Influence of Ribose Donors on the Action of 5-Fluorouracil

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SUMMARY

Although the most potent antimetabolite formed from 5-fluorouracil \textit{in vivo} is a deoxyribonucleotide, evidence has accumulated which suggests an antineoplastic role for drug ribonucleotides. A study of the consequences of stimulating 5-fluorouracil metabolism along pathways leading to drug ribonucleotides was therefore made, using murine leukemias. Appropriate ribose donors, e.g., glucose and inosine, strongly enhanced the conversion of 5-fluorouracil to ribonucleotides, both \textit{in vitro} and \textit{in vivo}, but this had little effect on drug-promoted survival of tumor-bearing animals.

INTRODUCTION

The antineoplastic agent 5-fluorouracil (FU) rapidly penetrates murine leukemia cells (11, 18, 22) and is subsequently converted to 5-fluorouridine-5'-monophosphate via uridine phosphorylase and uridine kinase (1, 6, 33), and via a pyrimidine-5'-pyrophosphorylase (20, 30). Di- and triphosphates of 5-fluorouridine are also formed (3, 12). The drug is also converted, to a minor extent, to 5'-fluoro-2'-deoxyuridine (FUdRP) (3), but not to deoxynucleoside-5'-di- or triphosphates. All of these derivatives of 5-fluorouracil are potential antimetabolites. The most potent appears to be FUdRP, which strongly inhibits thymidylate synthetase (1, 4, 12, 13), leading to interference with DNA synthesis, but incorporation of FU into RNA (3) and competition between FU and uracil for enzymes involved in uracil metabolism (7, 32) are possible additional sites of drug action. Many cell lines exposed to the drug develop "resistance" to FU by deletion or impairment of cellular enzymes needed for FU conversion to ribotides (7, 20, 28, 29). Presumably FUdRP formation was affected as well. Inherent response to FU in different murine leukemias has been related to cell capacity for conversion of FU to ribotides (23).

A number of reports have indicated that glucose, or other ribose donors, can enhance the rate of FU conversion to nucleotides and can potentiate the antimetabolic action of the drug in tumor cells (9, 26, 35). We have investigated the effects of glucose and of selected ribosides on FU anabolism and on the antitumor action of FU in several murine leukemias. The effect of ribose donors on the rate of FU transport was also measured.

MATERIALS AND METHODS

Labeled and Nonlabeled Compounds. FU-2\textsuperscript{14}C (12 mc/mmole) was purchased from Calbiochem. Nonlabeled FU was provided by Hoffman-LaRoche; inosine, deoxyinosine, and adenosine were purchased from Sigma Chemical Co.; N-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid buffer was purchased from Calbiochem.

Animal Tumors. These were provided by I. Wodinsky of the Arthur D. Little Corp., Cambridge, Massachusetts. The L1210/FU cell line was originally supplied by Dorris Hutchinson, Sloan-Kettering laboratories, and was carried in BDF\textsubscript{a} mice with daily injections (15 mg/kg) of FU. The Flexner-Jobling tumor was carried in Charles River CD random-bred rats. Methods of propagation of other cell lines have been described (31).

Isolation and Incubation of Cells. Ascitic tumor cells were isolated from tumor-bearing animals (31) and resuspended in a medium buffered with 75 mM N-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid (8) at pH 7.3, and containing, at levels found in Ehrlich ascitic fluid (16), 71 mM...
NaCl, 20 mM KCl, 1.5 mM MgCl₂, 1.3 mM CaCl₂ and 1 mM NaH₂PO₄. Substitution of tris(hydroxymethyl) aminomethane buffer (23) did not alter the results obtained. In our studies on human leukocytes, the medium contained 25% dialyzed calf serum (22). Results shown here were not altered by the addition of serum. Incubation tubes contained 200-μl aliquots of a 5-8% cell suspension. Addition of labeled and non-labeled compounds did not exceed 10 μl. Incubations were terminated by centrifugation of tubes at 500 x g for 35 seconds. For further details of these procedures see Reference 21.

