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Arabinofuranosyl-5-azacytosine: activity against human tumors in athymic mice

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Summary. Arabinofuranosyl-5-azacytosine (Ara-AC), a new compound structurally related to arabinofuranosylcytosine (Ara-C) and 5-azacytidine (5-AC), has demonstrated significant therapeutic activity against a wide spectrum of murine tumors and three human tumor xenografts in the NCI tumor panel. Studies on the activity of Ara-AC in these and other human tumor xenograft models were undertaken to define its potential anti-human-tumor profile more completely. Ara-AC demonstrated marked antitumor activity against human tumor xenografts, including leukemias and solid tumors that do not respond to Ara-C or 5-AC. An important finding was the demonstration that Ara-AC was as effective by the oral route as when given intraperitoneally. Furthermore, the compound demonstrated synergism when combined with cisplatin in the treatment of refractory solid tumors and also induced monocyte-type differentiation of promyelocytic leukemia (HL-60) cells in vitro. Ara-AC is a promising new compound that may have utility in the treatment of human cancer beyond that anticipated for a cytotoxic nucleoside.

Introduction

Arabinofuranosyl-5-azacytosine (Ara-AC; NSC 281272) is a new compound that combines the structural characteristics of arabinofuranosylcytosine (Ara-C) and 5-azacytidine (5-AC) [1, 2], both of which are clinically active in the treatment of human leukemia [3, 12]. Ara-AC has demonstrated significant antitumor activity against a wide spectrum of experimental murine tumors, and its striking activity in three human tumor xenograft models distinguishes this drug from Ara-C, 5-AC, and other structurally similar analogues [6]. An additional important feature of Ara-AC is its resistance to degradation by cytidine-deoxycytidine deaminase, the major enzyme responsible for the inactivation of Ara-C [18].

We studied the antitumor activity of Ara-AC against a variety of human tumor xenografts, including those previously reported [6]. The results of these studies as well as the present investigation on the cytological effects of Ara-AC in vitro are the subject of this report.

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Materials and methods

Drugs. Ara-AC was a gift from Dr. John S. Driscoll, Laboratory of Pharmacology and Experimental Therapeutics, NCI; Ara-C (Cytosar) was obtained from the UpJohn Co. (Kalamazoo, Mich), and 5-AC, from Sigma Chemical Co. (St. Louis, Mo). All drugs were dissolved and diluted in sterile saline; they were prepared fresh and held at +4° C for the duration of each experiment.

Tumors. The human tumors used in these studies are listed and described in Table 1. The lung LX-1, breast MX-1, and colon CX-1 tumors [9] were obtained through the Division of Cancer Treatment; breast tumors MX-2 and CooG, through the Breast Cancer Program, Division of Cancer Prevention and Control, NCI; and ovarian tumor MRI-H207, through Dr. A. E. Bogden, EG&G Mason Research Institute (Worcester, Mass). T-cell leukemias CCRF-CEM and T MOLT-4 [13], promyelocytic leukemia HL-60 [5], lung UCLA-P3 [15], breast MCF-7 [16] and melanomas SK MEL-3 [4] and SK MEL-28 [7] were obtained as cell lines from the laboratory of origin or from the American Type Culture Collection (Rockville, Md) and were established as subcutaneous transplant tumors in athymic mice. Lung tumor ACCO Lu-78, colon tumors ACCO Co-61, ACCO Co-65, and ACCO Co-77, and pancreatic tumor ACCO P-105 were established from fresh surgical specimens as subcutaneous transplant tumors in athymic mice in these laboratories. The histological diagnosis of the donor tumors, used to establish the ACCO transplant tumors, was done at the Department of Pathology, Valley Hospital (Ridgewood, NJ), and their histological characteristics were confirmed following passage in athymic mice by Dr. Richard McReynolds, Medical Research Division, American Cyanamid Co. All cell lines and tumors were tested and found free of microbial agents.

Evaluation of antitumor activity. Female athymic (HSD Nude-nu) mice weighing 20–23 g (Harlan Sprague-Dawley, Inc., Indianapolis, Ind) were used. The 5-week-old animals were shipped in filtered crates and maintained in barrier facilities for the duration of the experiments. Tumors were propagated in the mice as subcutaneous transplants.

