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Experimental Chemotherapy of Neuroblastoma

II. Increased Thymidylate Synthetase Activity in a 5-Fluorodeoxyuridine-Resistant Variant of Mouse Neuroblastoma

FRED BASKIN,[1] STEVEN C. CARLIN,[2] PATRICIA KRAUS,[2] MORRIS FRIEDKIN,[3] AND ROGER N. ROSENBERG[1,4]

Departments of Neurosciences and Biology, The University of California at San Diego School of Medicine, La Jolla, California 92037, and Department of Neurology, University of Texas Health Science Center at Dallas, Southwestern Medical School, Dallas, Texas 75235

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SUMMARY

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Mechanisms of resistance to chemotherapeutic agents have been investigated using mouse neuroblastoma cells grown in tissue culture as an experimental model. We report the characterization of a variant which can grow in the presence of 2000 times more 5-fluorodeoxyuridine (FUdR) than the sensitive parent strain. This mutant displays an 8-fold elevation in thymidylate synthetase, but its drug retention and pyrimidine kinase, phosphorylase, and phosphoribosyltransferase activities are identical with those of sensitive cells. Drug retention is temperature-dependent, inhibited by thymidine, and blocked by dinitrophenol but not ouabain, suggesting a need for cytoplasmic phosphorylation. Thymidylate synthetase isolated from either sensitive or resistant cells is strongly inhibited by 5-fluorodeoxyuridine 5'-phosphate, and thymidine protects cells against the toxic effects of FUdR. Both the resistance of the variant to FUdR and its elevated thymidylate synthetase specific activity are unstable, largely disappearing after 9 weeks (63 generations) without drug. Thymidine kinase is responsible for the phosphorylation of FUdR in this cell. Thus our FUdR-resistant neuroblastoma line differs from all corresponding mutants in other tumor cell lines.

INTRODUCTION

In recent years, neuronal functions differentiated at the molecular level have been successfully defined using the C1300 line of mouse neuroblastoma in culture (1, 2). Neuroblastoma is also one of the most common solid tumors of children (3) and remains largely refractory to all existing chemotherapeutic regimens; the best remission rates average 32-38%, with a mean duration of 3-12 months (4, 5). In an effort

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[1] Department of Neurology, University of Texas Health Science Center.

[2] Department of Neurosciences, University of California, San Diego.

[3] Department of Biology, University of California, San Diego.

[4] To whom requests for reprints should be addressed.

to propose better chemotherapeutic drug combinations for the management of this tumor, we have attempted to exploit the observation (6) that this tumor has high levels of thymidylate synthetase by investigating molecular mechanisms of resistance to various antifolates and pryimidine analogues that inhibit this key enzyme. We have used the C46 clone or C1300 mouse neuroblastoma grown in monolayer tissue culture as an experimental model. We feel that this model is useful because childhood neuroblastoma grows in thin sheets of rapidly dividing cells. By exposing cultured cells to stepwise increases in drug concentration, we have isolated a variant that is 2000 times more resistant than the parental strain to 5-fluorodeoxyuridine. We report here the biochemical characterization of this resistant variant.

MATERIALS AND METHODS

Chemicals

5-Fluorodeoxyuridine (Sigma Chemical Company) was dissolved in sterile water to make a 0.1 M stock solution, which was stored at −10°. The 2-amino-4-hydroxyquinazoline antifolate 5,8-deaza-10-methylfolate was made available to us by Dr. Lawrence Plante, Department of Biology, University of California, San Diego. 5-Fluoro-2′-deoxyuridine 5′-monophosphate was a generous gift of Dr. Daniel Santi, University of California, San Francisco, School of Medicine, to whom we are greatly indebted. [*methyl*-^{3}H]Thymidine (20 Ci/mmole), 5-fluoro[6-^{3}H]deoxyuridine (6.7 Ci/mmole), [5-^{3}H]deoxyuridine 5′-monophosphate, and 5-fluoro[6-^{3}H]uracil (14.7 Ci/mmole) were purchased from New England Nuclear and stored at 5° for no more than 60 days. 5-Fluoro[2-^{14}C]uracil (38 μCi/mmole) was purchased from Calatomic. Adenosine 5′-triphosphate, deoxyuridine 5′-monophosphate, and 5-phosphoribosyl 1-pyrophosphate were purchased from Sigma Chemical Company. Trypsin (2.5%), kanamycin, and spectinomycin were purchased from Grand Island Biological Company. Dulbecco's modified Eagle's medium was obtained in powdered form from Grand Island Biological Company, and fetal calf serum was purchased from Reheis Chemical Company; the same lot of serum was used for all experiments in this study.

Cell Lines

Clone C46 of the C1300 mouse neuroblastoma cell line was a gift of Dr. Gordon H. Sato, University of California, San Diego, and was initially sensitive to 5-fluorodeoxyuridine. A line resistant to FUdR[5] was derived from C46 cells by exposing rapidly dividing cells to 0.04 μM (0.01 μg/ml) FUdR in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Most of the cells exposed to FUdR showed cytotoxic effects and began floating off the substratum after 3 days. One month (approximately 20 cell generations) after the initial exposure to FUdR small colonies of viable cells began to appear. These colonies were dispersed by trypsinization, and the cells were allowed to grow to confluency. The cells were then subcultured into medium containing 0.4 μM FUdR, and the selection process was repeated. Most of the cells died at this higher FUdR concentration, but again colonies of viable cells developed. Eventually a line of cells that grew rapidly in 0.4 μM FUdR was established. This FUdR-resistant cell line, maintained in 0.4 μM FUdR for over 1 year (more than 240 generations), is morphologically very similar to the parental cell line from which it was derived; that is, under phase microscopy the cells appear dark, square, and without neurites. The cell number doubling time (generation time) of both the FUdR-sensitive and -resistant variants is 20 hr at 37°.

