A Comparative Study of the Antitumor and Antiviral Activity of 1-β-D-Arabinofuranosyl-5-fluorocytosine (FCA) and 1-β-D-Arabinofuranosylcytosine (CA) (33724)

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1-β-D-Arabinofuranosyl-5-fluorocytosine (FCA, ara-FC) was synthesized by Fox *et al.* (1) and subsequently evaluated for antileukemic activity in mice (2). These studies showed that FCA was superior to 1-β-D-arabinofuranosylcytosine (CA, ara-C) against leukemias P815 and P388 but was roughly equivalent in activity to CA when administered to mice bearing leukemias L-1210 and B82; both substances had equivalent effects against growing cell cultures of the P815 and P388 tumors *in vitro*. FCA was shown to produce unbalanced growth in HeLa cell cultures with regard to the ability to inhibit DNA synthesis without concurrent inhibition of the synthesis of either RNA or protein, in a manner similar to CA and 5-fluorodeoxyuridine (FUDR) (3). Since CA inhibits experimental tumors other than the murine leukemias (4) and is an effective chemotherapeutic agent in herpes simplex keratitis of the rabbit (5), it was felt that a more complete evaluation should be made of FCA with respect to additional transplanted tumors as well as both DNA and RNA viruses. Accordingly, FCA and CA were directly compared as to antitumor and antiviral activity in a series of experimental models.

Materials and Methods. Antitumor tests. The arabinosyl nucleosides were dissolved in water and tested against the following transplanted tumors by methods described in detail in previous publications (6, 7): leukemia L-1210, ascitic form (10^5 cells injected intraperitoneally into semi-isologous BDF_1 mice with survival recorded for 30 days), sarcoma 180 (tumor fragments implanted subcutaneously by trocar into albino mice with tumors excised and weighed at 8 days), Ehrlich carcinoma, solid form (5×10^6 cells injected subcutaneously into albino mice with tumors excised and weighed at 8 days), Ehrlich carcinoma, ascitic form (1×10^6 cells injected intraperitoneally into albino mice with survival recorded for 30 days), mammary adenocarcinoma EO 771 (tumor fragments implanted subcutaneously by trocar into C57 mice with tumors excised and weighed at 14 days), Walker carcinosarcoma 256 and Flexner-Jobling carcinoma (tumor fragments implanted subcutaneously by trocar into albino rats with tumors excised and weighed at 14 days).

In addition to the conventional leukemia L-1210 ascites experiments in BDF_1 mice, FCA and CA were evaluated as antileukemic agents against the solid form of this tumor. Solid tumors were produced by the implantation subcutaneously of approximately 3×10^6 ascitic tumor cells into either compatible BDF_1 mice or as a homograft into incompatible albino mice. In the former case, one group of mice were sacrificed after 8 days and the weight of the treated tumors compared to control tumors; a second group was allowed to live after completion of treatment and survival times were recorded up to 30 days. In the latter case, all mice were sacrificed 8 days after implantation and the tumors were excised and weighed as for the Ehrlich solid and S-180 experiments.

For the ascitic tumors, a 50% or greater increase in survival time ($T/C = 1.5$ or more) was considered to represent an antitumor effect. For the solid tumors, a tumor weight of 50% or less of the untreated controls ($C/T = 2.0$ or more) was considered to represent an antitumor effect.

All animals received their first treatment shortly after implantation and then were treated once daily. The mice bearing solid tumors were treated 8 or 14 times as indicated and the rats bearing the Walker-256 and Flexner-Jobling tumors were treated 14

times. Mice bearing ascitic tumors were treated 8 or 15 times. Mice bearing leukemia L-1210 in ascitic form were examined postmortem for gross signs of leukemia.

Antiviral tests. In vivo. The following viruses were used to infect mice: Columbia SK and herpes, Nutley strain (mouse brain homogenates), Coxsackie virus B1, GP strain (rhesus money kidney cell culture supernatant) and influenza A, PR8 (chick allantoic fluid). All mice were infected with approximately 10 LD_{50} of virus. Albino mice (Marland strain) weighing 9–12 g were infected intraperitoneally with Columbia SK virus and herpesvirus as previously described (8). Adult albino mice (CD-1 strain) weighing 18–20 g were infected intraperitoneally with Coxsackie virus B1 (9). Albino mice (Marland strain) weighing 9–12 g were infected intranasally with influenza A virus (PR8 strain) (10). All infected mice were treated by the intraperitoneal route according to dosage schedules described in an earlier publication (11).