To evaluate the sensitivity of tumors to drugs, mice received tumor implants by trocar (four to five 2-mm^3

tumor fragments, approximately 70 mg) subcutaneously in the axillary region. Mice were randomized and used when tumors reached a weight of 100–350 mg (staging day). Drugs were given i.p. or orally at several dose levels once daily for 9 days starting on staging day, with 5–6 mice per test and 10–12 animals in control groups. In some studies, drugs were given every 3×4 (q3h $\times$ 4) on days 1, 5, and 9 (q4d $\times$ 3) relative to staging day. To estimate drug toxicity, mice were weighed on staging day and on days 5 and 10 post-staging and were monitored daily for deaths.

Tumors were measured on days 11, 14, and 21 after staging by means of vernier calipers, and tumor weights were estimated from tumor diameters as described by Geran et al. [8] by the following formula:

$$\text{Tumor weight (mg)} = \frac{\text{tumor length (mm)} \times \text{tumor wight (mm)}^2}{2} .$$

The change (Δ) in tumor weight was calculated for each group by subtracting the initial mean group weight on staging day from the mean group weight on the day of evaluation. The percentage of treated divided by control weight change (% T/C) [or percentage of mean weight change over the initial mean weight for groups showing a negative number (regression)] was calculated for test groups with >65% survivors. For the plotting of tumor growth, actual mean tumor weights were used.

Induction of differentiation and cytolytic activity. Human promyelocytic (HL-60) cells [5] were propagated in a basal medium comprising RPMI 1640 [14] supplemented with 10% bovine fetal serum and 50 µg/ml gentamycin. For cytodifferentiation studies, cells were sedimented by centrifugation, adjusted to 2.5×10^5 cells/ml, and inoculated in 20-ml volumes into 75-cm^2 plastic culture flasks. Test drug or saline was added at 2.5 ml/flask, and cultures were placed in a humidified, 5% CO_2-in-air incubator at 37° C. After 5 days, cells were counted using a hemocytometer and viability was determined by trypan blue dye exclusion. To assess differentiation into monocyte-type cells, 330,000 viable cells in 0.3 ml were centrifuged onto 3×1-in. slides by means of a Shandon cytocentrifuge. Air-dried preparations were treated with pararosanilin-alpha-naphthyl acetate and with methyl green for the cytochemical demonstration of nonspecific esterase (NSE) [17].

Cytolytic activity in vitro was measured by a modification of the clonogenic assay of Hamburger et al. [11]. A cell line developed from the human small-cell lung carcinoma, LX-1, was used. A total of 2×10^4 cells in 1.0 ml basal medium were exposed to test drug at 37° C. After 1 h, 2×10^3 cells in 0.1-ml aliquots were resuspended in 1.0 ml basal medium containing 5.0 µg/ml insulin and 0.3% agarose and plated directly onto 2 ml solidified medium (McCoy's 5A medium containing 5% horse serum, 10% bovine fetal serum, 2 m*M* L-glutamine, 0.2% sodium pyruvate, 50 µg/ml gentamycin, and 0.5% agarose) in each gridded, 35×10 mm dish. Test cultures were prepared in triplicate and placed in a humidified, 5% CO_2-in-air incubator at 37° C and colonies were counted 14 days later.

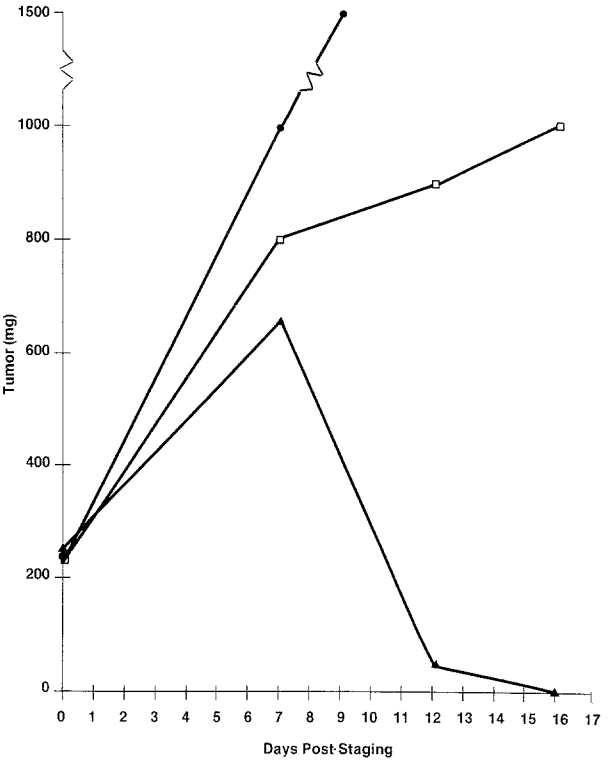


Fig. 1. Human promyelocytic leukemia (HL-60) xenograft: comparative response to Ara-AC and Ara-C. HSD Nude-nu athymic mice bearing subcutaneous tumors were treated i.p. daily for 9 days after tumor staging. ●, control; □, 40 mg/kg Ara-C; ▲, 50 mg/kg Ara-AC