Culture Conditions

Cell monolayers were routinely grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum plus 200 μg/ml of kanamycin and 125 μg/ml of spectinomycin. Cultures were grown in Falcon flasks or tissue culture dishes at 37° in an

[5] The abbreviations used are: FUdR, 5-fluorodeoxyuridine; FdUMP, 5-fluorodeoxyuridine 5′-phosphate; FU, 5-fluorouracil; FUMP, 5-fluorouridine 5′-phosphate.

atmosphere of 10% CO_2–90% air with 100% humidity. The cells were subcultured by trypsinization for 10 min at 23° with 0.25% trypsin, as previously described (7).

For growth curves, 1×10^6 cells from exponentially growing cultures were inoculated into each of a series of 100-mm tissue culture dishes. The concentrated cell suspension used as the inoculum was swirled between inoculations to keep the cells from settling, and samples of this suspension were taken from the beginning, middle, and end of the inoculation series and counted to ensure that all the dishes in the series had received equal numbers of cells. Each day during the growth period one plate was trypsinized and counted with a Coulter model Z_{BI} electronic cell counter, and two to six plates were used for making extracts as described below.

Determination of EC_{50} Values

Resistance to drug was quantitated in terms of EC_{50} values (7, 8). A series of 6–10 Linbro four-well, 60-mm cluster tissue culture dishes was prepared so that the wells of each successive dish contained medium with a higher concentration of drug, except for the first dish, which was a control with no drug. Each 60-mm well was then inoculated with 5×10^5 cells, and after 3 days the cells were trypsinized and counted with a Coulter counter. The average cell number at each drug concentration, expressed as a percentage of control, was then plotted against the logarithim of the drug concentration to determine the concentration of drug effective in inhibiting the growth by 50% (EC_{50}).

Protection by Thymidine against FUdR Growth Inhibition of FUdR-Sensitive and -Resistant Cells

FUdR-sensitive and -resistant neuroblastoma cells (5×10^5) were plated in 60-mm tissue culture dishes containing 5.0 ml of medium (Dulbecco's modified Eagle's medium plus 10% fetal calf serum, 200 μg/ml of kanamycin, and 125 μg/ml of spectinomycin) supplemented with thymidine in concentrations ranging from 2 to 32 μM and grown in the presence of either 2 nM (sensitive cells) or 3 μM (resistant cells) FUdR. Four dishes were plated with each combination of drugs, and the cells were grown for 3 days with daily medium changes. Cell numbers were determined on day 3 by dissociating the adherent cells for 10 min with 0.25% trypsin in medium without serum and counting the dissociated cells in a Coulter counter. The four plates for each combination were pooled prior to counting. The results are expressed as a percentage of the control with no thymidine or FUdR.

Enzyme Assays

Enzyme activities were measured in crude extracts prepared at daily intervals from parallel plates of FUdR-sensitive and -resistant C46 cells. Crude extracts were made by washing the cells twice with 0.9% sodium chloride and then scraping the cells from two to four 100-mm dishes into 4 ml of buffer with a rubber policeman. The buffer used contained 50 mM Tris (pH 7.4), 10 mM 2-mercaptoethanol, 1 mM sodium ethylenediaminetetraacetate, and 25% sucrose. The cells were disrupted by freezing and thawing three times, and the cell debris was removed by centrifugation at $12,000 \times g$ for 10 min at 4°. The supernatant fraction was removed and stored frozen. All enzymatic assays were performed in duplicate, all data pairs agreeing within 10%. Total protein in the crude homogenates and extract protein in the centrifuged supernatant fractions were assayed by the method of Lowry *et al.* (9).

Thymidylate synthetase was measured by a modification (6) of the method of Roberts (10). Samples were assayed in duplicate, with sufficient extract being used to record 5,000–50,000 cpm on a Beckman scintillation counter. Controls without enzyme averaged 1200±100 cpm. Duplicate samples agreed within ±10% in all cases. Dihydrofolate reductase was assayed spectrophotometrically as previously described (11). Thymidine kinase was quantitated by a modification of the method of Taylor *et al.* (12). Before freezing, 100 μl of 0.072 mM thymidine were added to a 500-μl portion of each enzyme

extract; this was done to stabilize the thymidine kinase activity (13). The final 120-μl reaction mixture contained 28 mM Tris buffer (pH 7.4), 9.4 mM $MgCl_2$, 0.06 mM ATP, 2.81 mM [*methyl*-^{3}H]thymidine (20 Ci/mmole), and 20 μl of enzyme (containing 12 μM unlabeled thymidine); the total concentration of thymidine in the reaction mixture was 3.17 μM. The reaction mixtures were incubated for 5 min at 37°, and the reaction was stopped by boiling for 5 min. After the addition of 10 μl of carrier containing 100 mM thymidine and 100 mM thymidylate, 10 μl of each reaction mixture were spotted on Whatman No. 1 paper, and the chromatograms were developed in 1-butanol–glacial acetic acid–water (12:3:5). The spots corresponding to thymidine (R_F 0.5) and thymidylate (R_F 0.1) were cut out, placed in scintillation vials, eluted with 0.5 ml of 0.2 N HCl for 30 min, and counted in a toluene-Triton X-100 (1:4) containing 9 g/l 2,5-diphenyloxazole scintillator. Thymidine kinase activity was calculated from the net conversion of thymidine to thymidylate after subtracting the conversion observed in the blank containing no ATP.

The phosphorylation of 5-fluorodeoxyuridine by crude neuroblastoma extracts was measured by substituting 4.18 μM[^{3}H]FUdR (6.7 Ci/mmole) for thymidine in the thymidine kinase assay; the reaction mixtures were chromatographed as above, and the spots corresponding to FUdR (R_F 0.57) and 5-fluorodeoxyuridylate (R_F 0.13) were cut out and counted. The possible conversion of 5-fluorouracil to 5-fluorouridine by a phosphorylase reaction was measured by adding 1 mM ribose 1-phosphate and substituting 4.62 μm fluoro-[^{3}H]uracil (14.7 Ci/mmole) for thymidine in the thymidine kinase assay. Radioactivity was measured in the spot corresponding to 5-fluorouracil plus 5-fluorouridine (R_F 0.56) and in that of 5-fluorouridylate (R_F 0.1). The amount of radioactivity present in the nucleotide form was used as an index of the extent of the pyrimidine nucleoside phosphorylase reaction. The hydrolysis of radioactive [6-^{3}H]FdUMP by sensitive and resistant extracts was measured in the same manner with and without the addition of ATP.

