5′-Nucleotide Phosphodiesterase Activity of Floxuridine-Resistant Mouse Glioma

ELIZABETH E. MILLER ˣ, STEPHEN L. LEDIS, K. W. LO, and K. C. TSOU

Abstract □ In tissue culture experiments, cells derived from glioma 26, a transplantable tumor of C57B1/6 mice, were sensitive to both floxuridine (5-fluorodeoxyuridine) and 5-fluorodeoxyuridine-5′-(5-iodo-3-indolyl) phosphate, an enzyme-mediated drug activated by 5′-nucleotide phosphodiesterase. When these compounds were tested on the tumor in the animals at a level of 5 mg/kg for 5 days, tumor growth was inhibited approximately 20% by both compounds. When higher levels of 5-fluorodeoxyuridine, 100 mg/kg four times weekly throughout the lifespan of the mouse, were given, the tumor, although inhibited at first, developed resistance and continued to grow until it killed the animal. Phosphodiesterase levels in the tumor rose as the tumor grew. On the other hand, thymidine kinase levels dropped, as anticipated from the known 5-fluorodeoxyuridine-resistant hepatoma tissue culture data. This enzyme pattern was maintained in transplantable mouse glioma lines established from the resistant tumors. One of these lines, tested at a level of 5 mg/kg for 5 days, showed no response to 5-fluorodeoxyuridine but was still sensitive to 5-fluorodeoxyuridine-5′-(5-iodo-3-indolyl) phosphate. These experiments, therefore, offer a model system and a rationale for the design and study of more compounds that could be activated by the enzyme phosphodiesterase. Such compounds might be used alternatively when resistance to 5-fluorodeoxyuridine develops, a common clinical experience in the use of this anticancer drug.

Keyphrases □ 5′-Nucleotide phosphodiesterase—activation of antitumor compounds, mice □ 5-Fluorodeoxyuridine-5′-(5-iodo-3-indolyl) phosphate—role of 5′-nucleotide phosphodiesterase in antitumor activity, mice □ Floxuridine—role of 5′-nucleotide phosphodiesterase in antitumor activity, mice □ Antitumor drugs—floxuridine and 5-fluorodeoxyuridine-5′-(5-iodo-3-indolyl) phosphate, role of 5′-nucleotide phosphodiesterase in antitumor activity, mice

An interest in the 5′-(5-iodo-3-indolyl) phosphodiester of floxuridine (5-fluorodeoxyuridine) (1, 2) as an enzyme-mediated drug led to the investigation of the 5′-nucleotide phosphodiesterase level of glioma 26, a tumor that showed sensitivity to this compound as well as 5-fluorodeoxyuridine[1]. In addition, the enzyme changes in the same tumor as it became resistant to treatment with 5-fluorodeoxyuridine were investigated.

Umeda and Heidelberger (3) found that Novikoff hepatoma cells, grown in tissue culture, that are resistant to 5-fluorodeoxyuridine have a low thymidine kinase activity. This finding suggested that if the 5′-nucleotide phosphodiesterase level could be maintained in such resistant tumors, the use of phosphodiesters of 5-fluorodeoxyuridine would provide a biochemical rationale for cancer chemotherapy of 5-fluorodeoxyuridine-resistant tumors. In other words, 5-fluorodeoxyuridine 5′-phosphate, the active intermediate (4), could be obtained through a different enzyme activation process.

The present work describes the phosphodiesterase and thymidine kinase activities of the mouse glioma tissue before, during, and after treatment with high levels of 5-fluorodeoxyuridine.

EXPERIMENTAL

Tumors and Animals—The mouse tumor, glioma 26, was originally obtained in the frozen state from the National Cancer Institute tumor bank. It is now maintained in C57B1/6 mice by sterile trocar transplant.

Tissue Cultures—A tissue culture line from glioma 26 was adapted to grow from minced tumor fragments in Eagles Minimal Essential Medium with Hanks base and 10% calf serum. Calf serum was heat inactivated 2–24 hr at 56° if phosphodiesterase was found to be present. When the cell line was established, the comparative cytotoxicity of the drugs on the glioma cells was tested by the method of Umeda and Heidelberger (3).

