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5-Fluorouracil and Derivatives in Cancer Chemotherapy III: *In Vivo* Enhancement of Antitumor Activity of 5-Fluorouracil (FU) and 5-Fluoro-2'-deoxyuridine (FUDR)

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Abstract □ The antitumor activity of 5-fluorouracil (FU) and 5-fluoro-2'-deoxyuridine (FUDR) has been enhanced in mice in the presence of deoxyribose donors such as 2'-deoxyuridine (UDR). Based upon *in vitro* data, it appears that the enhancement is due to the conversion of 5-fluorouracil to 5-fluoro-2'-deoxyuridine in blood and the stabilization of 5-fluoro-2'-deoxyuridine due to the presence of 2'-deoxyuridine.

Keyphrases □ 5-Fluorouracil (FU)—antitumor activity enhanced by deoxyribose donors (2'-deoxyuridine), mice □ 5-Fluoro-2'-deoxyuridine (FUDR)—antitumor activity enhanced by deoxyribose donors (2'-deoxyuridine), mice □ Antitumor activity, 5-fluorouracil (FU) and 5-fluoro-2'-deoxyuridine (FUDR)—enhanced in presence of deoxyribose donors (2'-deoxyuridine), mice □ Cancer chemotherapy—enhancement of 5-fluorouracil (FU) and 5-fluoro-2'-deoxyuridine (FUDR) activity by deoxyribose donors (2'-deoxyuridine), mice

A previous article (1) reported the *in vivo* stabilization of 5-fluoro-2'-deoxyuridine (FUDR) and its formation from 5-fluorouracil (FU) in the presence of nucleosides. The speculation was made that these findings could have marked effects on the clinical use of 5-fluorouracil and 5-fluoro-2'-deoxyuridine in cancer chemotherapy. Following the synthesis of 5-fluoro-2'-deoxyuridine (2), it was shown that the nucleoside was 10^3 times as effective as 5-fluorouracil *in vitro* (3). However, the relatively high anticipated effectiveness of 5-fluoro-2'-deoxyuridine as compared to 5-fluorouracil has not been borne out in clinical use. It was suggested (4) that the lower activity observed was due to the *in vivo* cleavage of 5-fluoro-2'-deoxyuridine to the pyrimidine base and the phosphorylated sugar moiety. The stabilization of 5-fluoro-2'-deoxyuridine by 2'-deoxyuridine (UDR) in blood (1) led to the speculation that this approach could lead to a marked clinical enhancement of 5-fluoro-2'-deoxyuridine activity. This article reports the effect of combining 5-fluorouracil and 5-fluoro-2'-deoxyuridine with 2'-deoxyuridine in protecting mice against L-1210 leukemia and adenocarcinoma 755.

EXPERIMENTAL

Materials and Instruments—The following were used: 5-fluoro-

uracil[1], 5-fluoro-2'-deoxyuridine[1], 2'-deoxyuridine[2], deoxycytidine[2] (CDR), and 5-trifluoromethyl-2'-deoxyuridine[3] (F_3TDR). All other substances used were either reagent grade or of the highest purity available.

A spectrophotofluorometer[4], a TLC scanner[5] modified to contain pendrive motor and range offset, and an *x-y* recorder were used.

In Vitro **Analysis of 5-Fluorouracil and 5-Fluoro-2'-deoxyuridine**—Drugs and additives in known initial concentrations in blood were dialyzed and evaporated by the method of Windheuser *et al.* (5). The dry samples were dissolved in a constant volume of ethanol. The samples were applied to a fluorescent TLC plate, which had previously been activated at 110° and ruled to restrict the lateral motion of the spots to within the range of the scanner optic. The components were resolved by multiple-pass discontinuous development (6). The developer for the process was composed of ethyl acetate–ethanol–10% ammonium hydroxide (200:5:2). The dried plates were scanned in the direction of development for fluorescence quenching at an activation maximum of 280 nm. and a fluorescence maximum of 530 nm., using a spectrophotofluorometer[4] with a thin-film scanner attachment. Quantities of substances present in the blood were computed by appropriate comparison of sample and standard peak areas obtained by planimetry. The method exhibited sufficient sensitivity to detect 10 ng. of 5-fluorouracil and 5-fluoro-2'-deoxyuridine.

