELL DNA, RNA AND PROTEIN*

Contact time† (min)	DNA‡ Per cent inhibition		RNA‡ Per cent inhibition		Protein‡ Per cent inhibition	
	AdO-*ara*-C§	*Ara*-C§	AdO-*ara*-C§	*Ara*-C§	AdO-*ara*-C§	*Ara*-C§
90	24	58	12	11	−8	−12
240	61	67	12	9	0	3

* Conditions, methods and treatment of data were similar to those of Fig. 2.

† Labeled precursors were added after 60 or 210 min of preincubation. Rates of label incorporation were determined over a 1-hr period. Contact time includes one-half the labeling period.

‡ Precursors were: DNA, ^{3}H-TdR (0·7 μc/30 μg/ml); RNA, ^{3}H-UR (1·25 μc/2·1 μg/ml); protein, ^{14}C-DL-valine (0·9 μc/200 μg/ml).

§ AdO-*ara*-C, 1·1 μM; *ara*-C, 0·05 μM.

*Metabolism of AdO-*ara*-C and* ara*-C in mammalian plasma.* Half-lives for hydrolysis of ^{3}H-AdO-*ara*-C (to ^{3}H-*ara*-C and adamantane 1-carboxylic acid) and for deamination of ^{3}H-*ara*-C (to ^{3}H-*ara*-U) by mammalian plasma are given in Table 3. Esterase activity was most pronounced in goat, guinea pig and rabbit plasmas (half-lives < 5 min). Hamster, mouse and rat plasmas also had high activity (half-lives of 5–12 min), while monkey, dog and human plasmas showed considerably less activity (half-lives of 70–240 min). However, the primates showed the highest plasma deaminase activities with that of the rhesus monkey (half-life of 10 min) being particularly notable. Human plasma had moderate deaminase activity but, of the other species, only the dog and guinea pig showed detectable (but small) activity.

TABLE 3. RATES OF AdO-*ara*-C HYDROLYSIS AND *ara*-C DEAMINATION BY MAMMALIAN PLASMA

Species	Half-lives (min)*	
	Hydrolysis of AdO-*ara*-C†	Deamination of *ara*-C‡
Goat	< 1	> 1200
Guinea pig	< 2	4200, 4600 (estimated)
Rabbit	2	> 1200
Hamster	5·5	> 1200
Mouse	5, 7	> 1200
Rat	10·5, 12	> 1200
Rhesus monkey	70, 80	10
Beagle dog	135, 240	> 1200
Human	160, 165	210, 300

* Half-lives were determined from first-order semilogarithmic plots of substrate concentration versus time.

† Initial ^{3}H-AdO-*ara*-C concentration was 3·8 to 5·1 μg/ml. THU (10 μg/ml) was included in the reaction mixture to prevent deamination.

‡ Initial ^{3}H-*ara*-C concentration was 20 μg/ml. Half-lives were determined or estimated by extrapolation only when at least 15% deamination was observed in 1200 min.

The nonenzymatic hydrolysis of ^{3}H-AdO-*ara*-C in buffered solutions (pH 2·2, 7·05, 7·6, 8·9 and 10·45) was also studied. AdO-*ara*-C was slightly hydrolyzed (< 10 per cent in 20 hr) at high pH (pH 8·0, 10·45) only.

The effect of a cholinesterase inhibitor (eserine sulfate) on the hydrolysis of ^{3}H-AdO-*ara*-C by mouse plasma is shown in Table 4. At relatively high concentrations (10^{-4} M), the rate of hydrolysis was inhibited markedly (84 per cent).

TABLE 4. ESERINE SULFATE INHIBITION OF THE HYDROLYSIS OF ^{3}H-AdO-*ara*-C BY MOUSE PLASMA*

Eserine sulfate (M)	Hydrolysis half-life (min)	Relative rate
0	5	1·0
10^{-6}	5	1·0
10^{-5}	11·5	0·43
10^{-4}	31	0·16

* ^{3}H-AdO-*ara*-C was incubated in mouse plasma with or without eserine sulfate. Conversion to *ara*-C and hydrolysis half-lives were determined as described in Materials and Methods.