Due to clumping of the cells, growth was determined by protein determinations instead of cell counts. The assays were carried out in Leighton tubes initially seeded with approximately 60,000 cells/ml of the medium. After incubation for 24 hr at 37° to allow cells to attach to the glass, drug dilutions were added and incubation was continued for 3 days. Cells were then prepared for protein determination by the Lowry *et al.* (5) method by pouring off the growth medium and rinsing the cells three times with 4.0 ml of 0.9% NaCl while they were still attached to the glass surface of the Leighton tube.

The cells were dissolved by adding 5.0 ml of the alkaline reagent used in the protein determination and allowing them to stand for 20–40 min. One milliliter of 0.9% NaCl was added to adjust the volume to that of the protein standard, and the color was developed by adding the phenol reagent directly to the Leighton tubes. In periods of maximum growth, when the amount of color developed exceeded the range of the colorimeter, aliquots were removed and treated like those used for the protein standard. In this way, the effect of clumping was minimized since the entire sample or a large portion of it was represented in the assay, rather than only the small drop from it used on the counting chamber.

The protein value for Day 0 was determined by centrifuging the cells from 4.0 ml of the cell suspension used for preparing the Leighton tubes, washing three times with 0.9% NaCl, dissolving the cell pellet, and making the protein determination as already described. Analyses were always carried out in duplicate, and the reliability of the method is indicated by the high reproducibility obtained for analyses on the duplicate Leighton culture tubes. The average deviation from the mean calculated from 19 pairs of control cultures (untreated with drug) was only 4%. In drug-treated culture tubes when cell growth was inhibited 0–60% by the drug, the average deviation also was only 4%. But when growth inhibition was greater than 60%, the average deviation was 10% (calculated from 132 pairs of drug-treated culture tubes).

Compounds and Methods of Administration—The synthesis of 5-fluorodeoxyuridine-5′-(5-iodo-3-indolyl) phosphate (I) and 5-fluorodeoxyuridine-3′-(5-iodo-3-indolyl) phosphate (II) was reported previously (1, 2). The effect of these drugs was tested on the tumor in the mice according to the following procedure. Twenty-seven mice were injected intraperitoneally with 20 mg/kg of 5-fluorodeoxyuridine, I, II, or 0.9% NaCl, starting on the day after tumor transplantation. Tumor size was calculated from measurements of the length, width, and depth of each tumor at weekly intervals made with centimeter calipers.

In efforts to produce a 5-fluorodeoxyuridine-resistant glioma line, preliminary experiments were carried out to find how much drug could be given without obtaining toxic deaths. Based on these experiments, a level of 100 mg/kg given four times weekly through-

[1] FUdR, Hoffmann-La Roche, Inc., Nutley, N.J.