Results

Antitumor activity

In comparative tests, Ara-AC and Ara-C were usually tested above and below their optimal doses (50 mg/kg Ara-AC, 40 mg/kg Ara-C) for the daily $\times 9$ schedule [6]. When 5-AC was included in the test, it was given i.p. on the same treatment schedule over a dose range of 0.8–4.0 mg/kg.

The therapeutic effectiveness of Ara-AC given i.p. on a daily $\times 9$ schedule against 18 human tumors growing as subcutaneous implants in athymic mice is summarized in Table 1. The drug was highly effective against the T-cell leukemias (CCRF-CEM and T Molt-4) and promyelocytic leukemia (HL-60), which grow relatively rapidly in these mice, progressing from 200-mg to >2-g tumors in 14 days. All animals with CCRF-CEM or HL-60 tumors that were treated with Ara-AC at 50–100 mg/kg daily for 9 days were completely free of tumors on the 14th day and remained healthy and tumor-free for the remainder of the observation period (21–28 days post-staging) (Fig. 1). At its optimal dose of 40 mg/kg, Ara-C also induced regression of CCRF-CEM tumors, although tumors in 3/6 mice were regrowing at day 20; 5-AC was ineffective against this tumor. Ara-C did not induce significant, sustained inhibition of tumor growth in animals bearing promyelocytic HL-60 leukemia (Fig. 1).

Both Ara-AC and Ara-C were highly active against T-cell leukemia T MOLT-4; both drugs induced complete tumor regression in the majority of animals following

Table 1. Comparative response of human tumor xenografts to Ara-AC and Ara-C

Human tumors	Type	Ara-AC T/C (%)[a] (at optimal dose[b])	Ara-C
CCRF-CEM	Acute T-cell leukemia	−100	−100
T MOLT-4	Acute T-cell leukemia	−100	−100
HL-60	Promyelocytic leukemia	−100	25, 30[3]
MRI-H207	Ovarian carcinoma	−100	−24, −18
LX-1	Lung small-cell carcinoma	−79, −82	neg[c]
UCLA-P3	Lung adenocarcinoma	neg	neg
ACCO LU-78	Lung squamous-cell carcinoma	neg	neg
MX-1	Breast duct-cell carcinoma	−55, −62	22, 31
MX-2	Breast undifferentiated carcinoma	04, 10	neg
MCF-7	Breast (ER+) adenocarcinoma	33, 28	neg
COOG	Breast carcinoma	neg	neg
CX-1	Colon adenocarcinoma	08, 10, 39	42, neg
ACCO CO-65	Colon adenocarcinoma	00, 09	−24, 12
ACCO CO-77	Colorectal adenocarcinoma	5, 29	neg
ACCO CO-61	Colorectal adenocarcinoma	neg	neg
SK MEL-28	Melanoma	neg	neg
SK MEL-3	Melanoma metastatic to lymph node	neg	neg
ACCO P-105	Pancreatic adenocarcinoma metastatic to liver	neg	neg

Groups of 5 or 6 (10 in control group) HSD Nude-nu athymic mice received five 2-mm^3 tumor fragments subcutaneously on day 0. Drug treatment given i.p. once daily for 9 days was started when tumors had reached an average weight of 100–350 mg (staging day)

[a] Percentage of T/C was calculated by dividing the change, Δ, in mean tumor weight of the treated group by the Δ in mean tumor weight of control animals. A T/C for a negative number (regression) was calculated by the formula:

$$\frac{\Delta \text{ mean tumor weight of test}}{\text{initial mean tumor weight}} \times 100$$

[b] The dose producing the greatest tumor response and >65% survivors during a 16- to 21-day observation period after staging day

[c] Compounds were considered inactive (*neg*) if T/C was >42%. For T/C values that were variable in a second or third test, all test values are given