The deamination of ^{3}H-AdO-*ara*-C to ^{3}H-AdO-*ara*-U by monkey plasma was examined (Table 5). When eserine sulfate was included in the reaction mixture (to prevent hydrolysis), less than 10 per cent of ^{3}H-AdO-*ara*-C was converted to ^{3}H-AdO-*ara*-U in 3 hr. Under these conditions, deamination of ^{3}H-*ara*-C is unaffected by eserine sulfate and is very rapid (half-life of ca. 10 min). The enzyme activities of monkey plasma represent a special and interesting case. In the absence of THU, very little *ara*-C was ever observed in the ^{3}H-AdO-*ara*-C hydrolysis mixture. This is due to the very rapid deamination of the *ara*-C formed by hydrolysis. Addition of THU (which virtually completely inhibits deamination of the hydrolysis product, *ara*-C) allowed the accumulation of *ara*-C.

TABLE 5. EFFECTS OF ESERINE SULFATE AND THU ON THE METABOLISM OF ^{3}H-AdO-*ara*-C IN RHESUS MONKEY PLASMA*

Substrate	Eserine sulfate (10^{-4} M)	THU (10 μg/ml)	Composition after 3 hr of incubation (%)			
			AdO-*ara*-C	*Ara*-C	AdO-*ara*-U	*Ara*-U
^{3}H-AdO-*ara*-C	−	−	24	6	3	67
	+	−	80	4	8	8
	−	+	26	70	0	4
	+	+	84	13	0	3

* ^{3}H-AdO-*ara*-C (20 μc/3·5 μg/ml) was incubated in rhesus monkey plasma (37°, 3 hr) in the presence of THU (10 μg/ml) and/or eserine sulfate (10^{-4} M). Metabolites were identified chromatographically in solvents I and II.

*Prevention of cytotoxicity of AdO-*ara*-C for L1210 cells by eserine sulfate.* Table 6 shows the effect of eserine sulfate on inhibition by AdO-*ara*-C of L1210 growth in culture. AdO-*ara*-C (0·74 μM) alone inhibited growth 90·1 per cent. Eserine sulfate at the concentrations employed (3·1–24·6 μM) was also growth inhibitory (17·6–55·1 per cent). In spite of this, combinations of AdO-*ara*-C (0·74 μM) and eserine sulfate were consistently less growth inhibitory than AdO-*ara*-C itself. The growth inhibition due to AdO-*ara*-C itself may be calculated if it is assumed that the inhibitions by AdO-*ara*-C and eserine sulfate are independent. When this is done, it is found (Table 6) that growth inhibition attributable to 0·74 μM AdO-*ara*-C is reduced to 44·2 (from 90·1 per cent) when 24·6 μM eserine sulfate is employed. This inhibition (44·2 per cent) is approximately that achieved with 0·19 μM AdO-*ara*-C alone.

Uptake studies. L1210 cells took up 2·8 per cent of added ^{3}H-*ara*-C (1 μg/ml), compared to 0·8 per cent of added ^{3}H-AdO-*ara*-C (1 μg/ml) after 2 hr of incubation. In both cases, ca. 90 per cent of the radioactivity was in the acid-soluble fraction while 10 per cent was associated with acid-insoluble material.

Plasma levels. In Fig. 3, plasma levels of *ara*-C at different times after a single i.p. dose (200 mg/kg) of ^{3}H-AdO-*ara*-C are compared to the plasma kinetics which would be expected after i.p. administration of a mole-equivalent dose (120 mg/kg) of *ara*-C. Both assay techniques gave similar results, except at large postadministration times,

TABLE 6. PREVENTION BY ESERINE SULFATE OF AdO-*ara*-C CYTOTOXICITY TOWARD L1210 CELLS

Eserine sulfate (μM)	Per cent inhibition of growth		
	Eserine sulfate alone*	Eserine sulfate + 0·74 μM Ado-*ara*-C†	AdO-*ara*-C (0·74 μM) (calculated)‡
0	0	90·1	90·1
3·1	17·6	85·3	82·0
6·2	27·4	82·6	76·0
12·3	41·0	77·0	60·8
24·6	55·1	74·9	44·2

* Per cent inhibition due to eserine sulfate (based on saline controls)

$$= 100 \left[\frac{1 - G_e}{G_c}\right].$$

† Per cent inhibition due to a combination of 0·74 μM AdO-*ara*-C and eserine sulfate (based on saline controls)

$$= 100 \left[\frac{1 - G_{e+d}}{G_c}\right].$$

‡ Calculated per cent inhibition due to 0·74 μM AdO-*ara*-C in the presence of eserine sulfate (based on tubes containing eserine sulfate as controls)

$$= 100 \left[\frac{1 - G_{e+d}}{G_e}\right].$$

where G_c = control growth (cells/ml) over the 3-day assay period; G_e = growth in the presence of a particular eserine sulfate concentration; G_{e+d} = growth in the presence of 0·74 μM AdO-*ara*-C and a particular eserine sulfate concentration.