**Measurement of 5-Fluorouracil Uptake.** Cells were incubated in medium containing labeled FU. The level varied from 0.1 to 50 mM. The 0.1 mM level was generally used since this was comparable to the drug level achieved in vitro during drug therapy programs and found pertinent for in vitro studies (23). Incubations were terminated by collection of cells by centrifugation. The supernatant fluid was removed, and the pellets were then blotted dry with filter paper and taken up in 250 μl of 0.9% NaCl. A 200-μl portion of this suspension was diluted with 10 ml of a liquid phosphor for determination of radioactivity by liquid scintillation techniques (23). The extent of intracellular conversion of FU to nucleotides was assessed by reincubation of pellets, obtained as described above, for 5 minutes at 37°C in fresh medium containing 10⁻⁴ M dinitrophenol. Addition of the latter compound prevented additional conversion of FU to nucleotides (17) during the second incubation, and free FU was washed from the cells (23). The cells were collected as before, and the residual cellular radioactivity, representing FU nucleotides and drug incorporated into RNA (23), was measured as described above.

**Determination of Water Space of Cell Pellets.** The amount of fluid trapped in cell pellets was determined by incubation in a medium containing labeled sulfate (24) to which the cells are impermeable. Radioactivity associated with the pellets was taken as a measure of extracellular fluid. Similar incubations with tritiated water yielded data on the total extracellular and intracellular water in the pellets. Previous studies (24) have shown that 200-μl aliquots of 5% cell suspensions (10 mg of cells) produce pellets containing 3.5 μl of intracellular water and 4 μl of extracellular water. After incubation at 37°C for 15 minutes, the extracellular space increases 10% at the expense of intracellular space. Appropriate corrections for these data were made in calculation of the apparent distribution of FU between cell water and the incubation medium.

**Dispersal of Solid Tumors.** The Flexner-Jobling tumor was cut into small pieces and forced through a fine wire mesh placed in the bottom of a 5-ml syringe. The cells were collected in 0.9% NaCl; large clumps of tissue were removed, and the suspended cells were collected by centrifugation at 250 x g for 5 minutes. The supernatant fluid was discarded.

**In Vivo Experiments.** Animals bearing ascitic leukemias were used 36 hours before death from the tumor was expected. Animals were treated by intraperitoneal injection with labeled FU and other compounds; the total injected volume was 0.3 ml or less in each animal. At measured intervals thereafter, 20- to 50-μl samples of ascitic fluid and cells were removed with a 26-gauge needle and syringe and used for determination of radioactivity (25). Specific details of washing procedures are given below.

**Estimation of FU and FU and Nucleotides after in Vivo Experiments.** Aliquots of about 20 μl of ascitic fluid were collected in chilled 10 x 30 mm glass tubes, and the cells were collected by centrifugation at 500 x g for 35 seconds. A 2- to 5-μl portion of the supernatant fluid was removed and diluted with 200 μl of water and 10 ml of a liquid phosphor for determination of radioactivity. The cell pellet was blotted dry and total radioactivity determined as described above. An appropriate correction was made, as determined by experiments using labeled sulfate, for contamination of the pellet with extracellular fluid. Duplicate samples of the ascitic fluid were pipetted into tubes containing 200 μl of incubation medium with 1 mM 2,4-dinitrophenol. The cells were collected by centrifugation, resuspended in 200 μl of medium containing 1 mM dinitrophenol, and incubated at 37°C for 5 minutes. This procedure washed free FU from the cells without depleting the cellular FU-nucleotide pool. The cells were then collected, and radioactivity was determined as before.

**Glucose Determinations.** A reduced triphosphopyridine nucleotide-coupled assay using hexokinase and glucose-6-phosphate dehydrogenase (Calbiochem) was used (19).

**Drug Survival Data.** Animals were treated by intraperitoneal injection with specified drug combinations from Day 1 to Day 10 after intraperitoneal inoculation with 10⁶ tumor cells; survival was compared with untreated control animals receiving similar tumor inoculations.