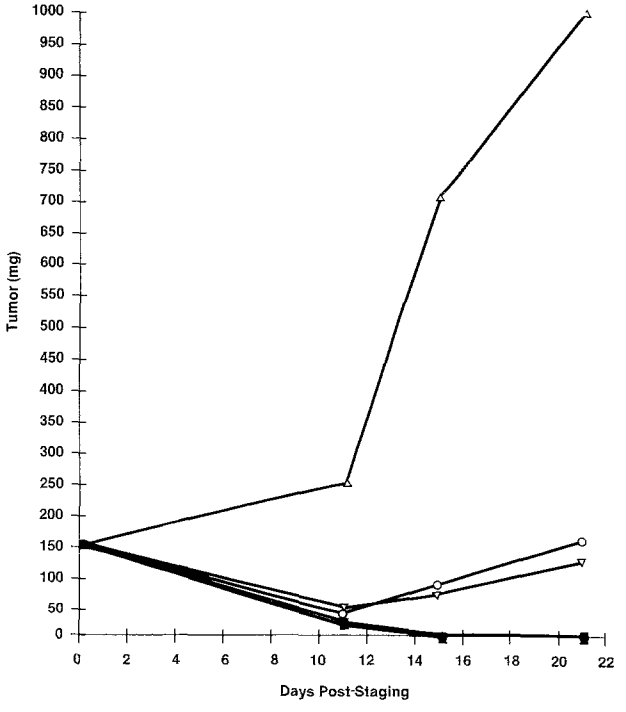
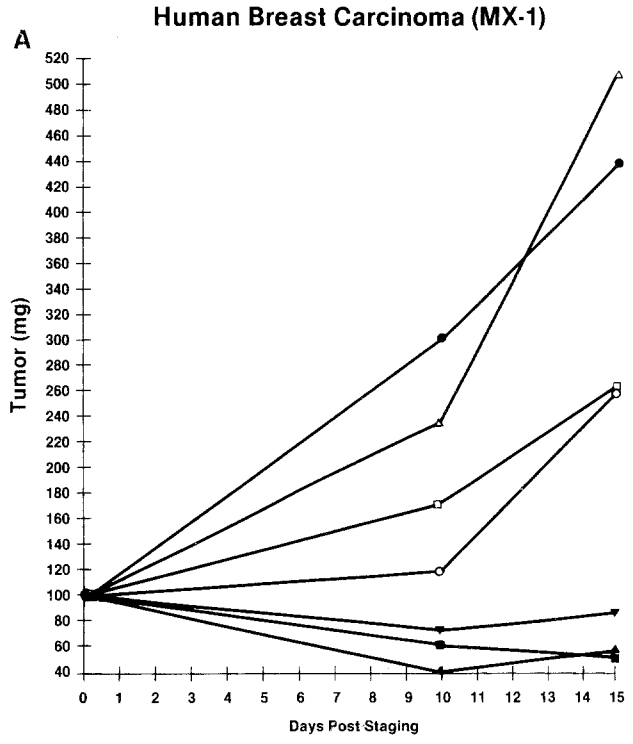


Fig. 2. Human ovarian carcinoma (MR-H207) xenograft: comparative response to Ara-AC and Ara-C. HSD Nude-nu mice bearing subcutaneous tumors were treated i.p. daily for 9 days after tumor staging. △, control; ▽, 50 mg/kg Ara-C; ○, 25 mg/kg Ara-C; ■, 50 mg/kg Ara-AC; ▲, 25 mg/kg Ara-AC

treatment at the optimal dose. Ara-AC was very active against the ovarian MRI-H-207 carcinoma, inducing complete tumor regression and tumor-free, healthy survivors in all groups treated over a dose range of 25–100 mg/kg. Ara-C was also active against MRI-H-207, inducing tumor regression but no tumor-free survivors. Regrowth of tumors was observed in animals treated with Ara-C but not in those treated with Ara-AC following cessation of drug treatment (Fig. 2).