Hypoxanthine-guanine phosphoribosyltransferase was measured by the method of Wood *et al.* (14). To prevent streaking on the thin-layer chromatograms used for separating hypoxanthine, inosine, and inosinic acid, the reaction mixtures were boiled for 5 min and centrifuged to remove excess protein prior to spotting on the chromatograms. Fluorouracil 5′-phosphoribosyltransferase was assayed essentially by the method of Kessel *et al.* (15). The 50-μl reaction mixtures contained 1 mM 5-phosphoribosyl 1-pyrophosphate, 1 mM $MgCl_2$, 0.5 mM mercaptoethanol, 100 mM glycine buffer (pH 9.0), 0.5 mM 5-fluoro[2-^{14}C]uracil (38 mCi/mmole), and 90–120 μg of crude enzyme protein. A reaction with 0.4 mM EDTA instead of $MgCl_2$ was carried out to control for the possible direct conversion of fluorouracil to fluorouridine by a pyrimidine nucleoside phosphorylase reaction. After incubation for 10 or 20 min at 37° the reaction mixtures were chilled and the products were separated on Whatman No. 1 paper using the chromatographic system described above.

Determination of Stability of Resistance to Growth Inhibition in the Absence of FUdR

Cells from a second FUdR-resistant population, which had been maintained in 50 nM FUdR for 6 months (180 generations) with a stable resistance to this drug (EC_{50} = 100 nM), were subcultured into flasks containing normal medium without FUdR. At 2-week intervals for 9 weeks (63 generations) these cells and the original resistant cell lines, continuously maintained in 50 nM FUdR, were subcultured into Petri dishes, and the degree of resistance to growth inhibition and thymidylate synthetase specific activities was determined coordinately as described above.

FUdR Retention Experiments

Cells in exponential growth were plated on a series of 100-mm plates (1×10^6 cells/plate). On each successive day two plates were removed and their cells were trypsinized and counted. Medium was aspirated from identical plates, which were rinsed with 10 ml of Tyrode's solution (8 g of

NaCl, 0.2 g of KCl, 0.2 g of $CaCl_2$, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.05 g of $NaH_2PO_4 \cdot H_2O$ and 1 g of glucose per liter, pH 7.2) at 23°. Transport was then initiated by adding 10 ml of Tyrode's solution containing 1 μCi (25 nM) of [6-^{3}H]FUdR and/or inhibitor. At various times the process was halted by carefully aspirating the labeled solution, cooling the plates on ice, and rinsing the plates three times with 10 ml of cold Tyrode's solution, taking care not to dislodge cells with each rinse. Plates were frozen once, and the cells were then scraped into 0.5 ml of distilled water with a rubber policeman. After 0.5 ml of rinse had been combined with the first material, the cells were disrupted with 20 strokes of an all-glass homogenizer; 0.1 ml was reserved for protein assay, and the remainder was counted in an Aquasol scintillator. Uptake inhibition studies were performed similarly, except that the cells were incubated with either 1 nM ouabain or 1 nM dinitrophenol for 30 min at 23° before addition of the labeled compound.

RESULTS

Degree of Resistance to FUdR and Cross-resistance to 5,8-Deaza-10-methylfolate

The EC_{50} data for sensitive and FUdR-resistant C46 neuroblastoma cells are shown in Fig. 1. As can be seen from the curves (each of which represents the average of two experiments), the FUdR-resistant cells (EC_{50} = 2 μM) are 2000-fold more resistant to FUdR than the sensitive cells (EC_{50} = 0.001 μM). It should be noted that the growth inhibition curve for sensitive cells appears to approach 20% survival asymptotically, perhaps explaining the relative ease experienced in obtaining a FUdR-resistant population. The FUdR-resistant cells are somewhat cross-resistant to 5,8-deaza-10-methylfolate, a 2-amino-4-hydroxyquinazoline antifolate that has been shown to be a potent inhibitor of thymidylate synthetase (7). The FUdR-resistant cells have an EC_{50} of 200 μM for this quinazoline antifolate, this is 14 times the EC_{50} of sensitive cells (14 μM) for the same drug. The slopes of the EC_{50} curves for both drugs with resistant cells are virtually identical, unlike the respective growth inhibition curves for the sensitive cells.

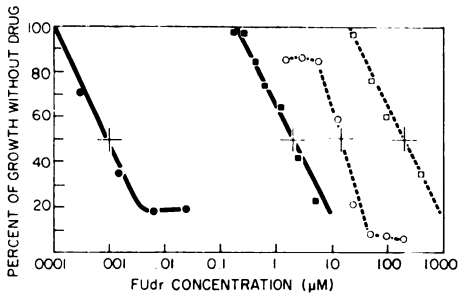

FIG. 1. *Inhibition of sensitive and FUdR-resistant neuroblastoma cell growth by FUdR and 5,8-deaza-10-methylfolate*

The cells for these EC_{50} determinations were plated and counted at day 3 as described under MATERIALS AND METHODS. The point where each curve crosses 50% on the ordinate (——) represents the EC_{50} value for the particular drug and cell line in question. Shown is the inhibition of sensitive (●——●) and FUdR-resistant (■——■)neuroblastoma cells by FUdR and of sensitive (O- - -O) and FUdR-resistant (□- - -□) cells by 5,8-deaza-10-methylfolate. Each curve represents the average of at least two experiments.

Stability of Resistance and Thymidylate Synthetase Specific Activity in the Absence of FUdR

Resistance to FUdR is lost exponentially in the absence of this drug, with a 50% decline in remaining resistance each 10 days (10 generations). By 9 weeks (63 generations) the population has returned to the resistance seen in the sensitive parental C46 clone. Thymidylate synthetase activity is lost with a similar but slower exponential decline, a 50% drop occurring in approximately 4 weeks (28 generations) (Fig. 2). The resistant population does not simply shift from maximal to minimal resistance in one step, but appears to lose this resistance to FUdR gradually (Fig. 3).