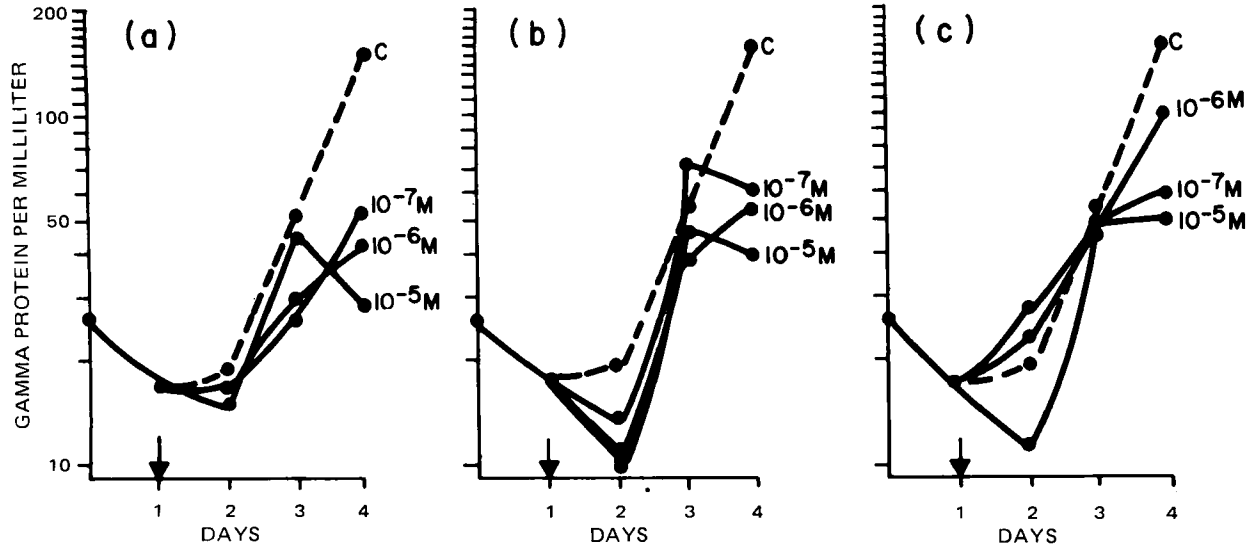

Figure 1—*Effect of 5-fluorodeoxyuridine* (a), *I* (b), *and II* (c) *at* 10^{-5}, 10^{-6}, *and* 10^{-7} M *concentrations on the growth of tissue-cultured mouse glioma cells compared with control cell growth* (*C*). *Compounds were added on Day 1* (↓).

out the tumor growth period was chosen. To study tumor enzyme levels, 37 glioma-bearing mice were treated in this manner. Mice were sacrificed at appropriate intervals to obtain tumor tissue for enzyme assays.

Extraction—The excised tumor tissue was minced, weighed, and frozen immediately in dry ice and alcohol and stored in the frozen state. Extracts from the thawed tissue were made in 0.25 *M* sucrose with a glass homogenizer in an ice bath. Samples of tissue weighing 0.5–1.0 g were extracted with 2 volumes of 0.25 *M* sucrose solution and centrifuged for 10 min at 2000 rpm. The protein concentration was determined by the Lowry *et al.* (5) method.

Enzyme Assays—5′-Nucleotide phosphodiesterase determinations were made by a previously reported method (6) using the fluorogenic substrate thymidine-5′-(4-methylumbelliferyl) phosphate[2].

Thymidine kinase was measured by a paper chromatographic method using the incubation conditions described by Breitman (7). The reaction mixture consisted of 2.0 μmoles of adenosine triphosphate, 2.0 μmoles of magnesium chloride, 0.04 μmole of thymidine, 2.5 μmoles of 3-phosphoglycerate, 33.75 μmoles of tromethamine buffer (pH 8.0), and 50.0 μl of tumor tissue extract in a total volume of 0.25 ml. Reactions were carried out in 2.0-ml screw-capped vials at 37° and were quenched at regular time intervals by placing the vials in a boiling water bath for 2 min.

Each entire reaction mixture was applied to Whatman No. 1 paper in a horizontal band 4.0 cm long and developed with ethanol–1 *M* CH_3COONH_4 (7:3, v/v). This solvent is commonly used in nucleoside and nucleotide separations (8). Several reactions could be chromatographed on each paper sheet. The thymidine bands were cleanly separated from the thymidylate bands by this method. The thymidine bands (higher R_f) were visualized by a short wavelength UV illumination and outlined in pencil. A blank solution consisted of the reaction mixture without the cell extract. The thymidine bands were cut out and quantitatively eluted with 2.0 ml of water.

The absorbances of the eluates were measured[3] at 267 nm (A_{267}). To correct for the small amount of UV-absorbing material in the paper, the absorbances at 300 nm (A_{300}) were also measured. Thus, the true absorbance due to thymidine is $A_{267} - A_{300}$ and the percent conversion can easily be calculated from:

$$\%\ \text{conversion at 10 min} = \left[1 - \frac{(A_{267} - A_{300})}{(A_{267} - A_{300})_{\text{blank}}}\right] \times 100 \quad \text{(Eq. 1)}$$

Because of interference by other enzymes, such as alkaline phosphatase and 5′-nucleotidase, the percent conversion deviated from linearity at about 20 min after incubation. Accordingly, the percent conversion at 10 min per milligram of protein in the extract was taken as the apparent activity of thymidine kinase. This useful and practical method can be done without radioactive thymidine, and it gives a comparative value of the activity of the different extracts.

[2] Fluorescence was measured in a Turner model 111 fluorometer.
[3] Beckman DB-G spectrophotometer.