Rabbits weighing approximately 2 kg were employed in experiments with herpes simplex keratitis. In these experiments, the eyes of the rabbits were scarified in crosshatch with a scalpel three times in each direction. Two drops of a 1:10 dilution of a rabbit kidney cell culture of herpes simplex (Smith strain) in saline were placed in each eye and treatment started 1 hr after infection. The drugs were instilled into each eye by administering two drops of a saline solution containing 1 mg/ml of the substance under test 3 times daily for 5 days and then twice daily for 2 days. The eyes were examined daily for evidence of keratitis. The results are expressed as percentage protection determined from the number of eyes which were free of infection after 21 days.

In vitro. The FCA and CA were dissolved in medium 199 and added to established tubes of rhesus monkey kidney cells incubated stationary at 36° and observed daily for cytotoxicity. The maximum tolerated tissue culture dose was determined at 3 and 7 days. The concentration in μg/ml tolerated at 3 days was employed in tests with the PR8 strain of influenza A virus and the concentration tolerated at 7 days was employed in tests with Columbia SK, Coxsackie virus B1 and vaccinia. The FCA and CA were added to the cell cultures immediately before inoculation with the virus under test and left in contact throughout the 3- or 7-day incubation period. Comparative infectivity titrations were performed in a parallel series of tubes with and without the antimetabolites. At the end of the incubation period the $TCID_{50}$ values were calculated using the method of Reed and Muench (12). A difference of 2 logs or more between the treated and the control cell cultures was taken to indicate an antiviral effect. When such a difference occurred the substance was titrated for antiviral activity by serial 2-fold dilutions against 10 $TCID_{50}$ of virus and the activity described in terms of the dose in μg/ml that protected 50% of the cultures (PD_{50}).

a. Influenza A virus. Tubes of African green monkey kidney cells previously grown in the presence of SV_5 antiserum were fed with Eagle's basal medium (Hank's base) supplemented with glutamine. Chlorioallantoic fluid from chick embryos was diluted in Eagle's basal medium and the various dilutions inoculated into the tubes (0.2 ml into each of 4 tubes/dilution). The cell cultures were incubated stationary for 3 days at 36°. After this time the fluid was poured off and to each tube was added 0.2 ml of a 0.4% suspension of washed guinea pig erythrocytes. The tubes were incubated for 20 min at 4° and then observed microscopically for hemadsorption. The $TCID_{50}$ values based upon this criterion were then calculated.

b. Columbia SK virus. The strain of Columbia Sk virus employed produced neither cytopathic effects (CPE) nor hemadsorption. Accordingly, cultures of rhesus monkey kidney cells fed with medium 199 (Earle's base) were incubated for 7 days in the presence of the nucleosides and various dilutions of virus (0.1 ml/tube) obtained from brain homogenates of paralyzed mice. After this time, the presence of virus was determined by intraperitoneal infection of 9–12 g albino mice with 0.1 ml of undiluted

TABLE I. Effect of FCA and CA against the Ascitic Form of Leukemia L-1210 in BDF_1 Mice.[a]

	T/C index[b]			
	8 Treatments		15 Treatments	
Dose (mg/kg i.p.)	FCA	CA	FCA	CA
20.0	2.64 (5/10)	2.79 (5/10)	2.68 (3/8)	1.82 (2/8)
10.0	2.73 (5/10)	2.85 (7/10)	1.78 (6/8)	2.35 (3/8)
5.0	2.49 (4/10)	1.98 (1/10)	2.69 (4/8)	2.84 (4/8)
2.0	1.89 (1/10)	1.24 (0/10)	2.08 (1/8)	1.53 (0/8)
1.0	1.37 (0/10)	1.09 (0/10)	1.91 (1/8)	1.05 (0/8)
0.5	1.18 (0/10)	1.07 (0/10)	1.25 (0/8)	0.95 (0/8)
0.25	1.07 (0/10)	0.95 (0/10)	0.98 (0/8)	0.85 (0/8)
Total no. of 30-day survivors/total no. of animals	15/70	13/70	15/56	9/56

[a] 10^5 cells intraperitoneally.