Ara-AC showed good activity against the three NCI human tumor xenograft models: breast MX-1, lung LX-1, and colon CX-1. Following daily i.p. administration for 9 days, this drug induced tumor regression in animals bearing breast MX-1 or lung LX-1 tumors (Fig. 3 A, B) and significant (61%–92%) inhibition of tumor growth in those with colon CX-1 tumors (Table 1). At its near-toxic dose of 80 mg/kg, Ara-C induced some delay in the growth of breast MX-1 tumors, but it was ineffective against this tumor at the optimal dose of 40 mg/kg (Fig. 3 A). Ara-C was also generally inactive against the lung LX-1 and colon CX-1 tumors (Table 1, Fig. 3 B). The effectiveness of Ara-AC against the colon CX-1 xenograft was not improved when the drug was injected i.v. instead of i.p. Ara-AC (but not Ara-C or 5-AC) produced significant inhibition of tumor growth (90%–96% and 67%–72%, respectively) when given i.p. to animals bearing breast carcinoma MX-2 or breast adenocarcinoma MCF-7 (Table 1).

Ara-AC and Ara-C were active when tested against colon ACCO Co-65; both drugs significantly inhibited tumor growth when given i.p. at their optimal doses. Ara-AC (but not Ara-C or 5-AC) was also active against colon



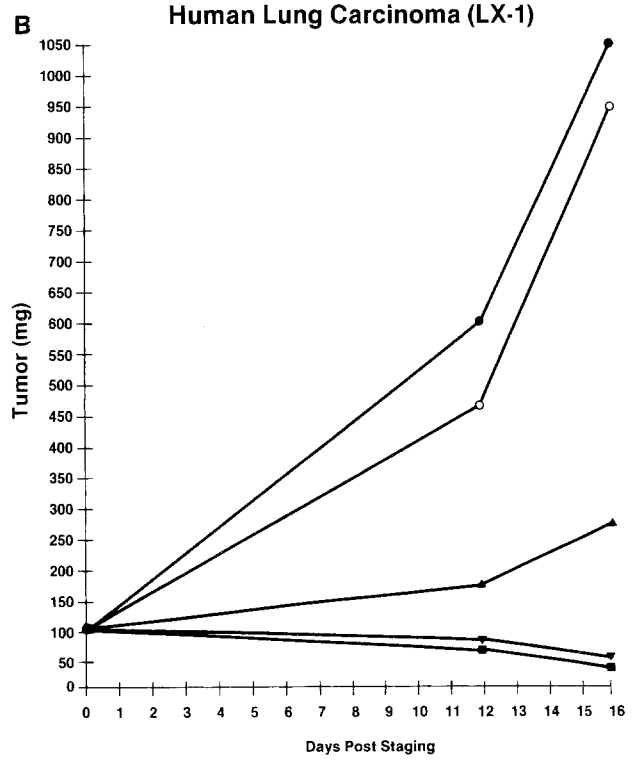


Fig. 3. Human breast carcinoma (MX-1) and human lung carcinoma (LX-1) xenografts: comparative response to Ara-AC and Ara-C. HSD Nude-nu athymic mice bearing subcutaneous tumors were treated i. p. daily for 9 days starting on staging day. **A** Breast MX-1: ●, control; ○, 80 mg/kg Ara-C; □, 40 mg/kg Ara-C; △, 20 mg/kg Ara-C; n , 100 mg/kg Ara-AC; ▲, 50 mg/kg Ara-AC; ▼, 25 mg/kg Ara-AC. **B** Lung LX-1: ●, control; ○, 50 mg/kg Ara-C; ■, 100 mg/kg Ara-AC; ▼, 50 mg/kg Ara-AC; ▲, 25 mg/kg Ara-AC

