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Determinants of Responsiveness to 5-Fluorouridine in Transplantable Murine Leukemias

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SUMMARY

The antineoplastic agent 5-fluorouridine was therapeutically effective against only the murine leukemias which contained low levels of uridine phosphorylase (EC 2.4.2.3). In such cell lines, degradation of 5-fluorouridine to 5-fluorouracil by uridine phosphorylase was insignificant. The selective toxicity of 5-fluorouridine was enhanced by this enzymatic deletion, since degradation to the less potent 5-fluorouracil is thereby prevented. 5-Fluorouridine, unlike 5-fluorouracil, is highly toxic to the host. Resistance to fluorouridine was characterized by the deletion of uridine kinase (EC 2.7.1.48), not by barriers to transport. On a molar basis, fluorouridine was more than 50 times as effective as fluorouracil in the inhibition of deoxyuridine incorporation into DNA by Leukemia P388 cells.

INTRODUCTION

The antimetabolite 5-fluorouracil can inhibit growth of several experimental animal neoplasms, and has proved useful in the treatment of human malignant disease (1). The selective toxicity of 5-fluorouracil against malignant cells has been ascribed to the absence of detoxification mechanisms in such cells (2), the drug being readily degraded by a ring cleavage reaction in normal cell types. The nucleoside 5-fluorouridine did not exhibit significant selective toxicity against murine leukemias, and was, in fact, found to be highly toxic to mice (3). In other studies, 5-fluorouridine was found to be a much more effective inhibitor of DNA synthesis than was FU[1] (4, 5). Heidelberger (1) suggested that the toxicity of FUR might be explained, in part, by the concentrative uptake of the compound into normal cells, followed by extensive incorporation of the drug into RNA.

We first examined responsiveness to FUR by using a subline of the L1210 murine leukemia selected for resistance to FU (6). The drug was extraordinarily effective, producing a large number of "cures." In the present study, we have examined enzymatic determinants of responsiveness to FUR in several transplantable murine leukemias. The results suggest that the drug might be useful in treatment of certain fluorouracil-resistant neoplasms.

MATERIALS AND METHODS

Murine leukemias. Sources of certain of these murine leukemias have been described

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[1] The abbreviations used are: FU, 5-fluorouracil; FUR, 5-fluorouridine; FUdR, 5-fluoro-2′-deoxyuridine.

(7). The P388/38280/FUR cell line was derived from P388/38280 by selection for resistance to fluorouridine, using a 1 mg/kg daily intraperitoneal dose after transplantation of 10^6 cells. The P388/FU cell line was similarly derived by selecting for resistance to daily doses of FU (25 mg/kg). Both lines were maintained in animals receiving these daily drug dosages. The P388 and P388/38280 lines were provided by Mr. I. Wodinsky, Arthur D. Little Corporation, Cambridge, Mass. All cell lines were carried in male CDF_1 animals.

Drug response studies. These were carried out by administration of FU (25 mg/kg), FUdR (80 mg/kg), or FUR (1 mg/kg) by intraperitoneal injection into animals from day 1 to day 10 following inoculation with 10^6 tumor cells. Groups of 10 animals were employed in each experiment, with another group used as untreated controls.

Drug transport studies. These were carried out as described previously (8, 9). Suspensions of freshly isolated cells were incubated in a buffered salts medium containing specified levels of 5-fluorouridine-2-^{14}C. In some cases, 0.2 mM Persantine [dipyridamole; 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido[5,4-*d*]pyrimidine] was added to block further uptake and exodus of FUR (9). The cells were collected by centrifugation, washed once in fresh medium containing 0.2 mM Persantine, and then dispersed in 0.9% NaCl for determination of radioactivity by liquid scintillation counting (10).

