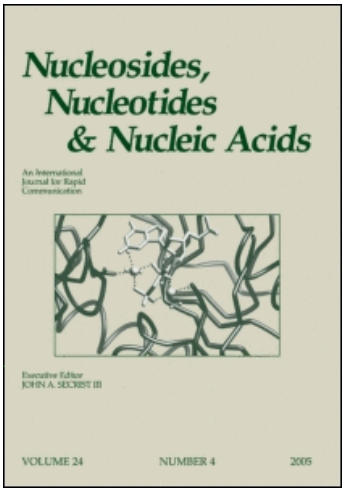

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Preclinical Antitumor Activity of 2-Chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine (C1-F-Ara-A)

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PRECLINICAL ANTITUMOR ACTIVITY OF 2-CHLORO-9-(2-DEOXY-2-FLUORO-β-D-ARABINOFURANOSYL)ADENINE (Cl-F-ARA-A)

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Dedicated to the memory of Dr. Gertrude B. Elion

ABSTRACT

Cl-F-ara-A, an analog of fludarabine, was evaluated against a spectrum of tumor systems in culture and in mice. The compound exhibited significant cytotoxicity against a variety of human tumor cell lines. More importantly, the compound showed selectivity *in vivo*, with excellent activity being demonstrated against human colon and renal tumors. Human nonsmall cell lung and prostate tumors were also sensitive *in vivo* to the compound, albeit at a reduced level.

INTRODUCTION

As part of our program to develop new anticancer drugs with improved activity, we prepared and examined three 2-halo-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) adenines.[1] Among these compounds 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) adenine (Cl-F-ara-A, **1**) was found to have promising initial *in vitro* and *in vivo* activity. Cl-F-ara-A, in contrast to fludarabine phosphate, an FDA-approved drug, has greater resistance to cleavage by *E. coli* purine nucleoside phosphorylase by virtue of the fluorine at the 2′-position.[1,2] This characteristic, along with other properties of this compound,[3] including its promising activity against P388 leukemia in mice, resulted in its selection for more advanced evaluation. We present herein data resulting from the evaluation of Cl-F-ara-A against a spectrum of human tumor systems in culture and in mice.[4]

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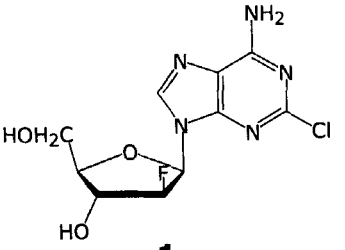

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MATERIALS AND METHODS

Drugs. Cl-F-ara-A was synthesized at Southern Research Institute.[1] Fludarabine phosphate was kindly provided by Schering AG (Berlin, Germany). PalmO-ara-C was synthesized using a literature procedure.[5] Doxorubicin, etoposide, paclitaxel, melphalan, and methotrexate were provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute (NCI), Bethesda, MD. For the *in vivo* studies Cl-F-ara-A and fludarabine phosphate were prepared fresh every five days in saline containing 0.05% tween 80 and kept at 2-8 °C between injections.

Cell Culture. The nine human cell lines used were obtained from the Developmental Therapeutics Program Tumor Repository, NCI (Frederick, MD). The cell lines were grown in RPMI 1640 medium containing 9% fetal bovine serum, 1% iron-supplemented calf serum, and 2 mM L-glutamine. For the *in vitro* evaluation of the sensitivity of the human cell lines to Cl-F-ara-A and fludarabine phosphate, cells were plated in 96-well microtiter plates and then were exposed continuously to various concentrations of the compounds for 72 h at 37°C. Cell viability was based on the reduction of XTT (in conjunction with phenazine methylsulfonate) to a water soluble formazan product, which was measured with a microplate reader at 450 nm. The background absorbance mean was subtracted from the data followed by conversion to percent of control. The drug concentrations producing survival just above and below the 50% level were used in a linear regression analysis to calculate the IC_{50}.