**RESULTS**

**Uptake of FU in Vitro.** Uptake of FU-¹⁴C was studied in the L1210/FU cell line in which intracellular conversion of the drug to nucleotides is absent. At 37°C, a distribution ratio (cell water/medium) of one was attained within 2 minutes, except at the highest extracellular drug level tested (50 μM). Drug to nucleotides is absent. At 37°C a distribution ratio is one obtained within 2 minutes, except at the highest extracellular drug level tested (50 μM). Drug uptake at 0°C was sufficiently slow to show the variation of uptake rate as a function of the external FU level (Table 1).

<table>
<thead>
<tr>
<th>Drug level in medium (μM)</th>
<th>0°C</th>
<th>37°C</th>
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</thead>
<tbody>
<tr>
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<td>5 min</td>
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<tr>
<td>0.1</td>
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<td>50</td>
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Table 1

Uptake of 5-fluorouracil (FU) by L1210/FU cells in vitro.

The time and temperature of incubations are indicated. Data represent the mean of five determinations. Results are given in terms of drug concentration in cell water divided by the drug level in incubation medium.
Approximate kinetic constants derived from rate measurements at 0°C indicate a $K_m$ of 40 mM and a $V_{max}$ of 5 mmol/kg cell water/minute. Addition of glucose (1-10 mM), inosine (1-20 mM), deoxyinosine (1-20 mM), adenosine (1-20 mM), or 2,4-dinitrophenol (1 mM) did not alter the rate of FU uptake by L1210/FU cells at 0°C or at 37°C.

**FU Conversion to Nucleotides in Vitro.** The relative extent of conversion of labeled FU into nucleotides and RNA by freshly isolated tumor cells is shown in Table 2. The effect of supplementation with glucose, inosine, or adenosine is also shown. Addition of 0.1-1 mM 2,4-dinitrophenol abolished FU incorporation into nucleotides in all cell lines tested. The stimulation obtained with glucose could be duplicated if fructose were substituted; galactose or ribose were ineffective. These data show that the capacity for FU conversion to nucleotides varied considerably among these cell lines as previously reported (23). The extent of stimulation of this conversion by the various sugars was peculiar to each line.

<table>
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<tr>
<th>Cell line</th>
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<th>Adenosine</th>
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<td>S178YF</td>
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<tr>
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<td>100</td>
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<td>17</td>
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*Conversion of 5-fluorouracil (FU) to nucleotides and RNA by tumor cells in vitro.*

*Data are in terms of μmol/kg cells/15 minutes. The rate of conversion was linear during the time. Values shown are the mean of five determinations. The medium contained 0.1 mM labeled FU. Glucose (5 mM), inosine (20 mM), or adenosine (20 mM) was added to the incubation medium.

**Influence of FU Concentration.** The incorporation of FU into nucleotides and RNA was measured in L1210 and P388 cells in vitro at various concentrations of FU. The process was saturated only at relatively high FU levels (5-10 mM). This is far in excess of the drug levels achieved in vivo during drug therapy programs (23). Addition of glucose (5-15 mM) enhanced the extent of FU incorporation but did not significantly alter the FU level required to saturate the process (Chart 1).

**Glucose Levels in Ascitic Fluids.** The glucose concentration in ascitic fluid of animals used in these experiments ranged from 0.02 to 0.1 mg/ml. This could be briefly elevated to 8-10 mg/ml by intraperitoneal injection of 2.5 gm of glucose per kg into animals bearing advanced ascitic leukemias. The glucose level in the ascitic fluid fell rapidly thereafter and had decreased to below 0.1 mg/ml within 2 hours. These data agree with a recent report (27) that free glucose in ascitic fluid of animals bearing the Ehrlich carcinoma could not be detected. The above data emphasize the rapid glycolysis carried out by most animal leukemias in vivo.