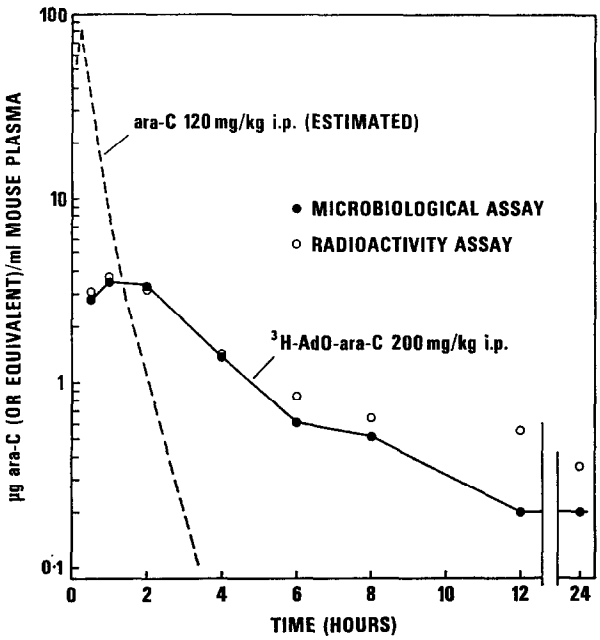

FIG. 3. *Ara*-C plasma levels after i.p. administration of ^{3}H-AdO-*ara*-C (200 mg/650 µc/kg) to BDF_1 mice. *Ara*-C levels were determined by microbiological assay and by a radioactivity assay using thin-layer chromatography. Also included is an estimate (based on a different dose[19]) of *ara*-C levels after i.p. administration of *ara*-C itself (120 mg/kg).

when levels were low and errors involved in their determination (particularly with the radioactive method) were relatively large.

Radiochromatography of plasma indicated that more than 90 per cent of the nondistillable tritium was present as ^{3}H-*ara*-C or ^{3}H-*ara*-U. For the first hour, *ara*-C predominated. However, after this time, the major component (53–67 per cent) was *ara*-U. Intact ^{3}H-AdO-*ara*-C was not detected.

Maximum *ara*-C levels (3–3·5 µg/ml) were achieved approximately 60–120 min after administration of ^{3}H-AdO-*ara*-C. Over the next 4–5 hr, levels decreased with a half-life of ca. 90 min. Subsequently, the half-life increased greatly. Levels greater than 0·1 µg/ml were detected at least 24 hr after administration. *Ara*-C itself has a half-life of ca. 20 min and plasma levels drop to less than 0·1 µg/ml within 3–4 hr after a dose of 120 mg/kg.

Excretion studies. After i.p. administration of 200 mg/kg of ^{3}H-AdO-*ara*-C to BDF_1 mice, only 21 per cent of the administered radioactivity was excreted in the urine in the first 24 hr. Excretion was even slower subsequently, and the cumulative urinary excretion was only 30 per cent (but still slowly increasing) at 7 days after treatment. Fecal radioactivity was low (less than ca. 10–20 per cent of that found in the urine) and was probably due to urine contamination. At 24 hr after administration, chromatography indicated that the urine tritium composition was 40–50 per cent *ara*-C and 50–60 per cent *ara*-U. ^{3}H-AdO-*ara*-C was not found.

Cell-kill by low levels of ara-C. The cell-kill achieved with various levels of *ara*-C in contact with L1210 cells growing in culture is shown as a function of contact time in