Enzyme Activities as a Function of Cell Division in Crude Extracts from Sensitive and FUdR-Resistant Neuroblastoma Cells

Thymidylate synthetase. The specific activity of thymidylate synthetase (Fig. 4) is significantly higher in FUdR-resistant cells than in sensitive C46 neuroblastoma cells throughout the entire growth curve,

varying from a minimum difference of 5-fold to a maximum of 9-fold. As previously reported (6), the activity of thymidylate synthetase is greatest in the early logarithmic phase of growth and decreases as the cells approach the stationary phase. The specific activity of this enzyme is quite high even in sensitive cells, and at the peak of activity in FUdR-resistant cells more

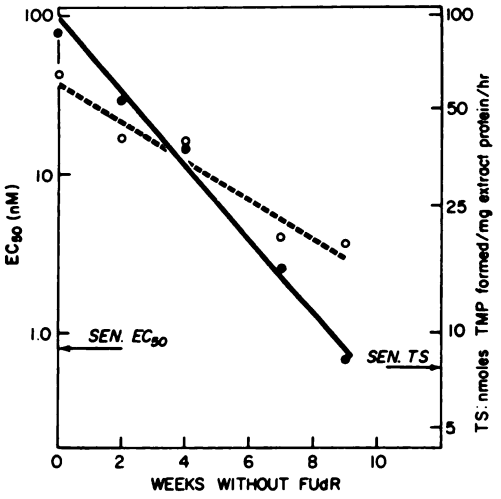

FIG. 2. *Coordinate loss of resistance to growth inhibition and thymidylate systhetase (TS) specific activity in the absence of FUdR*

FUdR-resistant cells were grown without drug, subcultures being assayed at the times indicated for their EC_{50} (●——●) and thymidylate synthetase specific activity (O- - -O). Arrows indicate the EC_{50} and thymidylate synthetase specific activity of the parental sensitive population. All points represent an average of two to four plates. One week corresponds to approximately seven generations.

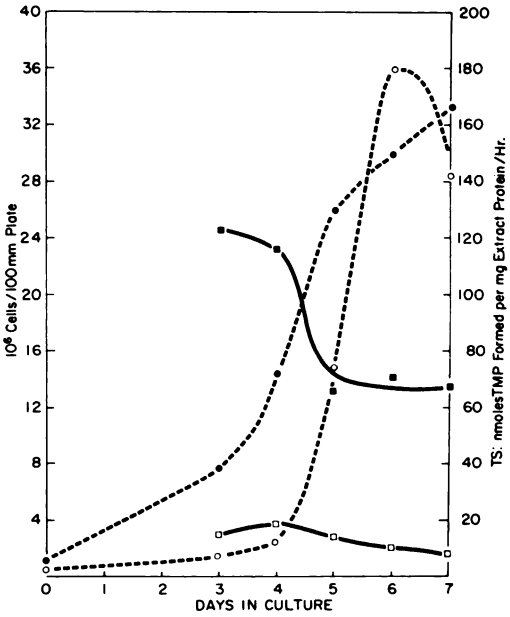

FIG. 4. *Thymidylate synthetase (TS) specific activity in relation to growth in sensitive and FUdR-resistant neuroblastoma*

Shown are the growth of sensitive (●- - -●) and FUdR-resistant (O- - -O) cells and the thymidylate synthetase specific activity of sensitive (□——□) and FUdR-resistant (■——■) cells. Each point represents the average of two plates.

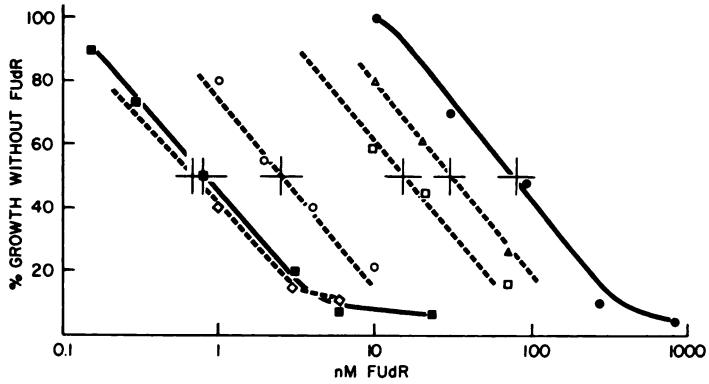

FIG. 3. *EC_{50} values of FUdR-resistant populations grown for various times without drug*

A FUdR variant, originally demonstrating an EC_{50} of 80 nM FUdR, was subcultured for 9 weeks without this drug. At 2 (Δ- - -Δ), 4 (□- - -□), 7 (O- - -O), and 9 (◇- - -◇) weeks the EC_{50} was redetermined and compared with the original resistant population (●——●) and the parental sensitive population (■——■). All data points represent the average of two to four plates.

than 120 nmoles of thymidylate are formed per milligram of extract protein per hour. When aliquots of sensitive and resistant cell extracts were mixed and then assayed, the resulting activities were exactly additive.

Thymidine kinase. As shown in Table 1, the specific activity of thymidine kinase is the same in sensitive and FUdR-resistant neuroblastoma cells. In both cell lines the specific activity of thymidine kinase reaches a peak at day 5 of growth and then declines. The specific activity of thymidine kinase is considerably lower than that of thymidylate synthetase in extracts from FUdR-resistant cells, the maximum ratio of thymidylate synthetase to thymidine kinase being 17 at day 3. The possibility that the relatively low activity measured in extracts was due to hydrolysis of the product TMP was excluded by adding [^{3}H]TMP and ATP to extracts, incubating, and observing no labeled thymidine.