Measurement of uridine phosphorylase (EC 2.4.2.3) and uridine kinase (EC 2.7.1.48). The enzymes were measured using cell-free extracts of freshly isolated cells. Uridine phosphorylase was measured at 37° (11) by observing the increase in optical density at 300 mμ during the conversion of fluorouridine to fluorouracil. Incubation mixtures contained 10 mM FUR, 50 mM potassium phosphate buffer at pH 7.5, and 5–10 mg of enzyme protein in a total volume of 0.5 ml. At intervals of 0, 10, 20, and 30 min after addition of enzyme, samples of 0.1 ml were removed and diluted with 0.1 ml of 0.6 M $HClO_4$. The denatured protein was removed by centrifugation, and a 0.1-ml portion of the supernatant fluid was mixed with 0.1 ml of 1 M KOH. The precipitate of $KClO_4$ was removed, and the optical density of the sample was measured at 300 mμ. Standards were run to determine extinction coefficients of FU and FUR. Uridine kinase was measured (12) at 37° in a mixture containing 1 mM FUR-2-^{14}C, 5 mM ATP, 6 mM $MgCl_2$, and 60 mM Tris chloride at pH 7.2. The reaction was initiated by addition of 0.05–0.1 mg of enzyme protein, and the final volume was 100 μl. At intervals of 0, 3, 10, and 15 min, 20-μl aliquots of the incubation mixture were removed and allowed to soak into DEAE-impregnated filter paper discs (2 cm in diameter). These discs were then washed for two 3-min periods in 10 mM citric acid, for two 1-min periods in water, and then for two 3-min periods in acetone. After drying in air, radioactivity absorbed by the discs was determined by liquid scintillation counting in a toluene-based liquid phosphor.

Studies on inhibition of DNA synthesis. Ascitic tumor cells were isolated from animals 24 hr following administration of FU (25 mg/kg) or FUR (1 mg/kg) in 0.1 ml of 0.9% NaCl by intraperitoneal injection. Animals were used on day 6 following transplantation of 10^6 tumor cells, and were not otherwise treated with any drugs. The cells were suspended in 10 volumes of 0.9% NaCl, and the resulting solution was diluted with 3 parts of cold distilled water. After mixing for 20 sec, 1 part of 3.6% NaCl was added, with mixing, to restore isotonicity. The cells were collected by centrifugation at 150 × *g* for 5 min, and the resulting top layer of ghosts was removed along with the hemoglobin-containing supernatant fluid. The cells were then resuspended in 0.9% NaCl for a final wash.

Incubation tubes contained 10 mg of cells and 1 ml of minimum Eagle's medium (Spinner), the latter being prepared with 10% horse serum. After warming at 37° for 10 min, 2-μl portions (0.1 μCi) of deoxyuridine-2-^{14}C solution (1 μM) were added, and incubations were continued for 10 min. Incubations were terminated by chilling the tubes, followed by centrifugation for 5 min at 0°. The cells were washed three times in 0.3 M $HClO_4$, and the DNA was isolated (13) by a procedure based on the preferen-

tial hydrolysis of RNA by treatment with dilute alkali for 1 hr at room temperature. A note on the accuracy of this method appears in ref. 13. DNA was dissolved in Nuclear-Chicago "NCS" solubilizer, and radioactivity was determined by liquid scintillation counting (10).

Substrates. Deoxyuridine-2-^{14}C (20 mCi/mmole) was purchased from New England Nuclear Corporation, and 5-fluorouridine-2-^{14}C (10 mCi/mmole) was purchased from Calbiochem. The latter was purified by descending chromatography on Whatman No. 1 paper, using 1-butanol–formic acid–water (77:10:13) as the solvent. Nonradioactive fluorouridine was provided by Hoffmann–La Roche.

RESULTS

Survival studies. The responsiveness of the various cell lines employed here to FU, FUR, and FUdR is shown in Table 1. The maximum tolerated drug dose (days 1–10) was used. These data differ slightly from a previous report (14), reflecting the gradual variations which occur during maintenance of transplantable murine leukemias. It is noteworthy that, in both P388/FU and P388/38280, decreased responsiveness to fluorouracil was accompanied by a corresponding increase in sensitivity to fluorouridine. The fluorouridine-resistant subline of P388/38280 was not responsive to any of the three drugs.