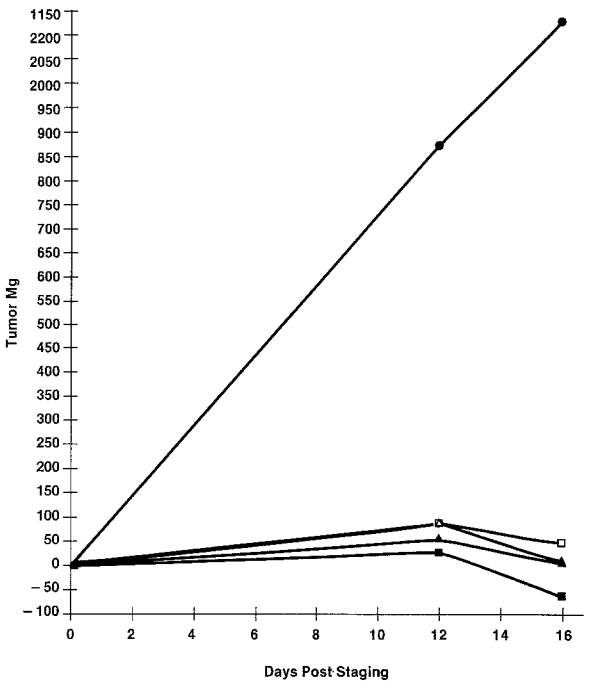


Fig. 4. Oral activity of Ara-AC against human lung (LX-1) carcinoma in athymic mice. HSD Nude-nu mice bearing subcutaneous tumors were treated i. p. or orally daily for 9 days after tumor staging. △, i. p. 100 mg/kg; □, i. p. 50 mg/kg; ■, p. o. 100 mg/kg; ▲, p. o. 50 mg/kg; ●, control

ACCO Co-77, inhibiting tumor growth by 65%–71% (Table 1). The superior activity of Ara-AC given once every 3 h (q3h × 8) in some tumor models [6] led us to compare this schedule of treatment with the once daily × 9 treatment in animals bearing colon ACCO CO-77 tumors. Results of this test indicated no greater response of colon ACCO Co-77 tumors in animals treated q3h × 8 on a 4d × 3 schedule than that of tumors in animals receiving a daily × 9 treatment starting on staging day.

Ara-AC was inactive against colon ACCO Co-61 adenocarcinoma, lung adenocarcinoma UCLA-P3, lung squamous-cell carcinoma ACCO Lu-78, breast carcinoma CooG, melanomas SK MEL-28 and SK MEL-3, and pancreatic carcinoma ACCO P-105 (Table 1). 5-AC was also inactive against lung squamous-cell carcinoma ACCO LU-78 and melanoma SK MEL-28.

When given orally Ara-AC showed antitumor activity against human tumor xenografts that was equal to or greater than that following its i. p. administration. This was observed in tests against human leukemias, i. e., T-cell leukemia CCRF-CEM, as well as in tests against solid tumors. In comparative tests in animals bearing subcutaneous tumors of lung LX-1 carcinoma, Ara-AC given i. p. at 50–100 mg/kg produced 90%–100% inhibition of tumor growth, whereas the oral preparation induced 97%–158% tumor inhibition (Fig. 4).

Ara-AC also enhanced the therapeutic response to cisplatin when the two drugs were used in combination in animals bearing refractory solid tumors (i. e., lung LX-1, colon ACCO CO-77, and melanoma SK MEL-28). Given i. p. q3h × 4 on a q4d × 3 schedule, cisplatin has shown antitumor effects on human solid tumors normally refractory to the drug on other treatment schedules in xenograft tests in our laboratory. In tests against melanoma SK MEL-28

Table 2. Response of human melanoma (SK-MEL-28) in athymic mice to cisplatin and Ara-AC combination chemotherapy

Drug	Dose (mg/kg)	Days after tumor staging:						
		11		15		21		
		Δtumor weight[a] (mg)	T/C[b] (%)	Δtumor weight (mg)	T/C (%)	Δtumor weight (mg)	T/C (%)	Survivors/ treated
Saline		98		184		345		10/10
Cisplatin	1.5	49	50	38	21	66	19	5/5
	0.75	108	110	167	91	331	96	5/5
Ara-AC	18	158	161	243	132	413	120	5/5
	14	38	60	72	39	157	45	5/5
Cisplatin + Ara-AC	1.5 + 14	−17	−45[c]	−21	−55	−14	−37	5/5
Cisplatin + Ara-AC	0.75 + 14	92	94	105	57	157	45	5/5

Athymic (HSD Nude-nu) mice were subcutaneously implanted with five 2-mm^3 tumor fragments. Drugs were given i. p. every 3 h × 4, every 4 days × 3, starting 10 days after tumor implantation (staging day)

[a] Final mean tumor weight minus the initial mean tumor weight

[b] Percentage of T/C weight change

[c] T/C for a negative number (regression) was calculated by the formula:

$$\frac{\Delta\text{mean tumor weight of test}}{\text{initial mean tumor weight}} \times 100$$

Table 3. Comparative effect of Ara-AC and Ara-C on the cytotoxicity and induction of differentiation of human promyelocytic leukemia (HL-60) cells in vitro