In Vivo **Mouse Studies—*L-1210 489 Leukemia*—A** standardized suspension prepared from 7-day-old tumors from BDF₁ mice was injected intraperitoneally. The volume was adjusted to provide 1 × 10⁶ cells/injection. Twenty-four hours after the transplant, the mice were divided into groups of 10 and each group was injected intraperitoneally for 7 days with one of the drug regimens listed in Table I.

The number of mice per group that died from the leukemia, as confirmed by autopsies, was recorded daily until all animals were dead. An average lifespan, that is, the average number of days that mice in each group survived after tumor transplant, was computed. The T/C value, which is the ratio of the lifespan of the experimental group to that of the control group, was calculated. An increase in lifespan representing the percent increase of the lifespan of each group relative to the control was determined from the T/C (Table I). The increase in lifespan is used as a measure of the effectivness of the drug regimen.

Adenocarcinoma 755—Donor BDF₁ mice bearing 10-day-old tumors were sacrificed, and the tumors were removed with sterile surgical instruments inside a tissue culture hood. Then the tumors

[1] Hoffmann–La Roche, Nutley, N. J.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Supplied by Ben Venue Laboratories, Bedford, Ohio, through Dr. William Wolberg, University of Wisconsin Hospitals, Madison, Wis.
[4] Aminco model 9-8106, American Instrument Co.
[5] American Instrument Co.

Table I—Effect of Various Drug Regimens on Lifespan of Mice with L-1210 Leukemia

Drug Regimen, mg./kg./day	Average Lifespan, days	T/C	Increase in Lifespan, %[a]
Control	10		
2'-Deoxyuridine			
200	9.9	0.99	−1
5-Fluorouracil			
10	12.0	1.20	20
12.5	10.9	1.09	9
20	14.9	1.49	49
25	14.1	1.41	41
5-Fluorouracil + 2'-deoxyuridine			
5-Fluorouracil, 10 / 2'-Deoxyuridine, 200	15.3	1.53	53
5-Fluorouracil, 12.5 / 2'-Deoxyuridine, 200	13.6	1.36	36
5-Fluoro-2'-deoxyuridine			
35	10.2	1.02	2
45	11.5	1.15	15
5-Fluoro-2'-deoxyuridine + 2'-deoxyuridine			
5-Fluoro-2'-deoxyuridine, 35 / 2'-Deoxyuridine, 200	11.8	1.18	18
5-Fluoro-2'-deoxyuridine, 45 / 2'-Deoxyuridine, 200	13.3	1.33	33

[a] Each increase in lifespan value represents the average for 10 mice.

were placed in ice-cold saline and cut into pieces about 2 mm. in diameter. Two pieces were transplanted bilaterally subcutaneously on the dorsal side of each BDF₁ mouse by means of a trochar.

Twenty-four hours after transplant, the mice were divided into groups of 10. Each group received, by intraperitoneal injections for 7 days, one of the drug regimens listed in Table II. These regimens were the same as those used for the L-1210 experiment, modified where necessary to minimize any observed toxic manifestations.

Twelve days after transplant, the diameter of each tumor was measured in two directions and the mean tumor radius for each group was calculated. The corresponding tumor volume was calculated, assuming a spherical shape. The volumes thus obtained for each experimental group were divided by the corresponding volumes for the control group to obtain the T/C. The percent inhibition of tumor growth was given by Eq. 1:

$$\text{percent inhibition} = 100 \left(1 - \frac{T}{C}\right) \qquad \text{(Eq. 1)}$$

Table II—Inhibition of Growth of Adenocarcinoma 755 in Mice by 5-Fluorouracil, 5-Fluoro-2'-deoxyuridine, and Their Combinations with 2-Deoxyuridine

Drug Regimen, mg./kg./day	Mean Tumor Volume, mm.³	Mean Weight Change, g.	T/C[a]	Tumor Inhibition, %
Control	1433	+0.03	1	0
2'-Deoxyuridine				
200	741	−0.37	0.52	48
5-Fluorouracil				
10	1161	−0.20	0.81	19
20	290	−3.17	0.20	80
5-Fluorouracil + 2'-deoxyuridine				
5-Fluorouracil, 10 / 2'-Deoxyuridine, 200	128	−3.57	0.09	91
5-Fluoro-2'-deoxyuridine				
40	692	−1.87	0.48	52
5-Fluoro-2'-deoxyuridine + 2'-deoxyuridine				
5-Fluoro-2'-deoxyuridine, 40 / 2'-Deoxyuridine, 200	17	−4.13	0.01	99

[a] Each T/C value is mean for 30 mice.