Experimental Chemotherapy. Mice, obtained from various commercial suppliers, were housed in microisolator cages. Mice were allowed commercial mouse food and water *ad libitum*. The various murine and human tumors were obtained from the Developmental Therapeutics Program Tumor Repository and were maintained in *in vivo* passage. The development of drug-resistant P388 murine leukemias at Southern Research Institute has

been described previously.[6] Only tumor lines that tested negative for antibodies to selected viruses were used. For the *in vivo* evaluation of the sensitivity of transplantable murine tumors to Cl-F-ara-A and fludarabine phosphate, $CD2F_1$ mice were implanted with 10^6 P388 leukemia cells (ip) or fragments of colon 36 adenocarcinoma (sc), whereas $B6C3F_1$ mice were inoculated with fragments of mammary 16/C adenocarcinoma (sc). For the *in vivo* evaluation of the sensitivity of human tumors to the compounds, NCr-nu athymic mice (scid mice for LNCAP) were implanted sc with tumor fragments (cultured cells in Matrigel™ for LNCAP). Tumor implantation day was designated Day 0. In each experiment, Cl-F-ara-A and fludarabine phosphate were tested at several dosage levels. Procedures were approved by the Institutional Animal Care and Use Committee, which conforms to the current Public Health Service *Policy on Humane Care and Use of Laboratory Animals* and the *Guide for the Care and Use of Laboratory Animals.*

For the P388 leukemias, antitumor activity was assessed on the basis of % median ILS (increase in life span) and net $\log_{10}$ cell kill. Calculations of net $\log_{10}$ cell kill were made using the tumor doubling-time that was determined from an internal tumor titration[7] or was based on historical data. Long-term survivors were excluded from calculations of % ILS and tumor cell kill. To assess tumor cell kill at the end of treatment, the survival time difference between treated and control groups was adjusted to account for regrowth of tumor cell populations that may occur between individual treatments.[8] The net $\log_{10}$ cell kill was calculated as described previously.[9] Cross-resistance was defined as a decrease in the sensitivity (> 2 $\log_{10}$ units of cell kill) of a P388/drug-resistant leukemia to Cl-F-ara-A as compared with that concurrently observed for parental P388 leukemia.

For the colon 36, mammary 16/C, and human tumors, antitumor activity was assessed on the basis of delay in tumor growth [(T-C)/C]. For early drug treatment (*e.g.*, beginning on Day 1), the delay in tumor growth is the unweighted average of the differences of the median times (days) postimplant for the treated (T) and control (C) groups to attain two evaluation sizes divided by the control value. For delayed drug treatment, the delay in tumor growth is the difference in the median of times poststaging for tumors of the treated and control groups to double in mass two, three, or four times (depending upon the growth rate of the tumor) divided by the control value. Drug deaths, tumor-free survivors, and any other animal whose tumor failed to attain the evaluation size were excluded. Tumors were measured in two dimensions (length and width) twice

weekly, and the tumor weight was calculated using the formula (length x width^2)/2 and assuming unit density.

RESULTS

***In Vitro* Evaluations.** The cytotoxicity of Cl-F-ara-A in nine human cell lines is shown in Table 1. The compound was cytotoxic to most of the human tumor cell lines at submicromolar concentrations. DLD-1 colon tumor cells and WI-38 normal fibroblasts were less sensitive to the compound. For comparison, the cytotoxicity of fludarabine phosphate in the same cell lines is also shown in Table 1. Fludarabine phosphate was less cytotoxic than Cl-F-ara-A, with IC_{50} values typically greater than 30 μM. Only the leukemias exhibited sensitivity at submicromolar concentrations.

***In Vivo* Antitumor Activity.** The therapeutic effectiveness of Cl-F-ara-A against ip implanted P388 leukemia is shown in Table 2. Moderate activity was observed for a dosage of 200 mg/kg/dose administered ip on Days 1-5 (ILS of 59% and a net cell kill of 1.6 $\log_{10}$ units). As is typical of antimetabolites, the activity was greatly enhanced by using a more intensive treatment schedule [*e.g.*, eight injections separated by 3 h intervals (q3h x 8) on Days 1, 5, and 9; or three injections separated by 4 h intervals (q4h x 3) on Days 1-9]. At a dosage of 20 mg/kg/dose the two treatment schedules yielded %ILS values of 220 and 300 and net cell kill values of 6.6 and 6.0 $\log_{10}$ units, respectively. The compound also exhibited antitumor activity orally (by gavage); however, the level was less than that observed for the ip route of administration.