RESULTS AND DISCUSSION

As shown in Fig. 1, when tested in the tissue culture system, the 5′-(5-iodo-3-indolyl) phosphodiester (I) was found to be more cytotoxic than the 3′-phosphodiester (II) and its activity was comparable to that of 5-fluorodeoxyuridine. In addition, the activity of I on the glioma cells was higher than in other tissue culture systems previously tested (1, 2).

Results obtained with the glioma cells in tissue culture indicated that it would be interesting to test these compounds in mice bearing the glioma. Again, the growth inhibitory effects of I and 5-fluorodeoxyuridine were comparable, a maximum of 20% of control tumor size, and II had little effect. The average survival time was 55 days for 5-fluorodeoxyuridine-treated mice and 58 days for I-treated mice, compared to 44 days for controls. Since clinically 5-fluorodeoxyuridine had been of limited use for human glioma, the mouse data indicated that further study was necessary.

In preliminary efforts to produce a resistant glioma line, low levels of 5-fluorodeoxyuridine were not effective. In studying the toxic effects of higher doses, it was found that 100 mg/kg could be given to normal mice for long periods if administered intermittently. In glioma-bearing mice treated with 100 mg/kg four times weekly, tumor growth was markedly inhibited at first. Under continued treatment, however, the tumor grew progressively until it killed the animals, presumably due to the development of drug resistance.

The phosphodiesterase and thymidine kinase concentrations of tumor tissue during treatment of the glioma with 5-fluorodeoxyuridine are shown in Fig. 2. The drug was administered as indicated by the arrows at the bottom of the chart, 100 mg/kg four times

Table I—Thymidine Kinase Activity of 5-Fluorodeoxyuridine-Resistant Mouse Glioma Tumor Lines

Tumor Line	Thymidine Kinase Activity[a] Experiment 1	Experiment 2
Resistant A	31	26
Resistant B	36	10
Resistant C	21	26
Original	110	97

[a] Percent conversion at 10 min per milligram of protein.