Table III—Effect of 5-Trifluoromethyl-2'-deoxyuridine on Recovery of 5-Fluoro-2'-deoxyuridine from Spiked Blood after 24-hr. Dialysis

5-Fluoro-2'-deoxyuridine Added, M	5-Trifluoromethyl-2'-deoxyuridine Added, M	5-Fluoro-2'-deoxyuridine Found, %
5.1×10^{-4}	0	37.1
5.1×10^{-4}	2.5×10^{-4}	49.5
5.1×10^{-4}	1×10^{-3}	87.3
5.1×10^{-4}	2.5×10^{-3}	90.4

Table IV—Effect of 5-Trifluoromethyl-2'-deoxyuridine on 5-Fluoro-2'-deoxyuridine Cleavage in the Presence of 5-Fluorouracil

Drug	Concentration in Blood before Dialysis, M	Concentration in Blood after Dialysis, M	Drug Found, %
5-Fluoro-2'-deoxyuridine	8.29×10^{-5}	1.73×10^{-4}	208.7
5-Fluorouracil	3.84×10^{-4}	7.60×10^{-6}	2.0
5-Trifluoromethyl-2'-deoxyuridine	2.03×10^{-3}	—	—

The percent inhibition is a measure of the effectiveness of the drug regimen.

RESULTS AND DISCUSSION

In Vitro Blood Studies—During efforts to develop analytical procedures for the determination of 5-fluoro-2'-deoxyuridine *in vivo*, using the dialysis method previously reported (5) for 5-fluorouracil, it was found that when 5-fluoro-2'-deoxyuridine was added to whole human blood samples only about 37% was recovered as 5-fluoro-2'-deoxyuridine after 24 hr. Attempts to increase recovery of 5-fluoro-2'-deoxyuridine by enzyme deactivation procedures, such as pH adjustment, protein precipitation, and increased temperature, failed. A survey of the literature indicated that the cleavage was thought to be mediated by nucleoside phosphorylase (7) and that inhibitors such as 5-trifluoromethyl-2'-deoxyuridine might be of some value.

Preliminary data indicated that the addition of 5-trifluoromethyl-2'-deoxyuridine did enhance the recovery of 5-fluoro-2'-deoxyuridine from "spiked" blood (Table III). Although the recovery was not quantitative, it is readily apparent that increased 5-trifluoromethyl-2'-deoxyuridine concentration appeared to reduce 5-fluoro-2'-deoxyuridine cleavage.

Since one would expect the presence of both 5-fluorouracil and 5-fluoro-2'-deoxyuridine in actual clinical samples, experiments were carried out with spiked blood containing 5-fluorouracil, 5-fluoro-2'-deoxyuridine, and 5-trifluoromethyl-2'-deoxyuridine. The results (Table IV) were startling in that 5-fluorouracil recoveries, which previously had been shown to be 95%, were very low and the 5-fluoro-2'-deoxyuridine concentration was increased to more than 208% of that initially added. These findings indicate that 5-trifluoromethyl-2'-deoxyuridine not only inhibits 5-fluoro-2'-deoxyuridine cleavage but is involved in a deoxyribosyl transfer giving rise to the reaction shown in Scheme I. This is, undoubtedly, an oversimplification of a complex enzyme-mediated reaction since a repetition in pH 7.4 buffered saline failed to show any conversion of 5-fluorouracil to 5-fluoro-2'-deoxyuridine in the presence of 5-trifluoromethyl-2'-deoxyuridine.