Hypoxanthine-guanine phosphoribosyltransferase. As shown in Table 1, the specific activity of hypoxanthine-guanine phosphoribosyltransferase is slightly higher in FUdR-resistant cells than in sensitive cells, but except for day 7, the difference is less than 2-fold. The specific activity of the phosphoribosyltransferase is intermediate between the activities of thymidine kinase and thymidylate synthetase.

Dihydrofolate reductase. As can be seen in Table 1, this enzyme is very active in both sensitive and FUdR-resistant cell extracts. As with thymidylate synthetase, its specific activity falls as the density of the culture increases and the growth rate slows. However, with the possible exception of day 3, there is no significant difference between the specific activity of this enzyme in resistant cells and that in sensitive cells.

Other enzymes. Other enzymatic reactions studied include the direct phosphorylation of FUdR to FdUMP, presumably by one or more of the pyrimidine kinases, the conversion of 5-fluorouracil to 5-fluorouridine by the reverse phosphorylase reaction, and the conversion of 5-fluorouracil directly to the nucleotide form by a novel pyrimidine phosphoribosyltransferase reaction (15). Each of these enzymatic activities was measured (see MATERIALS AND METHODS) in extracts from day 5 of growth of the sensitive and FUdR-resistant cells (Fig. 2). Only the phosphorylation of FUdR could be detected in our extracts, and this activity was approximately equal in both sensitive and resistant extracts (Table 2).

Interestingly, the specific activities of thymidine kinase (as measured with [methyl-^{3}H]thymidine) and the phosphorylation of [6-^{3}H]FUdR are approximately equal in both sensitive and resistant cell

TABLE 1

Enzyme levels during growth of cells

FUdR-sensitive and -resistant cells were plated and grown as described in the text. On the days shown cells were harvested and then frozen and thawed three times, and the resulting extracts were centrifuged at 10,000 rpm for 10 min at 3°. Assays for dihydrofolate reductase, hypoxanthine-guanine phosphoribosyltransferase, and thymidine kinase are described under MATERIALS AND METHODS. Values represent the average of at least two experiments, with each sample assayed in duplicate.

Day	Dihydrofolate reductase		Hypoxanthine-guanine phosphoribosyltransferase		Thymidine kinase	
	Sensitive cells	Resistant cells	Sensitive cells	Resistant cells	Sensitive cells	Resistant cells
	nmoles/mg protein/hr		*nmoles/mg protein/hr*		*nmoles/mg protein/hr*	
3	21,000	16,200	56	90	5.86	4.49
4	18,400	18,200	48	62	6.31	6.88
5	15,300	12,600	54	70	8.54	6.36
6	16,800		62	90	5.75	4.99
7	13,400	18,000	42	128	4.90	4.28
10		12,800				

extracts (see Table 1), and as the former reaction is entirely inhibited by 100 μM unlabeled FUdR, both presumably proceed via the same kinase. This conclusion is supported by the results of the reverse experiment, in which the phosphorylation of [6-^{3}H]FUdR was completely inhibited by 100 μM thymidine. Unlabeled uridine did not affect the phosphorylation of labeled thymidine at all, but did slightly inhibit the phosphorylation of labeled FUdR, suggesting a possible contribution of uridine kinase to the formation of FdUMP *in vivo*.

The rate of FUdR uptake (and phosphorylation) is given in Table 2 for both FUdR-sensitive and -resistant cells. The maximal rates measured at saturating levels of drug (100 μM), 3.0 and 4.0 nmoles of FUdR per milligram of protein per hour at 37°, are somewhat less than the corresponding phosphorylation rates for FUdR measured *in vitro*, 7.2 and 5.1 nmoles/mg of protein per hour.

TABLE 2

Metabolism of 5-fluorodeoxyuridine

Cell extracts sensitive and resistant to FUdR were prepared as described in Table 1. All values represent the average of two experiments.

Reaction	Sensitive cells	Resistant cells
	nmoles/mg protein/hr	
[6-^{3}H]FUdR + ATP → FdUMP	7.2	5.1
[*methyl*-^{3}H]Thymidine + ATP → TMP	8.5	6.9
[6-^{3}H]FUdR + ATP + 100 μM thymidine → FdUMP	0	0
[6-^{3}H]FUdR + ATP + 100 μM uridine → FdUMP	4.9	4.6
[*methyl*-^{3}H]Thymidine + ATP + 100 μM FUdR → TMP	0	0
[6-^{3}H]FU + ribose-1-P + ATP → FdUMP	0	0
[2-^{14}C]FU + PP-ribose-P → FUMP	0	0
Cells + [6-^{3}H]FUdR + 100 μM FUdR → FUdR retention by cells	3.0	4.0

Retention of Labeled FUdR by Sensitive and FUdR-Resistant Neuroblastoma Cells

The ability of both sensitive and FUdR-resistant cells to retain labeled FUdR falls as the culture density increases and the growth rate decreases (Fig. 5). However, at any given cell density, this activity is approximately the same in both cultures. Drug accumulation is linear for the first 10 min at room temperature, at this time reaching approximately 50% of the maximum uptake of labeled FUdR. This uptake plateau (saturation) was reached in approximately 40 min. Dinitrophenol, but not ouabain, inhibits this activity, which is strongly temperature-dependent (Table 3). Unlabeled 4 μM thymidine inhibits the transport of labeled FUdR as effectively as 4 μM unlabeled FUdR itself, by 32% and 46%, respectively. The fact that the inhibition of uptake of labeled FUdR is not proportional to its dilution with unlabeled FUdR suggests that the system is not saturated at concentrations below 40 μM.

Enzyme Inhibition Studies

Inhibition of neuroblastoma thymidylate synthetase by 5-fluorodeoxyuridine 5'-monophosphate and 5,8-deaza-10-methylfolate. After assaying the thymidylate synthetase in crude extracts from day 4 of growth for sensitive and FUdR-resistant neuroblastoma cells and adjusting the extracts to equal specific activity (by diluting the resistant extracts 10-fold), the amount of FdUMP necessary to inhibit thymidylate synthetase by 50% was determined by measuring the inhibition produced by graded concentrations of FdUMP and plotting the remaining thymidylate synthetase activity (expressed as a percentage of control) against the logarithm of the inhibitor concentration. In both sensitive and FUdR-resistant neuroblastoma cells 50% inhibition of thymidylate synthetase was produced by 0.02 μM FdUMP. Thymidylate synthetase was also inhibited 50% by 0.14 μM 5,8-deaza-10-methylfolate, as previously reported (7).