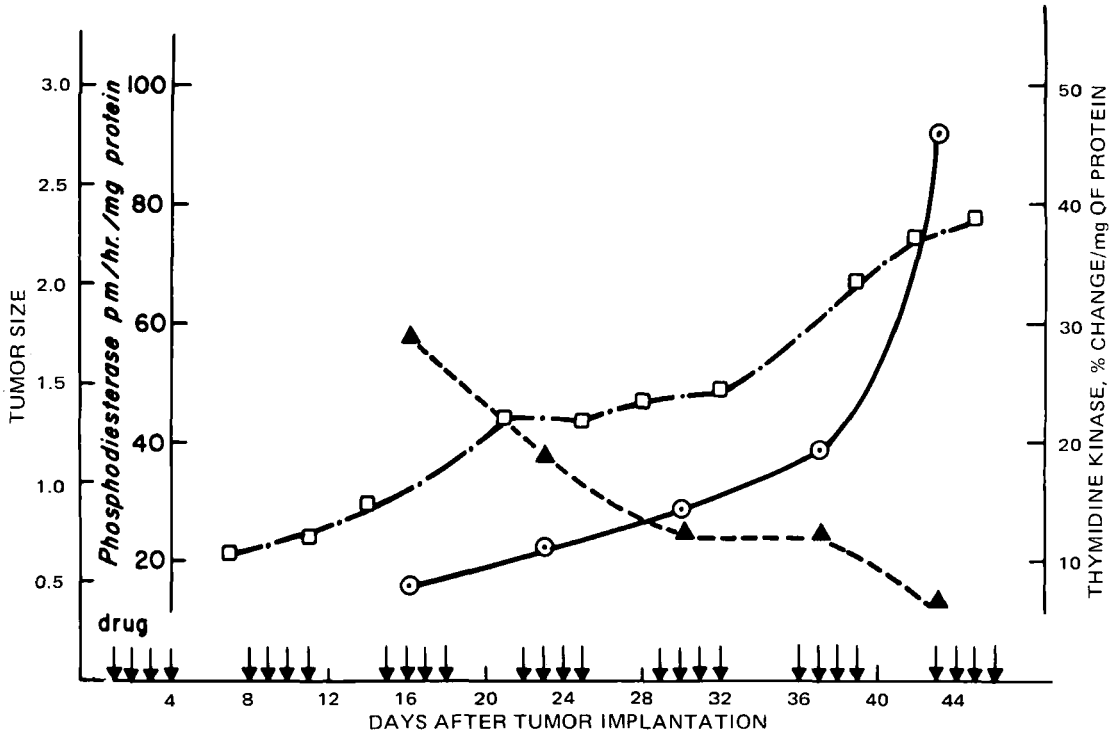

Figure 2—*Phosphodiesterase and thymidine kinase concentrations of glioma tumor tissue from mice during treatment with 5-fluorodeoxyuridine. Key: ⊙, phosphodiesterase; ▲, thymidine kinase; □, tumor growth; and ↓, drug administered intraperitoneally at 100 mg/kg/day.*

weekly. Phosphodiesterase activities and thymidine kinase activities were calculated in terms of milligrams of protein in the extracts. The progressive growth of the tumor is shown by the increase in tumor size plotted against time in days. The phosphodiesterase concentration in the tumor tissue rose as the tumor grew, while the thymidine kinase activity decreased on continued treatment with 5-fluorodeoxyuridine. The decrease in thymidine kinase is an indication of development of resistance to 5-fluorodeoxyuridine, while the maintenance of high levels of phosphodiesterase activity suggests that compounds whose action is mediated by this enzyme would still be effective in these resistant tumors.

In some of these mice receiving high levels of 5-fluorodeoxyuridine, the tumor tended to become very fluid. The fluid accumulated under the skin, and the head became very puffy. This fluid contained tumor cells and produced tumors when injected into mice. Histologically, the original and 5-fluorodeoxyuridine-resistant tumors looked different, the latter tumors tending to have more mitotic figures and a slightly more malignant appearance.

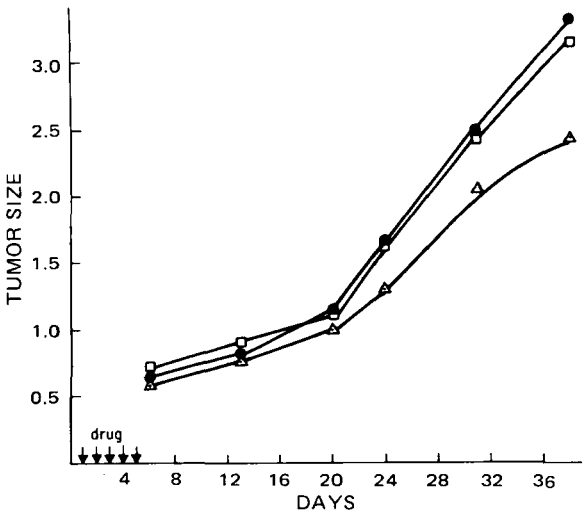

Figure 3—*Effect of 5-fluorodeoxyuridine and I on the 5-fluorodeoxyuridine-resistant glioma tumor line C in mice. Key: ●, control mice injected with 0.9% NaCl; □, mice injected with 5-fluorodeoxyuridine; △, mice injected with I; and ↓, drug administered intraperitoneally at 20 mg/kg or molar equivalent per day. Each point on the curves represents the average measurement of tumor size in six or seven mice.*

Several times during this experiment, animals were killed to obtain tissue for enzyme analysis. New transplants were made in other mice, and three of these tumor lines (A, B, and C) are now carried by transplanting from mouse to mouse at 3–4-week intervals. These lines are maintained with 5-fluorodeoxyuridine; after the tumor is transplanted, each mouse receives three or four weekly injections of 5-fluorodeoxyuridine, 100 mg/kg ip.

After the tumors had been transplanted several times, the enzyme determinations were again made on the tumor tissue taken from mice killed 20–30 days after tumor transplantation, corresponding in time to the midarea in Fig. 2. Table I shows values for thymidine kinase made on the three lines taken from two different transplant generations. Again, low values were obtained compared to those of the original tumors. Table II shows phosphodiesterase values obtained from the same tumor tissue extracts, indicating that the phosphodiesterase concentration was maintained at a level corresponding to that in the original tumor and did not drop under continued influence of high levels of 5-fluorodeoxyuridine.