Drug	Exposure dose (μg/ml)	Viable cells (% of control)	Cells positive for NSE (%)
Ara-AC	5.0	21	19
	2.5	41	42
	1.25	51	11
	0.62	62	7
	0.31	67	2
	0.15	100	2
Ara-C	0.25	0	–
	0.125	30	43
	0.06	33	32
	0.03	56	26
	0.015	73	13

Culture flasks (75 cm^2) were inoculated with 20 ml 2.5×10^5 HL-60 cells/ml, drug or saline was added, and cultures were incubated in a humidified 5% CO_2-in-air incubator at 37° C for 5 days. Cells were counted using a hemocytometer: viability was determined by trypan blue dye exclusion, and monocyte differentiation was assessed by the cytochemical demonstration of NSE

at its optimal dose, cisplatin produced a T/C of 50%–19% (50%–81% tumor inhibition), whereas Ara-AC was ineffective against this tumor. However, the combination of cisplatin and Ara-AC given at the optimal doses for each drug on the q3h × 4 schedule produced a significant increase in therapeutic response (tumor regression, T/C −37 – −45%; tumor inhibition, 137%–145%) (Table 2).

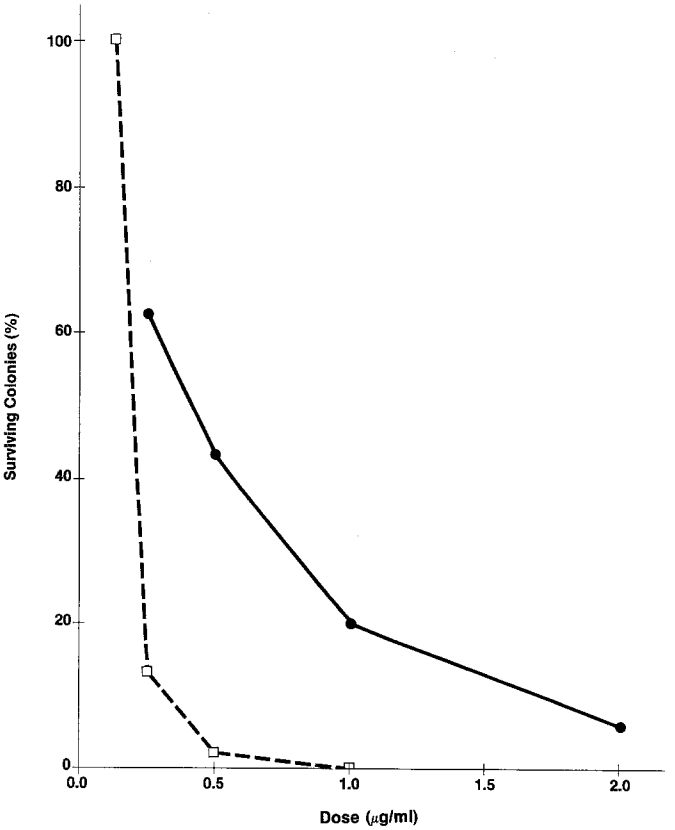


Fig. 5. Cytolytic effect of Ara-AC and Ara-C on human small-cell lung carcinoma (LX-1) in vitro. Cells (2×10^4) in 1.0 ml medium were exposed to drug for 1 h at 37° C. Cells (2×10^3) in 0.1-ml aliquots were resuspended in 1.0 ml warm agarose medium and plated onto 2 ml solidified agarose medium in each 35-mm dish. Colonies were counted after 14 days' incubation. □, Ara-C; ●, Ara-AC

In vitro effects

Ara-AC produced a concentration-dependent inhibition of the growth of human promyelocytic leukemia (HL-60) cells when cultures were exposed to the drug over a 5-day period. In these tests, the inhibitory (IC_{50}) concentration of Ara-AC was approximately 1.36 μg/ml, compared with 0.04 mg/ml for Ara-C (Table 3).

Ara-AC also induced differentiation of HL-60 cells into cells with characteristics of monocytes, as evidenced by the cytochemical demonstration of nonspecific esterase (NSE) and an increased ability of treated populations to adhere to the plastic surface of culture flasks. In the presence of 2.5 μg/ml Ara-AC, viable HL-60 cells increased in number from 2.5×10^5 to 6.6×10^5 over the 5-day incubation period, and 42% of this population was positive for NSE; HL-60 cells in control cultures reached a density of 1.3×10^6 viable cells/ml over this period, but $> 0.5\%$ of this population was positive for NSE. This represents a >10-fold increase in the number of NSE-positive cells in treated vs control cultures (Table 3).