The compound, administered ip, was evaluated against two murine solid tumors (colon 36 and mammary 16/C) implanted sc. Curative activity was observed against the colon 36 tumor (see Table 3), whereas moderate activity was noted against the mammary 16/C tumor (see Table 5). Cl-F-ara-A, administered ip, exhibited excellent activity against a variety of human tumor xenografts implanted sc in athymic nude or scid (LNCAP) mice. All of the eight human colon tumors tested were sensitive to the compound (see Table 3), with COLO 205, HCC-2998 (curative), HCT-15, and KM20L2 being the most sensitive. Three of the four renal tumors tested exhibited excellent sensitivity to the compound (see Table 4), with tumor growth delays of at least 200% and an occasional tumor-free survivor. The RXF 393 renal tumor was minimally responsive to the compound. The four nonsmall cell lung tumors tested were moderately sensitive to Cl-F-ara-A (see Table 4). Of the three

TABLE 1. Cytotoxicity of Cl-F-Ara-A and Fludarabine Phosphate in Nine Human Cell Lines

Cell Line	IC_{50}[a] (μM)	
	Cl-F-Ara-A	Fludarabine Phosphate
ACHN renal carcinoma	0.11	6.4
CAKI-1 renal carcinoma	0.29[b]	41[b]
SNB-7 CNS tumor	0.29	54
NCI-H23 nonsmall cell lung adenocarcinoma	0.29	45
DLD-1 colon adenocarcinoma	11	70
SK-MEL-28 melanoma	0.67	39
K-562 chronic myelogenous leukemia	0.028[b]	0.58[b]
CCRF-CEM acute lymphoblastic leukemia	0.15[b]	0.29[b]
WI-38 normal fibroblasts	6.8[b]	5.1[b]

[a] IC_{50} is defined as the concentration of drug inhibiting the growth of cells after 72 h to one-half that observed in the absence of drug. The values listed are the average of two or more determinations except where noted.

[b] Single determination.

human prostate tumor lines tested, PC-3 exhibited excellent sensitivity, whereas DU-145 and LNCAP were moderately sensitive.

For comparison, the *in vivo* activity of fludarabine phosphate (using an optimal treatment schedule) against the same murine and human solid tumors is shown in Tables 3-5. Cl-F-ara-A exhibited a greater spectrum of activity than fludarabine phosphate. Whereas the two compounds showed comparable activity in the two murine solid tumors, in three of the four human renal tumors, and in all of the three human prostate tumors, Cl-F-ara-A was clearly superior to fludarabine phosphate in the human colon and nonsmall cell lung tumors.

Cross-Resistance of Drug-Resistant P388 Leukemias to Cl-F-Ara-A. The cross-resistance profile of six drug-resistant P388 sublines to Cl-F-ara-A is shown in Table 6.

TABLE 2. Response of IP Implanted P388 Leukemia to Cl-F-Ara-A

Schedule	Route	Dosage range (mg/kg/dose)	Optimal dosage ($\leq LD_{10}$, mg/kg/dose)	Total dose (mg/kg/dose)	Median % ILS[a] (dying mice only)	Net $\log_{10}$ cell kill[b]	Tumor-free survivors/total
Days 1-5	ip	300-100	200	1000	+59	+1.6	0/3
q3h x 8, Days 1, 5, 9	ip	30-10	20	480	+220	+6.6	1/6
q4h x 3, Days 1-9	ip	20-8.9	20	540	+300	+6.0	3/6
q6h x 4, Days 1, 5, 9	po	150-45	67	804	+104	+1.7	0/6

[a] Median day of death of tumored control mice was 10-11 days.

[b] Net $\log_{10}$ reduction in the tumor cell population between the beginning and the end of therapy, based on the median day of death of the mice that died.