[b] *T/C* index = mean survival time in days of treated mice/mean survival time in days of control mice (no. of 30-day survivors in parentheses); a *T/C* value of 1.5 or more considered to represent an antitumor effect.

tissue culture fluid. Death of the animals was taken to indicate presence of virus in the cell cultures. The $TCID_{50}$ values based upon this end point were then calculated.

c. Coxsackievirus B1 (GP strain). Rhesus monkey kidney cell cultures fed with medium 199 were employed. The tubes were inoculated with various dilutions of virus (0.1 ml/tube) obtained from pooled monkey kidney cell cultures that had been infected with mouse liver homogenates (13). The $TCID_{50}$ values based upon CPE were calculated after an incubation period of 7 days.

d. Vaccinia virus. The virus was propagated in rhesus monkey kidney cell cultures fed with medium 199 and supplemented with horse serum to a final concentration of 10%. Tubes were inoculated with various dilutions of virus (0.1 ml/tube) and observed daily for CPE. Calculations of the $TCID_{50}$ were made on the basis of the degree of CPE detected at 7 days.

Results. Transplanted tumors. The results of experiments in which FCA and CA were administered intraperitoneally to BDF_1 mice bearing the ascitic form of leukemia L-1210 are summarized in Table I. Table I reveals that both of the arabinosyl nucleosides were similar in the degree of protection afforded to BDF_1 mice, the fluorinated derivative being the slightly more active of the two. The FCA also showed a tendency to superiority to CA in the 15-treatment group with regard to total survivors at 30 days (15/56 = 27% vs 9/56 = 16%). Amethopterin was active in this experimental model at doses of 1 and 2 mg/kg i.p. × 8 without producing any survivors at 30 days. The FUDR was inactive at 50 mg/kg i.p. × 8.

The FCA and CA were also tested for their activity against the solid form of leukemia L-1210. A series of experiments were performed in which the substances were administered to compatible BDF_1 and incompatible albino mice bearing the subcutaneous form of the L-1210 tumor. In the BDF_1 mice, tumor size at 8 days as well as survival time were studied. In the albino mice, which normally reject this tumor after an initial period of growth, the effects on tumor weight at 8 days were determined. The results of the BDF_1 and of the homograft experiments are summarized in Tables II and III, respectively.

Table II shows that a difference existed between the 2 substances when tested against the solid L-1210 tumor in BDF_1 mice. Employing criterion of a 50% or more increase in survival time (*T/C* = 1.5 or more), the minimum effective dose for FCA was 1.25 mg/kg or less as opposed to 5.0 mg/kg in the

TABLE II. Effect of FCA and CA against the Solid Form of Leukemia L-1210 in BDF_1 Mice.[a]

Dose (mg/kg i.p. × 8)	T/C index[b] FCA	CA
25.0	2.4 (15/18)	2.6 (11/24)
12.5	2.7 (17/20)	2.9 (17/24)
6.25	2.5 (13/23)	2.4 (11/24)
5.0	3.4 (14/24)	2.1 (4/24)
2.5	3.3 (14/24)	1.4 (1/24)
1.25	2.2 (7/24)	1.3 (1/24)
Total no. of 30-day survivors/total no. of mice	80/133 = 60%	45/144 = 31%

[a] Approximately 3×10^6 cells implanted subcutaneously.

[b] T/C index = mean survival time in days of treated mice/mean survival time in days of control mice observed 30 days for survival (30-day survivors in parentheses); a T/C value of 1.5 or more considered to represent an antitumor effect.

case of CA. The greater overall increase in survival time of mice treated with FCA was also reflected in the greater number of 30 day survivors or apparent "cures," 80/133 = 60% for FCA as opposed to 45/144 = 31% for CA ($p<.001$).

Employing the criterion of 50% or more inhibition of tumor growth in BDF_1 mice sacrificed at 8 days (C/T = 2.0 or more), the minimum effective dose for FCA was 2.5 mg/kg as opposed to 12.5 mg/kg for CA. The results summarized in Table III show that that minimum effective dose of FCA against the L-1210 homograft was 3.12 mg/kg as opposed to a value of 12.5 mg/kg for CA.

Table IV gives the results of experiments with the sarcoma-180, Ehrlich solid and EO 771 tumors in mice. The data in Table IV show that the sarcoma-180 tumor was somewhat more sensitive to FCA than to CA. Both compounds produced equivalent degrees of inhibition against the Ehrlich and EO 771 carcinomas but only at the highest tolerated dose. Both substances were inactive when administered orally to mice bearing the ascitic

TABLE IV. Effect of FCA and CA against Sarcoma-180, Ehrlich Solid Carcinoma and Mammary Adenocarcinoma EO 771 in Mice.