TABLE 1

Drug-promoted survival of tumor-bearing mice

Animals were treated with FUR (1 mg/kg), FU (25 mg/kg), or FUdR (80 mg/kg) from day 1 to day 10 after inoculation with 10^6 tumor cells.

Cell line	Increase in life span[a]		
	FU	FUR	FUdR
	%	%	%
P388	100	30	100
P388/38280	30	250	30
P388/38280/FUR	0	0	0
P388/VCR	75	20	60
P388/FU	0	>250[b]	0

[a] Percentage increase in life span of tumor-bearing animals (mean survival data).

[b] Three of 10 animals survived for more than 100 days.

TABLE 2

Enzyme levels in cell extracts

Cell extracts were incubated with FUR (10 mM) and 50 mM phosphate buffer at pH 7.5 for determination of uridine phosphorylase (11), or with FUR-2-^{14}C (1 mM), ATP (5 mM), $MgCl_2$ (6 mM), and Tris chloride at pH 7.2 (60 mM) for determination of uridine kinase (12). The data represent mean values of five experiments, with a variation at ±10%.

Cell line	Uridine kinase	Uridine phosphorylase
	μmoles/g protein/hr	
P388	100	50
P388/38280	84	5
P388/38280/FUR	<1	3
P388/VCR	103	255
P388/FU	33	2

Enzyme studies. Levels of uridine phosphorylase and uridine kinase in the different cell lines are shown in Table 2. Uridine phosphorylase is essentially absent from P388/38280, P388/FU, and P388/38280/FUR, but is greatly elevated in P388/VCR. Uridine kinase is absent from P388/38280/FUR, but present in the other cell lines.

Incorporation studies. When tumor-bearing animals received injections of FUR (1 mg/kg) or FU (25 mg/kg), incorporation of deoxyuridine into DNA was, in general, inhibited. The data are summarized in Table 3. Several conclusions can be drawn from these data. (a) Both drugs are inactive against P388/38280/FUR. (b) FUR causes considerably more inhibition of deoxyuridine incorporation than does FU in P388/FU and P388/38280, and at markedly lower dosage. (c) This difference between the two drugs is less marked in P388 and P388/VCR. (d) FUR, at 2% of the molar level of FU, causes an equivalent degree of inhibition of DNA synthesis in P388.

In other studies, we found that FUR was much more effective than FU as an inducer of thymidine kinase in P388 cells. A dose of 25 mg/kg of FU elevated cellular levels of this enzyme by 100% within 24 hr, whereas a much smaller dose of FUR (1 mg/kg)

TABLE 3

Drug effects on incorporation of deoxyuridine into DNA

Tumor-bearing animals were treated with FUR (1 mg/kg) or FU (25 mg/kg) on day 6 after implantation of 10^6 tumor cells. Cells were isolated 24 hr later and incubated with labeled deoxyuridine for 10 min as described in the text. Incorporation of label into DNA was measured. The data represent the average of six experiments, with figures rounded off to the nearest 50. The range of values differed from the averages by no more than ±12%.

Cell line	DNA labeling following treatment with		
	Controls	FU	FUR
	cpm/mg wet cell wt		
P388	5400	1850	2100
P388/38280	3400	2900	950
P388/38280/FUR	1950	1700	1800
P388/VCR	4900	2400	2250
P388/FU	2250	2200	600

elevated thymidine kinase by 200% over the same interval.