These findings led to the consideration that the mechanism of action of so-called nucleoside phosphorylase inhibitors actually functioned in part by reversal of the cleavage equilibrium. It was further rationalized that compounds of similar structures such as 2'-deoxyuridine and deoxycytidine should function in an analogous manner in blood. The effect of 5-trifluoromethyl-2'-deoxyuridine, 2'-deoxyuridine, and deoxycytidine on 5-fluoro-2'-deoxyuridine

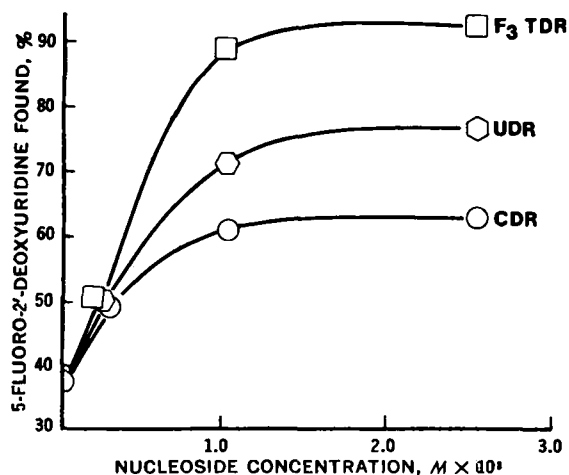$$FU + F_3TDR \rightleftharpoons FUDR + F_3T$$
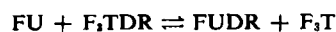
Scheme I

Figure 1—*5-Fluoro-2'-deoxyuridine found in blood in the presence of various concentrations of added nucleosides after 24-hr. dialysis. (Initial 5-fluoro-2'-deoxyuridine concentration was 5.1 × 10⁻⁴ M.)*

cleavage is summarized in Fig. 1. In all three cases, the apparent cleavage of 5-fluoro-2'-deoxyuridine was reduced as nucleoside concentration increased. A similar experiment was conducted to determine the interaction of 5-fluorouracil with 5-trifluoromethyl-2'-deoxyuridine, 2'-deoxyuridine, and deoxycytidine to form 5-fluoro-2'-deoxyuridine. The results (Fig. 2) clearly show that in all cases there was significant conversion of 5-fluorouracil to 5-fluoro-2'-deoxyuridine. The sugar moiety alone, deoxyribose, did not react with 5-fluorouracil.

The importance of these interactions to the clinical use of 5-fluorouracil and 5-fluoro-2'-deoxyuridine is immediately apparent.

In Vivo Mouse Studies—The preliminary *in vitro* studies raised the question that, if the reduced activity of 5-fluoro-2'-deoxyuridine was truly due to *in vivo* cleavage to 5-fluorouracil and deoxyribose, could this reaction be reversed *in vivo* by the coadministration of a suitable nucleoside. Of the three compounds tested *in vitro*, 2'-deoxyuridine appeared to be the most promising candidate for the following reasons:

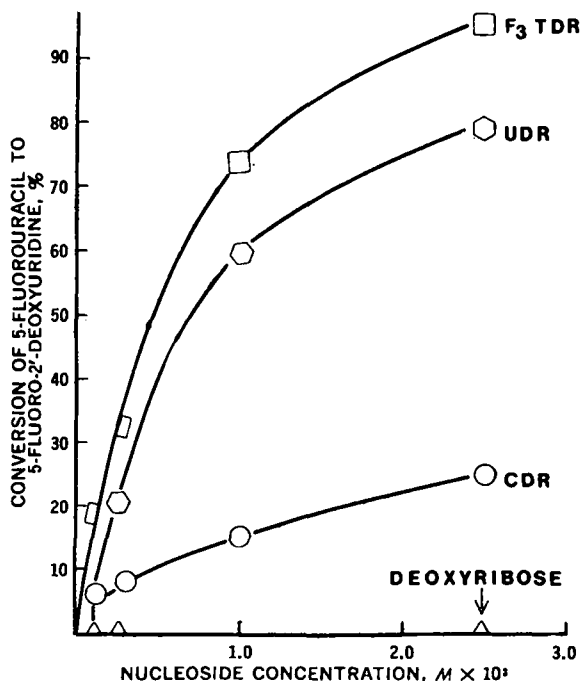1. 2'-Deoxyuridine is of lower order toxicity and has no antitumor activity of its own.