TABLE 3. Response of SC Implanted Colon Tumors to Cl-F-Ara-A and Fludarabine Phosphate

Tumor	Schedule	Cl-F-Ara-A			Fludarabine Phosphate		
		Optimal ip dosage (mg/kg/dose)	Growth delay [(T-C)/C, %]	Tumor-free survivors/total	Optimal ip dosage (mg/kg/dose)	Growth delay [(T-C)/C, %]	Tumor-free survivors/total
murine 36	Day 1 q4h x 3, Days 1-9 q4h x 3, Days 14-22	210[a] 20(LD_{17}) 20	27[b] —[c] 788[d]	1/6 5/6 5/6	420[a] 90 90	28[b] —[c] 761	1/6 6/6 5/6
human COLO 205	q4h x 3, Days 9-17	20	>325[d]	1/6	90	>325[d]	1/6
human DLD-1	q4h x 3, Days 11-19	20	181[d]	1/6	60	21[d]	0/6
human HCC-2998	q4h x 3, Days 9-17	20	—[c]	6/6	90	262[d]	1/6
human HCT-15	q4h x 3, Days 8-16	20	356[e]	1/6	90	101[e]	0/6
human HCT-116	q4h x 3, Days 6-14	20	198[d]	0/6	90	48[d]	0/6
human HT29	q4h x 3, Days 15-23	20	164[d]	2/6	90	81[d]	0/6
human KM20L2	q4h x 3, Days 16-24	20	310[d]	0/6	90	150[d]	0/6
human SW-620	q4h x 3, Days 6-14	13.3	179[f]	0/6	90	67[f]	0/6

[a] Highest dosage tested.
[b] Using 100 and 200 mg as the evaluation sizes.
[c] Unevaluable (none of the surviving mice had tumors).
[d] Using two doublings in tumor mass as the evaluation size.
[e] Using three doublings in tumor mass as the evaluation size.
[f] Using four doublings in tumor mass as the evaluation size.

TABLE 4. Response of SC Implanted Human Renal and Lung Tumors to Cl-F-Ara-A and Fludarabine Phosphate

Tumor	Schedule	Cl-F-Ara-A			Fludarabine Phosphate		
		Optimal ip dosage (mg/kg/dose)	Growth delay [(T-C)/C, %]	Tumor-free survivors/total	Optimal ip dosage (mg/kg/dose)	Growth delay [(T-C)/C, %]	Tumor-free survivors/total
renal A498	q4h x 3, Days 7-15	13.3[a]	200[b]	1/6	90	110[b]	0/6
	q4h x 3, Days 13-21	20	245[c]	0/6	90	73[c]	0/6
renal CAKI-1	q4h x 3, Days 16-24	20	307[b]	1/6	90	229[b]	0/6
	q4h x 3, Days 19-27	13.3	>535[b]	1/6	90	>535[b]	1/6
renal RXF 393	q4h x 3, Days 6-14	13.3[a]	70[c]	0/6	90 (LD_{17})	59[c]	0/6
	q4h x 3, Days 6-14	20	35[b]	0/6	90	13[b]	0/6
renal SN12C	q4h x 3, Days 14-22	13.3[a]	252[c]	1/6	90 (LD_{33})	>296[c]	2/6
	q4h x 3, Days 14-22	13.3	>187[b]	2/6	90	>187[b]	3/6
lung A549	q4h x 3, Days 12-20	13.3	165[b]	0/6	90	91[b]	0/6
lung NCI-H23	q4h x 3, Days 16-24	30	61[b]	0/6	—	—	—
	q4h x 3, Days 19-27	20 (LD_{17})	12[b]	0/6	90	-57[b]	1/6
	q4h x 3, Days 14-22	20	146[b]	0/6	90	37[b]	0/6
lung NCI-H322M	q4h x 3, Days 23-31	20	65[d]	0/6	90	27[d]	0/6
lung NCI-H460	q4h x 3, Days 7-15	20	127[d]	0/6	90	22[d]	0/6

[a] Highest dosage tested.
[b] Using two doublings in tumor mass as the evaluation size.
[c] Using three doublings in tumor mass as the evaluation size.
[d] Using four doublings in tumor mass as the evaluation size.