Hydrolysis of 5-fluorodeoxyuridine 5'-monophosphate in vitro, and lack of inhibition of neuroblastoma thymidine ki-

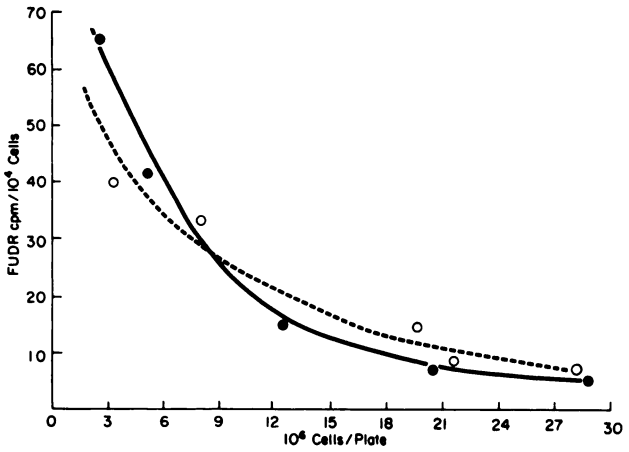

FIG. 5. *FUdR retention in relation to growth in sensitive and FUdR-resistant cells*

FUdR-sensitive (O---O) and -resistant (●——●) cells were plated, grown, and counted as described under MATERIALS AND METHODS. When cell densities reached the levels indicated, plates were aspirated and 10 ml of Tyrode's solution containing 25 nM FUdR (1 μCi/plate) were added. After 40 min at 23° uptake was halted by aspiration, rapid cooling, and rinsing as described in the text. Plates were frozen and scraped, and the resulting cell suspension was homogenized and assayed for radioactivity in a scintillation counter.

TABLE 3

5-Fluorodeoxyuridine retention by sensitive cells

Cells were grown on 100-mm Falcon plates as described under MATERIALS AND METHODS. Uptake was initiated by the addition of 10 ml of Tyrode's solution containing 1 μCi of [6-^{3}H]FUdR and was halted after 40 min at 22° by aspirating, cooling, and rinsing the plates. Uptake is presented as the percentage of radioactivity measured in the homogenized cells compared with controls. All plates contained 12 × 10^4 cells in the midlogarithmic phase. Values represent the average of four plates.

[6-^{3}H] FUdR	Unlabeled FUdR	Temperature	Inhibitor	Transport
nM	*nM*			%
2.5		22°		12
25	400	22°		100
25	4,000	22°		80
25	40,000	22°		54
25		22°	4 μM thymidine	68
25		22°	40 μM thymidine	11
25		5°		18
25		22°	1 mM ouabain	75
25		22°	1 mM dinitrophenol	0.3

nase. Thymidine kinase from FUdR-sensitive neuroblastoma cells was not inhibited by FdUMP or TMP at 100 μM. Slow hydrolysis of FdUMP could be detected *in vitro*, with sensitive and resistant cell extracts showing equal specific activities. No hydrolysis could be observed with crude extracts in the presence of ATP.

Protection by Thymidine against Toxic Effects of FUdR

Thymidine at concentrations of 2, 8, or 32 μM protected both sensitive and FUdR-resistant neuroblastoma cells against doses of FUdR (2 nM and 3 μM, respectively) that produced 75% and 46% inhibition of growth during the 3-day assay period. (See Table 4).

DISCUSSION

FUdR itself is believed to be noncytotoxic, requiring phosphorylation to the 5'-monophosphate (FdUMP) by a pyrimidine kinase, generally thymidine kinase, to exert its principal effect, the reversible (16), nonreversible (17), or quasireversible (18) inhibition of thymidylate synthetase.

The effects of thymidine kinase are paradoxical. On the one hand, it is this enzyme which is believed to activate FUdR and whose activity is often inversely correlated with resistance to this drug (19, 20), but in a milieu of endogenous thymidine *in vivo* it is also this salvage enzyme which can

TABLE 4

Protection by thymidine of FUdR-sensitive and -resistant cells against growth inhibition by FUdR

Cells were grown and counted as described under MATERIALS AND METHODS. Values represent the percentage of growth found without drug and are the average of two plates.

Drug	Sensitive cells	Resistant cells
	%	%
None	100	100
2 μM thymidine	100	102
8 μM thymidine	92	93
32 μM thymidine	88	89
2 nM FUdR	25	
3 μM FUdR		54
8 μM thymidine + 2 nM FUdR	85	
8 μM thymidine + 3 μM FUdR		90

provide an alternative biosynthetic pathway to thymidylate and prevent thymineless death (21, 22). The radioactivity of labeled FUdR is accumulated in the cell by an energy-requiring system, probably involving phosphorylation to FdUMP, but if this process is analogous to uridine transport, the rate-limiting step may not involve uridine or thymidine kinase (23). This reaction can be prevented if FUdR is instead hydrolyzed by one of the pyrimidine phosphorylases to 5-fluorouracil, and in fact an inverse correlation has been shown in one system between the level of this enzyme and the toxicity of FUdR (19).

FU may then be converted to FUMP by (a) the sequential actions of one of the phosphorylases (in the presence of high concentrations of ribose 1-phosphate), followed by a pyrimidine kinase (24), or (b) a novel reaction involving a pyrimidine phosphoribosyltransferase and 5-phosphoribosyl 1-pyrophosphate (15). As FUMP has been shown to be ineffective as an inhibitor of thymidylate synthetase in both bacterial (17) and mammalian (16) systems, further phosphorylation and reduction to FdUMP would be required before it could block DNA synthesis and again cause thymineless death.