Figure 2—*Percent conversion of 5-fluorouracil to 5-fluoro-2'-deoxyuridine at various concentrations of four deoxyribosyl donors in blood during 24-hr. dialysis. (Initial 5-fluorouracil concentration was 4.1 × 10⁻⁴ M.)*
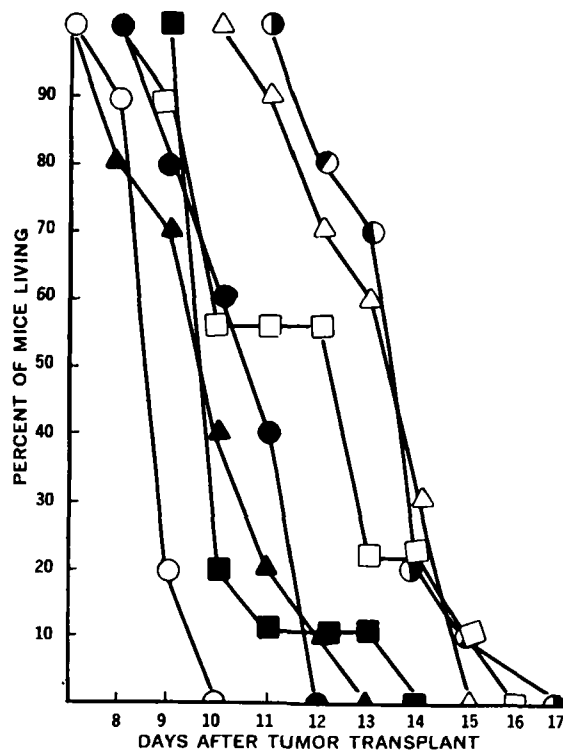

Figure 3—*Survival of mice during chemotherapy of L-1210 leukemia with 5-fluorouracil, 5-fluoro-2'-deoxyuridine, and their combinations with 2'-deoxyuridine. Key: ◑, 5-fluorouracil (10 mg./kg./day) plus 2'-deoxyuridine (200 mg./kg./day); △, 5-fluorouracil (20 mg./kg./day); □, 5-fluoro-2'-deoxyuridine (45 mg./kg./day) plus 2'-deoxyuridine (200 mg./kg./day); ●, 5-fluorouracil (10 mg./kg./day); ▲, 2'-deoxyuridine (200 mg./kg./day); ■, 5-fluoro-2'-deoxyuridine (45 mg./kg./day); and ○, control, 0.9% sodium chloride solution.*

2. Although 5-trifluoromethyl-2'-deoxyuridine appears to be the most active in preventing or reversing cleavage, it is an antitumor agent and toxic.

3. Deoxycytidine is less effective in preventing 5-fluoro-2'-deoxy-uridine cleavage.

Based upon the concentration-conversion ratios from Figs. 1 and 2, it appears that 2'-deoxyuridine exhibits its maximum "anti-cleavage" activity at a concentration approximately 5 times that of the 5-fluoro-2'-deoxyuridine and remains invariant thereafter. Based upon an average dose of 40 mg./kg. 5-fluoro-2'-deoxyuridine, a standard dose of 200 mg./kg. 2'-deoxyuridine was used in all combinations.

Chemotherapy of Mouse L-1210 489 Leukemia with Combinations of 5-Fluorouracil or 5-Fluoro-2'-deoxyuridine and 2'-Deoxyuridine— As noted under *Experimental*, 70 mice (divided into seven groups of 10 each) were inoculated with a standardized suspension of L-1210 leukemia cells. The animals were randomized into groups and subjected to various drug regimens alone and in combination with 200 mg./kg. 2'-deoxyuridine. The drugs were administered once a day by intraperitoneal injection, using normal saline as a control. The drug activity was determined grossly by recording a survivial time of each animal (Table I).