One resistant glioma line was tested under the conditions used previously with the sensitive glioma line; the compounds were in-

Table II—Phosphodiesterase Activity of 5-Fluorodeoxyuridine-Resistant Mouse Glioma Lines

Tumor Line	Phosphodiesterase Activity[a] Experiment 1	Experiment 2
Resistant A	28.4	28.3
Resistant B	24.2	34.1
Resistant C	22.2	58.1
Original	34.0	28.6

[a] Picomoles per hour per milligram of protein.

jected intraperitoneally for 5 days, starting on the 1st day after tumor transplantation (Fig. 3). On this resistant line, 5-fluorodeoxyuridine had no effect at this level while I still inhibited tumor growth. Obviously, the effects of other nucleoside phosphodiesters whose action also would be aided by high levels of phosphodiesterase in these resistant tumors should be studied.

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[x] To whom inquiries should be directed.

GLC Determination of Plasma Concentrations of Phenprocoumon

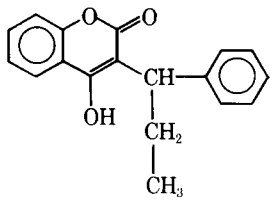
K. K. MIDHA [*x], J. W. HUBBARD [‡], J. K. COOPER [*], and I. J. McGILVERAY [*]

Abstract □ A GLC method for the quantitative estimation of phenprocoumon from plasma is described. Plasma containing phenprocoumon, to which a known amount of phenytoin is added as the internal standard, is acidified and extracted with ethylene dichloride. The drug and the internal standard are then back-extracted into alkali, which is acidified and reextracted with ethylene dichloride. The organic extract is evaporated, and the evaporated residue is mixed with 50 μl of trimethylanilinium hydroxide in methanol. Aliquots (1–2 μl) are injected into a gas chromatograph equipped with a flame-ionization detector in which the injection port is held at 325°. The methyl derivatives of phenprocoumon and the internal standard give sharp, well-separated, symmetrical peaks. The method is of sufficient sensitivity to determine 0.125 μg/ml of the drug in plasma with a coefficient of variation of 7%.

Keyphrases □ Phenprocoumon—GLC determination from human plasma, phenytoin as internal standard □ GLC—analysis, phenprocoumon in plasma, phenytoin as internal standard

Phenprocoumon (I) is an orally administered anticoagulant with action and uses similar to those of phenindione (1). Phenprocoumon is structurally related to warfarin; both are oral coumarin anticoagulants. Several analytical procedures (2–8) were described for the estimation of warfarin and its metabolites from biological fluids, but only one fluorometric method (9) was reported for phenprocoumon. The latter is subject to interference from plasma constituents at low values and from any metabolites present.

The GLC behavior of several coumarin anticoagulants including phenprocoumon, as well as their trimethylsilyl ethers, acetates, trichloroacetates, and trifluoroacetates, was reported (10, 11). These results suggest that derivatization of coumarin drugs might be useful for measuring their concentrations in biological fluids. To facilitate study of the pharmacokinetics and potential interactions with other drugs



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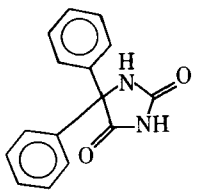

II

after single- and multiple-dose oral administration of phenprocoumon, a sensitive and specific GLC procedure was developed for the measurement of intact drug in plasma.

EXPERIMENTAL

Reagents—Ethylene dichloride[1] and ether[2] were glass distilled prior to use. Stock solutions containing 100 μg/ml of phenprocoumon[3] were prepared daily in 0.1 *N* NaOH and stored in the dark in a refrigerator. Appropriate dilutions (0.125–4.00 μg/ml) were made immediately before use. Aqueous solutions containing 100 μg/ml of the internal standard, phenytoin[4] (II), were prepared weekly by

[1] Caledon Laboratories Ltd., Georgetown, Ontario, Canada.
[2] Mallinckrodt Chemical Works Ltd., Montréal, Québec, Canada.
[3] Hoffmann-La Roche Ltd., Vaudreuil, Québec, Canada.
[4] USP reference standard.