Ara-AC was cytolytic to human small-cell lung carcinoma (LX-1) when these cells were exposed to the drug for a 1-h period prior to plating in a clonogenic assay. Ara-C was also cytolytic to LX-1 cells in this assay and, as previously observed by Dalal et al. [6] in an in vitro system Ara-C IC_{50} values were considerably lower than those of Ara-AC (Fig. 5).

Discussion

Ara-AC demonstrated marked antitumor activity against several human tumors of different histologic origins in xenograft models and, in comparative tests, showed therapeutic activity superior to its structural analogues, Ara-C and 5-AC. Ara-AC induced complete regression of tumor (cures) in all treated mice bearing T-cell leukemias T Molt-4 or CCRF-CEM, promyelocytic leukemia HL-60, or ovarian carcinoma MRI-H207. In contrast, Ara-C induced complete cures only in animals bearing T-cell leukemia T Molt-4 and was ineffective in the treatment of mice bearing the promyelocytic HL-60 leukemia.

Ara-AC also induced regression of the breast MX-1 and lung LX-1 tumors and significantly inhibited the growth of the colon CX-1 tumor, whereas Ara-C was ineffective in the same tests. Results of these tests appear to confirm those reported by Dalal et al. [6] in studies on the antitumor effect of Ara-AC and Ara-C on the breast MX-1, lung LX-1, and colon CX-1 tumors in xenograft models using the subcutaneous route for drug administration and the subrenal capsule as the site of tumor implantation. In this xenograft model, Ara-AC induced regression of the breast MX-1 and lung LX-1 tumors and inhibited the colon CX-1 tumor by 93%; Ara-C and 5-AC were ineffective against LX-1 and CX-1 in these studies, and, although the breast MX-1 tumor was sensitive to Ara-C and 5-AC, only Ara-AC induced its regression [6].

The activity of Ara-AC against the LX-1 and CX-1 tumors is of some interest. The LX-1 small-cell lung carcinoma and the CX-1 colon adenocarcinoma in xenograft systems are relatively insensitive to most anticancer drugs. Of 22 clinically active agents tested against these tumors in xenografts using the subcutaneous site for tumor implantation, none were active against the CX-1 tumor, and only CCNU and procarbazine induced $>85\%$ inhibition of LX-1 tumor growth [9].

Although the question as to the predictability of human tumor xenograft models for activity in the clinic remains unanswered, Golden et al. [9] have noted that in agreement with clinical experience, most human solid tumors in xenografts appear to be relatively resistant to therapy [9]. Its activity against these refractory human tumor xenografts suggests that Ara-AC may have value in the clinic as an antineoplastic agent in the treatment of solid tumors as well as leukemias. Ara-AC was at least as effective against human tumor xenografts when given orally as when injected i.p. This would support and extend the results of studies showing that both bioavailability and efficacy are retained by oral administration of Ara-AC for treatment of i.p. implanted murine L1210 leukemia [10].

The synergistic combination therapy using Ara-AC and cisplatin in advanced L1210 leukemia has been reported (Daniel Griswold, Southern Research Institute, Birmingham, Ala, personal communication). In the present studies, Ara-AC enhanced the therapeutic response to cisplatin when the two drugs were used in combination treatment of typically refractory human solid tumors (melanoma, non-small-cell lung carcinoma and colon carcinoma).

The Ara-AC IC_{50} for human promyelocytic HL-60 and human small-cell lung carcinoma LX-1 cells in vitro was considerably higher than comparable values for Ara-C. A similar disparity between IC_{50} values for these agents was noted by Dalal et al. in murine tumor cells in vitro [6]. However, the optimally effective dose of Ara-C in vivo is only slightly lower than that of Ara-AC. Both Ara-AC and Ara-C induced differentiation of HL-60 cells into monocyte-type cells; Ara-AC is also reported to actively induce the differentiation of HL-60 cells into NBT-positive cells with characteristics of granulocytes [6]. Whether or not its activity as an inducer of differentiation plays a role in the remarkable antitumor activity of Ara-AC against myeloid and T-cell leukemias in xenografts has not been determined. In view of its superior therapeutic activity against experimental human tumors, Ara-AC appears to be an interesting new compound that may have utility in the treatment of human cancer beyond that anticipated for a cytotoxic nucleoside.

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