TABLE 5. Response of SC Implanted Prostate and Mammary Tumors to Cl-F-Ara-A and Fludarabine Phosphate

Tumor	Schedule	Cl-F-Ara-A			Fludarabine Phosphate		
		Optimal ip dosage (mg/kg/dose)	Growth delay [(T-C)/C, %]	Tumor-free survivors/ total	Optimal ip dosage (mg/kg/dose)	Growth delay [(T-C)/C, %]	Tumor-free survivors/ total
human prostate DU-145	q4h x 3, Days 11-19	20	116[b]	0/6	90	74[b]	0/6
human prostate LNCAP	q4h x 3, Days 12-20	13.3	98[a]	0/6	90	108[a]	0/6
human prostate PC-3	q4h x 3, Days 9-17	13.3	497[b]	0/6	90	470[b]	0/6
murine mammary 16/C	Days 1-9 q4h x 3, Days 1-9	67 20	25[c] 88[c]	0/6 0/6	200 60	58[c] 35[c]	0/6 0/6

[a] Using one doubling in tumor mass as the evaluation size.
[b] Using two doublings in tumor mass as the evaluation size.
[c] Using 500 and 1000 mg as the evaluation sizes.

TABLE 6. Activity of Cl-F-Ara-A against Drug-resistant P388 Leukemias *In Vivo*

			Therapeutic response						
			Parental P388 Leukemia			Resistant Leukemia[d]			
Expt. No.	Resistant leukemia[a]	Optimal ip dosage[b] (≤LD_{10}, mg/kg/dose)	Median % ILS	Net $\log_{10}$ cell kill[c]	60-Day survivors/ total	Median % ILS	Net $\log_{10}$ cell kill[c]	60-Day survivors/ total	Cross-resistance?
1	P388/ADR	13.3	+252	+6.6	2/5	+170	+6.8	0/5	No
3	P388/VP-16	13.3	+240	+7.0	1/5	+107	+4.1	0/5	Marginal
1	P388/Taxol	13.3	+252	+6.6	2/5	+162	+6.6	3/5	No
3	P388/L-PAM	13.3	+240	+7.0	1/5	–	–	5/5	No
2	P388/ARA-C	13.3	+305	+6.7	3/5	–30	–2.3	0/5	Yes
2	P388/MTX	13.3	+305	+6.7	3/5	+287	+6.4	3/5	No

[a] ADR = doxorubicin, VP-16 = etoposide, Taxol = paclitaxel, L-PAM = melphalan, ARA-C = 1-β-D-arabinofuranosylcytosine, MTX = methotrexate.

[b] Cl-F-ara-A was administered every 4 h for 3 injections on days 1-9 using an injection volume of 0.25 mL/10 g animal body weight.

[c] Net $\log_{10}$ reduction in the tumor cell population between the beginning and the end of therapy, based on the median day of death of mice that died.

[d] In these studies, the degree of resistance of a drug-resistant subline in comparison to the parental line was as follows: ADR, 5-$\log_{10}$ units; VP-16, 9-$\log_{10}$ units; Taxol, 2-$\log_{10}$ units; L-PAM, 7-$\log_{10}$ units; ARA-C, 8-$\log_{10}$ units; and MTX, 2-$\log_{10}$ units.

Treatment of ADR-, VP-16-, Taxol-, L-PAM-, ARA-C-, and MTX-resistant P388 leukemias with the compound resulted, with few exceptions, in net cell kills comparable to that obtained by treating the parental sensitive line. However, treatment of VP-16-resistant P388 leukemia with the compound resulted in approximately 3 $\log_{10}$ units less of cell kill than observed with the parental P388 leukemia, indicating marginal cross-resistance. As expected, the compound was ineffective against ARA-C-resistant P388 leukemia. Treatment of ARA-C-resistant P388 leukemia with the compound resulted in approximately 9 $\log_{10}$ units less of cell kill than observed with the parental P388 leukemia, indicating marked cross-resistance.