Transport studies. We examined the transport of FUR in the P388/38280/FUR subline, which lacks both uridine phosphorylase and uridine kinase (see above), and is therefore unable to metabolize this compound. The rate of uptake of FUR was found to be temperature-sensitive, with a Q_{10} of 1.3. No net concentration of fluorouridine was found. Drug uptake was inhibited competitively by other pyrimidine nucleosides, but not by purine nucleosides or pyrimidines. Dinitrophenol (1 mM) did not inhibit FUR uptake, nor did UO_2^{++} (Table 4). In other studies, we found that the approximate K_m for FUR, measured at 27°, was 2.5 mM.

TABLE 4

Studies on transport of FUR-2-^{14}C by P388/38280/FUR cells

Cells were incubated under specified conditions with the labeled drug, then collected by centrifugation, and washed once in medium containing 0.2 mM Persantine to eliminate loss of drug (9).

FUR concentration	Time	Temperature	Additions	Distribution ratio[a]
mM	*min*	°C		
1	1	37	None	1.03
1	2	37	None	0.97
1	1	37	1 mM 2,4-dinitrophenol	0.95
1	1	37	5 mM UO_2^{++}	0.96
0.1	3	37	None	1.02
0.1	3	37	10 mM fluorouridine[b]	0.26
0.1	3	37	10 mM deoxycytidine	0.29
0.1	3	37	10 mM uridine	0.28
0.1	3	37	10 mM adenosine	0.96
0.1	3	37	10 mM fluorouracil	0.99
1	0.1[c]	37	None	0.55
1	0.1	27	None	0.42
1	0.1	17	None	0.26
1	0.1	7	None	0.13
1	0.1	0	None	0.03

[a] Distribution ratio of drug between cell water and extracellular water.

[b] Nonradioactive.

[c] To carry out incubations for 0.1 min, Persantine was added to a final level of 0.2 mM 6 sec after addition of labeled FUR to the cell suspensions.

DISCUSSION

The present data suggest that the ability of FUR to prolong lives of tumor-bearing animals is inversely related to levels of uridine phosphorylase in the tumor cells. FUR is clearly a potent antimetabolite and can readily inhibit incorporation of deoxyuridine into DNA at lower dosage levels than can FU. But a high degree of host toxicity effectively limits the daily dosage of FUR to the vicinity of 1 mg/kg, while FU can be provided at a daily level of 25 mg/kg. FUdR can be given at a still higher level, 80 mg/kg, although this has not resulted in a significantly enhanced antineoplastic effect.

Heidelberger's group established (15) that FUR was readily phosphorylated by liver, spleen, kidney, intestine, and marrow of mice to form pharmacologically active nucleotides. This provided an explanation for the observed lack of selective toxicity of the drug (3).

FUR (1 mg/kg) was more effective than

FU (25 mg/kg) as an inhibitor of deoxyuridine incorporation into DNA, presumably reflecting a greater inhibition of thymidylate synthetase (1). FUR was also more effective in inducing thymidine kinase, an effect presumably related to a lowering of the cellular pool of thymidine phosphates (16).

Heidelberger found evidence to suggest active transport of fluorouridine in Ehrlich ascites cells (15), although Jacquez (17) characterized FUR transport as nonconcentrative facilitated diffusion. In the present study, we examined fluorouridine transport in a cell line lacking both uridine phosphorylase and uridine kinase. The proposal by Jacquez appears to be confirmed here: transport of the drug was mediated by a nonconcentrative, temperature-sensitive process of broad specificity for pyrimidine nucleosides. It is possible, however, that an active transport process might have been lost during the selection for fluorouridine resistance.

We conclude that selective toxicity of FUR against malignant cell types is enhanced by the deletion of uridine phosphorylase, an enzyme involved in degradation of the drug to the less potent FU (18). A cell line lacking uridine phosphorylase, but with adequate levels of uridine kinase, could therefore convert fluorouridine efficiently into pharmacologically active nucleotides with minimal formation of fluorouracil. Such a cell would therefore be considerably more sensitive to a low dose of FUR than would a cell in which extensive degradation to FU could occur. These data suggest that fluorouridine might be an effective agent against certain examples of fluorouracil-resistant neoplasms.

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