Our neuroblastoma C46 clone produces colonies of cells resistant to FUdR in less than 4 weeks, perhaps because considerable numbers of cells are spontaneously resistant to the metabolite in this presumably pure clone. This contention is supported by the shape of the EC_{50} curve for sensitive cells (Fig. 1), wherein a small portion of the plated sensitive cells demonstrate an ability to grow in the presence of 200 times the FUdR concentration suppressing growth by 50%. A threshold concentration of 0.1 nM FUdR produces no inhibition in the sensitive line, but 1.0 nM drug causes 50% inhibition. For the resistant line, concentrations below 200 nM produce no growth inhibition, and 2 μM FUdR is required for 50% inhibition. Thus, this line is 2000-fold resistant to the antimetabolite.

A second FUdR-resistant variant of C46 neuroblastoma, with an initial EC_{50} of 80 nM FUdR when subcultured in the absence of this drug, demonstrated a coordinate exponential lability in both growth inhibition and thymidylate synthetase specific activity. The rate of loss was sufficiently great to place in doubt the genetic origin of the acquisition of resistance (25), but the exponential nature of the loss suggests a highly revertant mutant more than it does the loss of an induced phenotype (26). The resistance to growth inhibition by FUdR was lost at approximately 3 times the rate of loss of thymidylate synthetase activity. This is very similar to the rapid loss of resistance to methotrexate (99% in 35 days, approximately 70 generations) and the slower loss of dihydrofolate reductase activity (99% in 90 days, approximately 180 generations) seen in L5178 mouse leukemia cells grown *in vitro* (26). Courtenay and Robins (26) suggested the presence of several mutations that might elevate the enzyme level; some were thought to be more stable than others, and each would affect the over-all resistance of the cell differently. By analogy we would have to propose that at least two mutations contribute to the elevated thymidylate synthetase activity in FUdR-resistant neuroblastoma, one of which is more stable but does not significantly contribute to the resistance of the cell.

Both the FUdR-resistant and -sensitive

cell lines demonstrate similar retention of labeled drug, reaching saturation in approximately 40 min at room temperature. This uptake is competitively inhibited by thymidine and is blocked by dinitrophenol but not ouabain, suggesting a first step involving cytoplasmic phosphorylation and a similar accumulation system for thymidine and FUdR. It is unclear which thymidine kinase is involved (27), but thymidine kinase activities, as assayed with labeled thymidine, and the kinase activity capable of phosphorylating labeled FUdR itself in experiments *in vitro* are present in equal quantities in sensitive and resistant cells. FUdR has been reported to be a weak inhibitor of thymidine kinase (28) in other lines. However, the phosphorylation of labeled thymidine by our extracts *in vitro* is competitively inhibited only by FUdR and not by FdUMP, suggesting that the phosphorylation of labeled FUdR proceeds with this kinase. This possibility is supported by the reverse observation that the phosphorylation of labeled FUdR is competitively inhibited by thymidine. Although the ability of C46 neuroblastoma cells to hydrolyze FUdR to fluorouracil has been neither implicated nor detected in these cells, an attempt was made to detect a ribose 1-phosphate-dependent pathway to 5-fluoro-5'-uridylic acid (29). No activity could be detected in either the sensitive or resistant cell extracts under conditions promoting vigorous FdUMP synthesis. The virtual absence of this activity toward FUdR is in agreement with the extremely low threshold for sensitive cells, 0.1 nM FUdR.

A pyrimidine phosphoribosyltransferase enzyme, normally utilizing orotic acid as its substrate, was demonstrated in one system to convert 5-fluorouracil directly to FUMP (15, 19). In fact, this activity, the absence of which was cited as the mechanism of resistance in a fluorouracil-resistant cell line, could not be detected in sensitive or resistant neuroblastoma extracts. However, we have demonstrated an average 8-fold increase in thymidylate synthetase activity, the presumed target of FdUMP, in our resistant cell line. This increase is relatively uniform across the entire growth cycle from the logarithmic to the stationary phase. Moreover, the FUdR-resistant cells are 14 times more resistant than the sensitive cells to 5,8-deaza-10 methylfolate, an antifolate shown to be a potent inhibitor of thymidylate synthetase (7).

It may be argued that an increase in resistance to a drug need not be mirrored by a corresponding increase in the level of an enzyme inhibited by that drug, as has been reported for many, but not all, methotrexate-resistant cancer lines (30, 31). Specifically, in the case of FUdR the degree of drug resistance might be greater than the observed elevation in thymidylate synthetase if the uptake and/or enzymatic activation of FdUMP were not linear with the concentration of FUdR in the growth medium. The critical factor is the apparent need for 2000 times more FUdR in the tissue culture medium to produce the 8-fold increase in intracellular FdUMP necessary to inactivate the 8-fold increased thymidylate synthetase found in the resistant cells. Our sensitive and FUdR-resistant lines appear to have equal uptake and FUdR phosphorylation activities, as measured *in vitro* using labeled FUdR in micromolar concentrations, rendering a change in K_m or V_{max} in these systems unlikely. These uptake data would also rule out the presence in the resistant cells of a FUdR-binding protein activity not found in the sensitive cell lines, as this would be expected to increase the accumulation rate markedly. No net hydrolysis of FdUMP could be demonstrated *in vitro* in the presence of ATP in sensitive or resistant extracts.

An increase in the rate of hydrolysis of FUdR to the less toxic fluorouracil seems unlikely in view of the virtual absence of any FUdR phosphorylase activity as measured in the forward direction with labeled FU and ribose 1-phosphate. Furthermore, FdUMP, which has been reported (18) to bind specifically the active sites of thymidylate synthetase in the presence of tetrahydrofolate, appears to cause the same strong inhibition of equal activities of the thymidylate synthetase present in extracts from both sensitive and resistant cells, 20 nM FdUMP causing 50% inhibition in both

cases. This suggests a similar activity for each active site, that is, a similar V_{max} for both enzymes. Furthermore, the observation that the assay of mixed sensitive and resistant extracts produced strictly additive thymidylate synthetase activities renders the possibility of cytoplasmic inhibitors or activators unlikely.