Dose (mg/kg i.p.)	C/T index[a] S-180[b] FCA	CA	Ehrlich solid[b] FCA	CA	EO 771[c] FCA	CA
40–50	16.7	10.9	2.4	2.9	Toxic	Toxic
20–25	5.1	2.1	1.7	1.9	4.1	2.7
10–12.5	2.1	1.4	1.4	0.9	1.3	0.7
6.25	1.3	1.4	—	—	—	—

[a] C/T value of 2.0 or more considered to represent antitumor effect.

[b] Eight treatments.

[c] Fourteen treatments.

TABLE III. Effect of FCA and CA against the Solid Leukemia L-1210 Homograft in Albino Mice.[a]

Dose (mg/kg i.p. × 8)	FCA C/T index[b]	FCA No. of survivors /no. treated	CA C/T index[b]	CA No. of survivors /no. treated
50.0	462.0	18/24	127.3	5/8
25.0	43.3	38/40	14.2	24/24
12.5	23.5	28/32	3.9	21/24
6.25	8.4	30/32	1.7	15/16
3.12	2.8	16/16	1.0	8/8
2.5	1.4	8/8	1.1	8/8
1.25	1.4	8/8	1.3	8/8

[a] Approximately 3×10^6 cells implanted subcutaneously.

[b] C/T index = average weight of control tumors/average weight of treated tumors in mice sacrificed 8 days after implantation; a C/T value of 2.0 or more considered to represent an antitumor effect.

TABLE V. Effect of FCA and CA against Walker Carcinosarcoma 256 in Albino Rats.

Dose (mg/kg i.p. × 14)	FCA		CA	
	C/T index[a]	No. of survivors /no. treated	*C/T* index[a]	No. of survivors /no. treated
160	26.7	8/8	3.1	8/8
80	4.5	8/8	1.9	7/8
40	2.7	8/8	1.2	8/8
20	3.2	8/8	1.8	8/8
10	1.3	8/8	1.4	7/8

[a] *C/T* value at 2.0 or more considered to represent an antitumor effect.

form of the Ehrlich carcinoma at a dose of 62.5 mg/kg.

Table V summarizes the comparative antitumor effect of the substances against the Walker-256 tumor in adult rats employing doses as high as 160 mg/kg, since both FCA and CA were better tolerated in rats than in mice. The data in Table V reveal that a major difference exists between FCA and CA with regard to activity against the Walker carcinosarcoma 256 in rats. The inactivity of CA at doses of 80 mg/kg or less is in agreement with the findings of Evans *et al.* (4). However, at a dose of 160 mg/kg, CA exerted a detectable effect. In comparison, FCA exerted activity at a dose as low as 20 mg/kg. Neither arabinosyl nucleoside produced an antitumor effect against the Flexner-Jobling carcinoma in rats at the highest dose tested (80 mg/kg).

Antiviral tests in vivo. FCA and CA were active against only a single viral experimental model, herpes simplex keratitis in the rabbit. These data are summarized in Table VI. FCA and CA showed similar degrees of activity against herpes keratitis. FCA and CA were without antiviral effect when administered intraperitoneally to mice infected with the following RNA-containing viruses: Columbia SK, Coxsackie virus B1 (GP) and influenza A (PR8).

Antiviral tests in vitro. Table VII shows the degree of cytotoxicity produced by FCA and CA when incubated with established normal mammalian cell cultures. From the comparative point of view, FCA was substantially less toxic than CA to both monkey and rabbit kidney cells on continuous exposure. The low order of toxicity of both substances is in contrast to the rather marked effects (50% inhibition at <1.0 μg/ml) reported by other investigators when these substances were tested against proliferating malignant cells in culture (2, 3). Dollinger *et al.* reported that the highly sensitive Burkitt's tumor and leukemia L-1210 cells lack the ability to deaminate CA *in vitro*, whereas both CA and FCA are rapidly deaminated to the corresponding inactive uracil nucleosides by both human liver and mouse kidney enzyme systems *in vitro* (14). The disparity between high cytotoxicity for malignant cells *in vitro*, and the low degree of cytotoxicity for normal resting mammalian cells reported here, may

TABLE VI. Effect of FCA and CA against Experimental Herpes Simplex Keratitis in Rabbits.

Drug[a]	Duration of treatment (days)	No. of rabbits	No. of eyes negative at 21 days/no. of eyes infected[b]	Percentage negative
FCA	5	11	12/22	55
CA	5	12	10/24	41
Saline	5	7	1/14	7

[a] One mg/ml solution; 2 drops t.i.d. for 5 days then 2 drops b.i.d. for 2 days.

[b] Both eyes scarified and infected with 2 drops of tissue culture fluid.