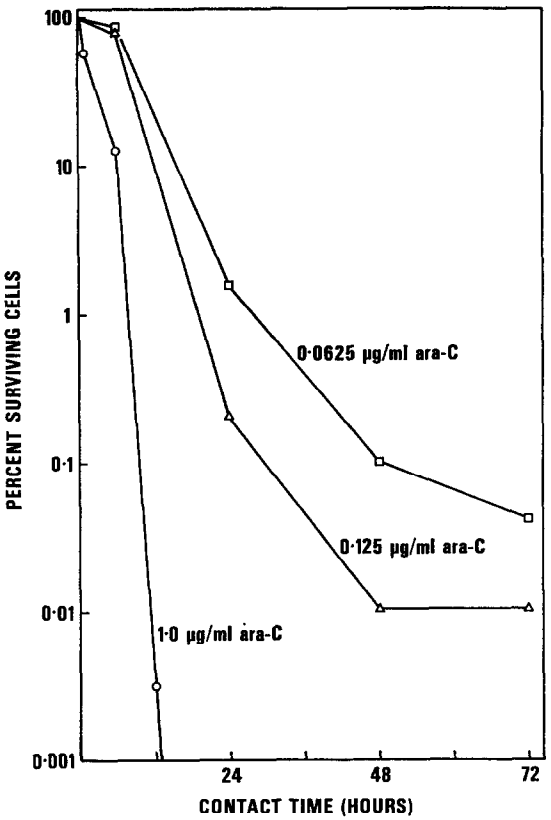

FIG. 4. Survival of L1210 cells (in culture) in contact with low levels of *ara*-C for prolonged contact times. A modification of the cloning method of Himmelfarb *et al.*[20] was used.

Fig. 4. A "high" level of *ara*-C (e.g. 1 μg/ml) was capable of killing close to 90 per cent of the cells after an exposure of 6 hr. On the other hand, 0·0625 μg of *ara*-C/ml gave little cell-kill (< 10 per cent) in that time. However, a 48-hr exposure to this low level resulted in a three-log (99·9 per cent) cell-kill.

DISCUSSION

The differences in antitumor activity between *ara*-C and AdO-*ara*-C can best be explained by the hypothesis that the latter is a depot form of the parent compound, releasing *ara*-C over a prolonged period of time. *Ara*-C, an S-phase specific agent, has a short plasma half-life in the mouse,[18,19] and thus, is most active when administered in courses of multiple, closely spaced doses or as constant parenteral infusions.[21] AdO-*ara*-C, however, has high antitumor activity when administered as a single dose,[2] and, unlike *ara*-C, is not schedule-sensitive.[21] Also, AdO-*ara*-C administered up to 48 hr prior to tumor inoculation was therapeutically effective, indicating that the agent (or a cytotoxic metabolite such as *ara*-C) was present in the mouse at least for that period of time.

One question to be answered is, "Is AdO-*ara*-C active *per se* or is hydrolysis to *ara*-C required for cytotoxic activity?" Gerzon and Kau,[22] after studying several

nucleoside 5′-adamantoates, suggested that the increases in biological activity observed could not be explained solely on the basis of the influence of the adamantane moiety on metabolic factors, but that at least in some cases, the adamantoates may exert their biological effects as intact molecules. The data in this report suggest that this is not true with the *ara*-C derivative.

It is generally accepted that phosphorylation of *ara*-C at the 5′-position is necessary for cytotoxic activity.[23–27] Since AdO-*ara*-C is already substituted at the 5′-position, precluding 5′-phosphorylation, it would have to have a rather unique biochemical mode of action if it were to act as an intact molecule. We report here many similarities in the biological and biochemical activities of *ara*-C and the derivative, which suggest that *ara*-C is indeed the active form. The shapes of cytotoxicity dose–response curves have been shown to be quite characteristic of a particular agent.[8] The dose–response curves obtained for L1210 and KB cytotoxicity of *ara*-C and AdO-*ara*-C (Fig. 1) were almost identical. Like *ara*-C, AdO-*ara*-C had little effect on RNA and protein synthesis at levels where DNA synthesis was greatly inhibited (Table 2). As was found with *ara*-C,[23] it was possible to prevent the cytotoxicity of AdO-*ara*-C *in vitro* with CdR (Fig. 1). The cross-resistance observed with L1210 cells in culture with the two agents (Table 1) also indicates similar modes of action.

The differences observed between *ara*-C and the derivative (for the most part, quantitative ones) are certainly consistent with the hypothesis that AdO-*ara*-C is hydrolyzed to *ara*-C. The lower intrinsic cytotoxicity (Fig. 1) and ability to inhibit DNA synthesis (Table 2) could be explained by lack of complete hydrolysis during the time of the assay. In addition, the time course of inhibition of DNA synthesis by AdO-*ara*-C (Fig. 2) also suggests a slow hydrolytic release of *ara*-C. The reversal of cytotoxicity by an esterase inhibitor, eserine (Table 6), is perhaps the strongest argument against intrinsic cytotoxicity of the intact molecule. The results of metabolism studies *in vitro* in mammalian plasma (Table 2) offer direct evidence that the hydrolysis can indeed occur.