Although antifolates have been reported to select for large increases in thymidylate synthetase as well as the directly inhibited dihydrofolate reductase (32), no increase in the latter enzyme and only a small increase in hypoxanthine-guanine phosphoribosyltransferase could be detected here.

Since the C46 variant cell possesses moderate levels of thymidine kinase activity, and no inhibition of thymidine kinase by FdUMP could be detected *in vitro*, growth inhibition due to the presumed inhibition of thymidylate synthetase *in vivo* should be at least partially overcome by this salvage pathway (7). The fact that thymidine concentrations as low as 2 μM did protect our FUdR-sensitive and -resistant cells from growth inhibition by 2 nM and 3 μM FUdR, respectively, is of considerable importance, further supporting the contention that elevation of thymidylate synthetase activity is the principal mechanism of resistance to FUdR in neuroblastoma.

All available evidence supports the conclusion that the elevated activities of thymidylate synthetase seen in our resistant variants are responsible for their increased resistance to growth inhibition by FUdR. Only the large discrepancy between the level of resistance (2000-fold) and the relatively small increase in thymidylate synthetase activity (8-fold) raises the possibility that an additional toxic or a very novel detoxification mechanism is present. Perhaps FdUMP is inserted into RNA molecules only in sensitive neuroblastoma cells (33), producing inhibition of protein synthesis or the synthesis of faulty proteins. This and other possibilities are currently under investigation in our laboratory.

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REFERENCES

1. Amano, T., Richelson, E. & Nirenberg, M. W. (1972) *Proc. Natl. Acad. Sci. U. S. A.*, **69**, 258-263.
2. Rosenberg, R. N. (1973) *Tissue Culture of the Nervous System*, Plenum Press, New York.
3. Steward, J. K. (1964) *J. Clin. Pathol. (Lond.)*, **17**, 407-410.
4. Evans, A. E., Heyn, R. M. Newton, W. A. & Leiken, S. L. (1969) *J. Am. Med. Assoc.*, **207**, 1325-1327.
5. James, D. H., Hustu, O., Wrenn, E. L. & Pinkel, D. (1965) *J. Am. Med. Assoc.*, **194**, 123-126.
6. Rosenberg, R. N., VandeVenter, L., DeFrancesco, L. & Friedkin, M. (1971) *Proc. Natl. Acad. Sci. U. S. A.*, **68**, 1436-1440.
7. Carlin, S. C., Rosenberg, R. N., VandeVenter, L. & Friedkin, M. (1974) *Mol. Pharmacol.*, **10**, 194-203.
8. Biedler, J. L. & Riehm, H. (1970) *Cancer Res.*, **30**, 1174-1184.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265-275.
10. Roberts, D. (1966) *Biochemistry*, **5**, 3546-3548.
11. Friedkin, M., Crawford, E., Humphreys, P. & Goldin, A. (1962) *Cancer Res.*, **22**, 600-606.
12. Taylor, A. T., Stafford, M. A. & Jones, O. W. (1972) *J. Biol. Chem.*, **247**, 1930-1935.
13. Littlefield, J. W. (1965) *Biochim. Biophys. Acta*, **95**, 14-22.
14. Wood, A. W., Becker, M. A., Minna, J. D. & Seegmiller, J. E. (1973) *Proc. Natl. Acad. Sci. U. S. A.*, **70**, 3380-3883.
15. Kessel, D., Deacon, J., Coffey, B. & Bakamjian, A. (1972) *Mol. Pharmacol.*, **8**, 731-739.
16. Hartman, K.-U. & Heidelberger, C. (1961) *J. Biol. Chem.*, **236**, 3006-3013.
17. Cohen, S. S., Flaks, J. G., Barner, H. D., Loeb, M. R. & Lichtenstein, J. (1958) *Proc. Natl. Acad. Sci. U. S. A.*, **44**, 1004-1012.
18. Santi, D. V. & McHenry, C. (1972) *Proc. Natl. Acad. Sci. U. S. A.*, **69**, 1855-1857.
19. Kessel, D. & Wodinski, I. (1970) *Mol. Pharmacol.*, **6**, 251-254.
20. Dubbs, D. R. & Kit, S. (1963) *Proc. Am. Assoc. Cancer Res.*, **4**, 17.
21. Cohen, S. S. (1971) *Ann. N. Y. Acad. Sci.*, **186**, 292-301.
22. Borsa, J. & Whitmore, G. F. (1969) *Cancer Res.*, **29**, 737-744.
23. Plagemann, P. G. W. & Roth, M. F. (1969) *Biochemistry*, **8**, 4782-4789.
24. Skold, O. (1958) *Biochim. Biophys. Acta*, **29**, 651.
25. Sharp, J. D., Capecchi, N. E. & Capecchi, M. R. (1973) *Proc. Natl. Acad. Sci. U. S. A.*, **70**, 3145-3149.
26. Courtenay, V. D. & Robins, A. B. (1972) *J. Natl. Cancer Inst.*, **49**, 45-53.

27. Okuda, H., Arima, T., Hashimoto, T. & Fujii, S. *Cancer Res.*, **32,** 791-794.
28. Bresnick, E. (1974) *The Molecular Biology of Cancer*, p. 290, Academic Press, New York.
29. Kessel, D., Bruns, R. & Hall, T. C. (1971) *Mol. Pharmacol.*, **7,** 117-121.
30. Biedler, J. L., Albrecht, A. M., Hutchison, D. J. & Spengler, B. A. (1972) *Cancer Res.*, **32,** 153-161.
31. Fischer, G. A. (1962) *Biochem. Pharmacol.*, **11,** 1233-1234.
32. Dunlap, R. B., Harding, N. G. L. & Huennekens, F. M. (1971) *Ann. N. Y. Acad. Sci.*, **186,** 153-165.
33. Goldberg, A. R., Machledt, J. H. & Pardee, A. B. (1966) *Cancer Res.*, **26,** 1611-1615.

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