**FU Conversion to Nucleotides in Vivo.** An intraperitoneal injection of a therapeutic dose of labeled FU (15 mg/kg) into mice bearing advanced P388 leukemia led to rapid drug incorporation into nucleotides and RNA (Chart 2). Within 30 minutes, no free drug could be detected in the tumor cells. Simultaneous injection of glucose (500 mg/kg) or inosine (100 mg/kg) significantly increased the total FU incorporation. Further elevation of the glucose or inosine levels did not enhance FU incorporation. Similar data were obtained with L1210 or P1534Ja cells although the extent of FU incorporation and the stimulation afforded by glucose or inosine varied. If administration of glucose or inosine were delayed for 30

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3 Data to be published separately. The single exception to this finding occurred in animals bearing the Gardner leukemia; elevated glucose levels produced by administration of 2.5 gm/kg of the compound persisted for several hours.

4 The extent of FU incorporation increased linearly as the FU dose was varied between 1 and 15 mg/kg. The labeled products were identified according to the procedure described in Reference 23.
Chart 2. Incorporation of 5-fluorouracil (FU) into nucleotides and RNA of P388 cells in vivo. Tumor-bearing animals were given 15 mg/kg of the labeled drug by intraperitoneal injection, given concurrently with glucose (500 mg/kg) or inosine (100 mg/kg) as shown. The dashed line indicates that no glucose or inosine was added. Sequential samples of ascitic fluid were removed, and the level of drug incorporation was measured. A typical experiment is shown here.

Table 3

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Table 4

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</table>

Effect of inosine on the antitumor response to 5-fluorouracil (FU) in murine leukemias. Tumor-bearing animals were treated from Day 1 to Day 10 with specified levels of FU and/or inosine, following inoculation with 10⁶ tumor cells. Both inoculation and drug treatment were via intraperitoneal injection.

Data are expressed as defined in Footnote a, Table 3.

Drug toxicity was observed in these animals as shown by decreased survival compared with control animals.

4. The P388 and P1534J_a tumor lines were chosen because of the striking increase in FU conversion to nucleotides produced by glucose or inosine in vitro. This phenomenon had only minor therapeutic consequences. There was no evidence that glucose or inosine exerted a "sparking" effect on FU, i.e., a low, ineffective dose of the drug was not made more effective by concurrent glucose or inosine administration. The major effect of inosine was an increase in drug toxicity as shown by the decreased survival time of animals receiving FU and inosine (Table 4).

Although glucose and inosine were ineffective in promoting antitumor responses to FU, these compounds did cause an increase in the rate of FU incorporation into cell nucleotides and RNA in vivo. A representative experiment, the effect of 30 mg/kg of inosine on the incorporation of 1.5 mg/kg of FU-¹⁴C by P388 cells over a 25-hour period, is shown in Chart 3. Administration of inosine resulted in a 2-fold initial elevation of FU incorporation. After 25 hours, the cells treated with labeled FU and inosine retained 2-fold more radioactivity (identified as FU incorporated into cell RNA) than cells treated with labeled FU alone. This level of inosine did not potentiate the antitumor action of FU (Table 4).

Identification of Intracellular Radioactivity. The "incorporated" radioactivity which could not readily be washed from
tumor cells was identified chromatographically as the mono-, di-, and triphosphates of 5-fluorouridine and FU incorporated into RNA (23). After short incubations (10-60 minutes), the acid-soluble radioactivity was mainly localized in the triphosphate, and FU incorporation into RNA represented 25-35% of the total nondiffusible radioactivity. After the longer incubation times (10-25 hours in vivo), the nondiffusible label was all found in the acid-insoluble RNA fraction. Incorporation of FU into FUdRP was not detectable by the methods used here. Our studies on metabolic transformations of FU will be reported in detail in a separate publication.

**DISCUSSION**

The experiments described here were designed to assess the possibilities of potentiating the antineoplastic action of FU by ribose donors. A previous study (23) had shown that the rate of conversion of FU to ribonucleotides and RNA, measured in vitro, was strongly correlated with drug response in different murine leukemias. This finding suggested that metabolism of FU along ribonucleotide-forming pathways could contribute to drug action. Another report (7) postulated that the main site of action of FU was on the salvage pathway from uracil to RNA, in which case competition between FU and uracil for ribotide-forming enzymes would be a major site of drug action. Related studies showed that supplementation with glucose or nucleosides increased cellular rates of nucleotide formation from uracil by Ehrlich carcinoma cells (34); that glucose potentiated the action of FU in decreasing transplantability of murine leukemias (35); and that inosine and glucose enhanced the rate of incorporation of FU into nucleic acids by the Ehrlich cell (9). Finally, potentiation by glucose of the antitumor effect of FU against the Flexner-Jobling rat carcinoma was reported (26).