TABLE VII. Cytotoxicity[a] of FCA and CA after Incubation (7 days) with Normal Mammalian Cells.

	Cell line[b]			
	Rhesus monkey kidney cells		Rabbit kidney cells	
Drug	Toxic dose (μg/ml)	Tolerated dose (μg/ml)	Toxic dose (μg/ml)	Tolerated dose (μg/ml)
FCA	>4000	4000[c]	2000	1000
CA	250	125[d]	500	250

[a] Based on appearance of extensive granulation, vacuolization, rounding and disintegration.
[b] Established tube cultures fed with mixture 199 with Earle's base.
[c] This dose and 2000 μg/ml cytotoxic at 10 days.
[d] 500 μg/ml tolerated at 3 days.

possibly be explained by either the preferential lethality of these antimetabolites for rapidly growing cells and/or inactivation by normal cells through deamination of the cytosine moiety in both molecules. Additional work would be required to elucidate this point further.

Of the various viruses tested under *in vitro* conditions, only vaccinia was sensitive to these agents, as might be expected from the nature of its nucleic acid. FCA at a dose of 1000 μg/ml and CA at a dose of 125 μg/ml both produced Δ $TCID_{50}$ values between treated and control tubes of >1.7 logs. When both substances were titrated for their antiviral end point against 10 $TCID_{50}$ of vaccinia virus, FCA and CA gave PD_{50} values of 0.975 and 1.55 μg/ml, respectively. These values indicate that the effect against vaccinia virus was marked and essentially similar for both substances. Furthermore, if the monkey kidney cells deaminated these two substances, sufficient unaltered antimetabolite was left to prevent the host cell synthesis of viral DNA.

FCA and CA were inactive in tissue culture against the following RNA-containing viruses: Columbia SK, Coxsackie virus B1 (GP), and influenza A (PR8).

Discussion. The literature to date on the antitumor activity of FCA indicates that it is similar or slightly superior to CA with regard to ascitic murine leukemias. The present report confirms the finding in the ascitic leukemia L-1210 model and extends these data to show that with nonleukemic tumors such as sarcoma-180, Ehrlich carcinoma and mammary adenocarcinoma EO 771, FCA is similar or slightly superior to CA. Both arabinosyl nucleosides were inactive against Flexner-Jobling carcinoma. The present report also shows that both FCA and CA are equivalent in their effectiveness *in vivo* against herpes simplex virus and *in vitro* against vaccinia virus. Not unexpectedly, both substances were inactive *in vivo* and *in vitro* against all RNA viruses tested.

However, when one studies the solid form of leukemia L-1210 in mice and the Walker carcinosarcoma 256 in rats, appreciable differences in the antitumor effectiveness of these two substances can be detected.

With a tumor such as leukemia L-1210, one that is highly sensitive to CA, the addition of fluorine produced a marked quantitative difference in regard to inhibition of the solid forms. Thus, FCA was 4–5 times more active on a mg/kg basis than CA. With Walker 256, a tumor of low sensitivity to CA, the addition of fluorine produced an even greater difference, FCA being 8 times more active than CA.

With regard to its mode of action, FCA, like CA, inhibits deoxycytidilate synthetase *in vitro*, and thus blocks the reduction of cytidine to deoxycytidine (2, 3). Although replacement of a hydrogen atom at the 5-position of the cytosine moiety by the more electronegative fluorine atom does not alter its site of action, it enhances its activity

against certain transplanted tumors. Whether FCA in these tumor systems enters the cells more readily, has a greater avidity for the enzyme that it inhibits, or is deaminated by the host animals to a lesser extent, is not known.

Summary. FCA was similar or slightly superior in activity to CA with regard to antitumor effects when tested against the following experimental tumors: ascitic leukemia L-1210, sarcoma-180, Ehrlich solid carcinoma and mammary adenocarcinoma EO 771. FCA was appreciably more active on a mg/kg basis than CA with regard to its effect against the solid forms of leukemia L-1210 in both semi-isologous BDF_1 and homologous albino mice, as well as against Walker carcinosarcoma 256. Both substances were without effect against the Flexner-Jobling carcinoma in rats. FCA and CA exerted similar degrees of antiviral activity against herpes simplex keratitis in the rabbit and vaccinia virus in rhesus monkey kidney cell cultures. Both substances were inactive *in vivo* and *in vitro* against the following RNA-containing viruses: Columbia SK, Coxsackie virus B1 (GP), and influenza A (PR8).

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