AdO-*ara*-C was not present in plasma after i.p. administration of the agent to mice; only *ara*-C and *ara*-U were found (Fig. 3). Recent studies[28] have also shown that other 5′-acylates of *ara*-C (for example, the 5′-palmitate) have properties very similar to those of AdO-*ara*-C. These data argue against a unique role of the adamantane moiety.

If *ara*-C is the active "principle" of AdO-*ara*-C, what then accounts for the distinctive pharmacologic properties of the derivative? The possibility of slow hydrolytic release of *ara*-C being responsible for a sustained effect has been alluded to above. This may, indeed, represent a reasonable explanation for the results obtained *in vitro* with cells in culture. However, *in vivo*, *ara*-C once liberated by hydrolysis would be subject to the rapid degradation and excretion observed for *ara*-C itself[18,19] (approximately 20-min biological half-life in plasma). Since hydrolysis by mouse plasma is rapid, some other step must represent the critical factor in determining the depot effect. In all likelihood, dissolution of the suspended AdO-*ara*-C at the injection site is this rate-determining step. AdO-*ara*-C has very low water solubility (approximately 20 μg/ml) and is administered to animals as a suspension. In the disposition studies, white deposits were found in the peritoneal cavity up to 24 hr after i.p. administration of tritiated material. Chromatographic analysis identified this material as ^{3}H-AdO-*ara*-C.

AdO-*ara*-C is resistant to deamination (Table 5). This protection of *ara*-C from inactivation, however, is present only so long as the ester linkage is intact. Only if the adamantoate has a physiological distribution pattern which is qualitatively different from that of *ara*-C (e.g. a preferential uptake of the intact ester by tumor cells such that hydrolysis and phosphorylation might occur before deamination, or deposition in other sites *via*, e.g. a phagocytotic process) might this deaminase resistance be important. It should be noted, however, that with L1210 cells there is no preferential uptake of the ester.

The relative rates of hydrolysis (of AdO-*ara*-C to *ara*-C) and of deamination (of *ara*-C to a biologically inactive species, *ara*-U) are important factors. Where the deamination is much more rapid than hydrolysis, little *ara*-C will exist in the steady state.

The nature of the esterase activity which is responsible for the hydrolysis observed cannot be unequivocally determined on the basis of the results reported here, but the inhibition by eserine sulfate of mouse plasma hydrolysis suggests the participation of a cholinesterase.[29] It should be emphasized that the esterase activity of only one tissue has been studied, and considerable variation in activity between tissues in the same species might be expected. This has clearly been shown for *ara*-C deaminase activity.[30]

It is obviously impossible on the basis of our knowledge of hydrolysis and deamination alone to choose a model animal system which would be predictive for possible human studies with depot forms of *ara*-C. Variation of activities between tissues, absorption and excretion rates, activities of other enzymes (for example, deoxycytidine kinase, the enzyme required for cytotoxic activity of *ara*-C itself[31]), as well as other factors, will greatly influence the metabolic pattern. Even in terms of the plasma metabolism of AdO-*ara*-C alone, no species studied appears to represent a particularly good model for man. In terms of hydrolysis, only rhesus monkey or dog plasmas approximate the activity observed in human plasma. The high deaminase activity in the rhesus monkey plasma and the low activity in that of the dog would, however, seem to argue against these species as good models. Enzymatic studies with other tissues and blood level studies may provide further information useful in the choice of an appropriate system.

The cytotoxic effects of low levels of *ara*-C in prolonged contact with L1210 cells in culture (Fig. 4) are in excellent agreement with the mouse plasma level data *in vivo* (Fig. 3) and therapy data.[2] The cell-kill data suggest strongly that plasma levels of *ara*-C of the order of (and even lower than) the sensitivity of the assays employed are quite capable of yielding therapeutically effective cell-kill if, as is the case with AdO-*ara*-C administration, these levels persist long enough. These results also lend support for the use in man of constant i.v. infusions of *ara*-C over prolonged periods.

In conclusion, the results of this study have shown that AdO-*ara*-C represents a molecular depot form of *ara*-C and that the sustained release achieved by administration to mice yields persistent cytotoxic levels of *ara*-C which result in the marked therapeutic effects previously observed.

Acknowledgements—We wish to acknowledge the excellent technical assistance of Mrs. M. E. Hall, Miss L. G. Scheidt and Mr. T. J. Fraser. We thank also Dr. P. F. Wiley and Mr. M. E. Herr of these laboratories for the AdO-*ara*-C used. The editorial assistance of Mrs. E. L. Clark is greatly appreciated.

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