In the present study, it was found that conversion of FU into ribonucleotides (and subsequently into RNA) by freshly isolated tumor cells could be strongly stimulated by glucose or by other ribose donors. The extent of this stimulation was peculiar to each cell line and apparently did not involve an increase in the rate of FU transport. The latter was characterized, in a cell line incapable of further FU metabolism, as a relatively temperature-insensitive, not easily saturable process which was not enhanced by addition of glucose or ribosides. The freshly isolated tumor cells used in this and previous (23) experiments were found deficient in ribose donors needed for optimal cellular synthesis of FU ribonucleotides because of the low glucose content of ascitic fluid reported here and elsewhere (26).

Although supplementation by glucose or certain nucleosides strongly stimulated the rate of FU ribonucleotide formation, especially in the P388 or P1534Ja leukemias, a corresponding potentiation of the antitumor action of FU was not observed, nor was a “sparing” effect on FU produced. The only effect of inosine observed was a potentiation of drug toxicity. Our experiments showed that conditions for these in vivo trials were defined by the very rapid disappearance of glucose and FU from the ascitic fluid. Therapeutic doses of FU were lost within 30 minutes by conversion to nucleotides or by catabolism. Intraperitoneal injections of glucose led to only transitory elevations in the glucose level of the ascitic fluid. Therefore, in our studies, FU and glucose (or inosine) were injected simultaneously. Even under these conditions, wherein stimulation of FU conversion into ribonucleotides and into RNA could be demonstrated, the antitumor action of the drug was not increased. Certain FU-inosine combinations resulted in increased drug toxicity. It is noteworthy that attempts to promote the antineoplastic action of FU in man by glucose administration were ineffective (5, 10).

The present data suggest to us that the rate of incorporation of FU into ribonucleotides and RNA is not, in itself, a determinant of cell responsiveness to FU but that the latter is related to cellular production of the potent FU antimetabolite FUdRP (3, 4, 12, 13). Measurements of FU conversion to ribonucleotides in the absence of added ribose donors are believed to be an index of FUdRP formation. Addition of ribose donors presumably stimulates formation of FU ribonucleotides but not of FUdRP. FU conversion to ribotides occurs by both sequential enzymatic addition of ribose and phosphate (33) and via a pyrimidine-5'-pyrophosphorylase (20, 30). Either could be stimulated by providing sources of ribose-1-phosphate or of 5-phosphoribosyl-1-pyrophosphate (14, 15), such as glucose or ribosides.

In a limited number of preliminary tests, we have unsuccessfully attempted to promote the action of FU against murine leukemias by several deoxyribonucleosides on the assumption that this might enhance the formation of FUdRP. This procedure, previously useful in increasing incorporation of thymine into leukocyte DNA (2), was ineffective here.
A comment should be made regarding the present method of assessing drug responsiveness, namely, drug-promoted survival time of tumor-bearing animals. This is not necessarily a measure of drug-induced inhibition of tumor cell division. We have previously observed, for example, death of tumor-bearing animals when drug therapy had apparently eliminated all tumor cells from the peritoneal cavity.

We conclude from the present study that the conversion of FU into drug ribonucleotides in murine leukemias may be necessary, but it is apparently not sufficient for an antineoplastic drug response as indicated by drug-promoted survival of tumor-bearing animals. Deletion of enzymes involved in FU conversion to ribonucleotides (7, 20, 28-30) may be an important factor in drug resistance only because subsequent conversion of drug ribotides to FUdRP is thereby prevented. The next step in these investigations is a study of pathways and control mechanisms involved in FUdRP formation.

REFERENCES


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