Although fluorinated pyrimidines are considered to be less effective against leukemias than against other forms of cancer, such as solid tumors, the data obtained here indicate that their effectiveness can be enhanced by the presence of 2'-deoxyuridine in the drug regimen. For example, the addition of 2'-deoxyuridine to the 12.5-mg. regimen of 5-fluorouracil quadrupled its effectiveness against L-1210 leukemia. The addition of 2'-deoxyuridine to the 10-mg. regimen enhanced its effectiveness over 2.5 times.

Autopsies of mice given these drugs, singly or in combination with 2'-deoxyuridine, indicated that death was partly due to toxicity. However, enhancement of 5-fluorouracil activity at the lower dose levels, such as 10 mg./kg./day, by the addition of 2'-deoxyuridine is significant. Further clarification of the optimum dosage level is necessary.

An alternative way of presenting the survival data is shown in Fig. 3. The percentage of animals alive in each group was computed for each day of the experiment, and this information was graphed. The data indicate the superiority of the drug combinations to the single drugs.

Chemotherapy of Mouse Adenocarcinoma 755 with Combinations of 5-Fluorouracil or 5-Fluoro-2'-deoxyuridine and 2'-Deoxyuridine—Experiments dealing with solid tumors were carried out using adenocarcinoma 755. The tumor was grown in BDF_1 mice and transplanted to 70 normal mice. After allowing 24 hr. for the tumors to acclimate to the host, drug therapy was started using drugs alone or in combination with 200 mg./kg. 2'-deoxyuridine. Therapy was continued for 7 days using daily intraperitoneal injections. Twelve days after the tumor transplants, the average tumor volumes were determined for each group. The results given in Table II compare the tumor volumes of the treated groups to those of saline controls.

The reduction in tumor volumes of the mice treated with 10 mg./kg. 5-fluorouracil and 200 mg./kg. 2'-deoxyuridine and 40 mg./kg. 5-fluoro-2'-deoxyuridine and 200 mg./kg. 2'-deoxyuridine as compared to each drug alone or to the control is dramatic. Although there is an apparent tumor inhibition due to 2'-deoxyuridine alone, the apparent diminutions are not significant. Based on the data, it can be shown that the drug-2'-deoxyuridine combinations are synergistic and not simply additive.

The loss in weight of the mice appears to correlate with the percent tumor inhibition. This may be indicative of increased toxicity because of the higher effective titer of 5-fluoro-2'-deoxyuridine in the body due to the presence of the deoxyribosyl donor. There are some indications that the loss in weight was partly associated with the mouse colony used. Further toxicity studies are being carried out and will be reported later.

These *in vivo* data tend to support the *in vitro* findings that 5-fluorouracil is converted to 5-fluoro-2'-deoxyuridine by deoxyribosyl transfer in the presence of an excess donor compound such as 2'-deoxyuridine, and the activity therefore is enhanced. Also the apparent stabilization of 5-fluoro-2'-deoxyuridine has a dramatic effect on antitumor activity. In light of the present results, the observation by Birnie *et al.* (4) of great toxicity in the use of uridine with 5-fluoro-2'-deoxyuridine may be explained in terms of the exchange reaction where one product is the very toxic 5-fluorouridine.

SUMMARY AND CONCLUSIONS

In vitro experiments in human blood showed that deoxyribonucleosides such as 5-trifluoromethyl-2'-deoxyuridine, 2'-deoxyuridine, and deoxycytidine can react with 5-fluorouracil to form 5-

fluoro-2'-deoxyuridine. A prediction that deoxyribosyl transfer reaction can enhance the antitumor activity of 5-fluorouracil and 5-fluoro-2'-deoxyuridine was tested *in vivo* in mice. When survival time and decreased tumor growth are used as measures of effectiveness, results from mouse leukemia L-1210 and adenocarcinoma 755 indicate that 5-fluorouracil or 5-fluoro-2'-deoxyuridine coadministered with excess 2'-deoxyuridine has higher antitumor activity than either of the pure drugs alone.

The data are consistent with the prediction of enhanced antitumor activity due to the deoxyribosyl transfer reaction, although other contributory mechanisms are possible.

Deoxyribosyl transfer reactions may provide a rationale for efficacy of some drug combinations used clinically.

Further work to determine toxicity and efficacy of the combinations is underway.

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