DISCUSSION AND CONCLUSIONS

In recent years three nucleoside analogs (cladribine, fludarabine phosphate, and gemcitabine) have received FDA approval for the treatment of cancer. We have a continuing program designed to look for nucleosides with improved properties, agents that might have either an increased selectivity or a broader application to different types of tumors. Toward that end, a series of analogs of fludarabine were prepared. Cl-F-ara-A appeared to have the best antitumor effect in initial screens. The compound was evaluated against a spectrum of tumor systems in culture and in mice. Cl-F-ara-A exhibited significant cytotoxicity against a variety of human tumor cell lines. More importantly, the compound showed selectivity in the *in vivo* tumor studies. All of the human colon tumors tested were sensitive to the compound, with cures being observed in at least one-half of the models. Similarly, three of the four renal tumors tested exhibited excellent sensitivity to the compound, with cures being observed. The nonsmall cell lung and prostate tumors tested were sensitive to the compound, albeit at a reduced level.

This broad spectrum of *in vivo* antitumor activity suggests that Cl-F-ara-A should be considered seriously for advancement to clinical trials. Also noteworthy is the oral activity of Cl-F-ara-A. Studies by Takahashi and co-workers have shown that the compound was extremely effective against four human colon tumor xenografts (HT29, WiDr, Co-3, and COLO-320DM), the LX-1 human lung tumor xenograft, and the MX-1 human breast tumor xenograft when administered orally using a 5-day daily treatment schedule.[10] There is concern that Cl-F-ara-A will exhibit the same type of interspecies differences in maximum tolerated dosage (MTD) between humans and mice as observed

for fludarabine phosphate and cladribine[11, 12] and therefore will not exhibit the level of antitumor activity in humans observed in the xenograft models. Encouraging is the fact that Takahashi and co-workers have shown potent antitumor activity of Cl-F-ara-A at oral dosages one-sixteenth of the MTD using a 10-day daily treatment schedule.[10]

Even though Cl-F-ara-A exhibited superior activity to fludarabine phosphate in the human colon and nonsmall cell lung tumors, fludarabine phosphate did exhibit a broad spectrum of *in vivo* activity in the colon, renal, and prostate tumor lines. Because fludarabine phosphate has FDA approval, consideration should be given to evaluating the compound in one or more of these three tumor types. However, the large difference in MTD for fludarabine phosphate between humans and mice may prevent the observation of efficacy in humans.[11]

We have shown that Cl-F-ara-A is phosphorylated via deoxycytidine kinase to the corresponding triphosphate, which potently inhibits ribonucleotide reductase as an allosteric regulator.[3] Cl-F-ara-A also serves as a substrate for DNA polymerase α, and its incorporation into the 3'-end of the growing DNA chain causes disruption of further DNA synthesis. Because Cl-F-ara-A competes with dATP for incorporation into DNA, the decline in dNTP levels (including dATP) caused by the inhibition of ribonucleotide reductase potentiates the activity of Cl-F-ara-A against DNA polymerases. These findings are similar to those observed for fludarabine phosphate and cladribine; however, the degree of inhibition of ribonucleotide reductase and DNA polymerase α by the triphosphates of these nucleosides is quite different. The inhibition of ribonucleotide reductase by Cl-F-ara-A and cladribine are similar and greater than that observed with fludarabine phosphate. The inhibition of DNA polymerase α by Cl-F-ara-A and fludarabine phosphate are similar and greater than that observed with cladribine. Therefore, Cl-F-ara-A incorporates the best properties of both fludarabine phosphate and cladribine.

As new agents enter phase II and III clinical trials, the selection of patients, most of whom have been treated previously with one or more drugs, may be critical to the success of the trials.[13] Information on the patterns of cross-resistance among various antitumor agents may be helpful in the selection of patients for treatment with Cl-F-ara-A. For these trials, it may be important to exclude or to monitor with extra care patients who have been treated previously with either ara-C or etoposide. The observation of a lack of cross-resistance of P388/ADR, P388/Taxol, P388/L-PAM, and P388/MTX leukemias to

Cl-F-ara-A suggests that a combination of doxorubicin, paclitaxel, melphalan, or methotrexate with Cl-F-ara-A might exhibit antitumor activity greater than that observed for the corresponding single agents.

On the basis of these results, Cl-F-ara-A appears to have a profile distinct from other nucleoside antitumor agents and warrants serious consideration for advancement to clinical trials.

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