Comparison of the Actions of 5-Fluorouracil and Ftorafur in *Escherichia coli*

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The actions of 5-fluorouracil (FU) and Ftorafur (NCS 148958) were compared in *Escherichia coli* B3 to determine if Ftorafur can act in ways other than through the production of FU. Very high concentrations of Ftorafur relative to FU were necessary to achieve a comparable inhibition of cell growth. Inhibition by both agents could be reversed by uracil. An FU-resistant strain and an Ftorafur-resistant strain were isolated and found to be cross-resistant to Ftorafur and FU, respectively.

A derivative of 5-fluorouracil (FU), 1-(2-tetrahydrofuranyl)5-fluorouracil (Ftorafur [NSC 148958]), has been synthesized in the Soviet Union and shown to be an effective antitumor agent. The spectrum of antineoplastic activity of Ftorafur is similar to FU, but it appears to be less myelosuppressive (2). It has been suggested that the cytotoxic activity of Ftorafur in murine L1210 leukemia cells may reflect cleavage of the drug with the subsequent release of FU (5). In this report we describe experiments that were designed to compare the mechanisms of action of FU and Ftorafur in *Escherichia coli* B3. Three types of comparative experiments were carried out: (i) growth inhibition; (ii) reversal of growth inhibition by uracil; and (iii) isolation of drug-resistant strains and determination of cross-resistance.

MATERIALS AND METHODS

Chemicals. Ftorafur was obtained through T. L. Loo from the Drug Research and Development Branch of the National Cancer Institute, Bethesda, Md. All other chemicals were purchased from appropriate commercial sources.

Growth of cells. *E. coli* B3, a thymidine-requiring strain, was grown in a previously described minimal medium (7) that was supplemented with 2 μ g of thymidine per ml. Growth curves were routinely carried out in 250-ml nephelometer flasks (19 by 130 mm, with side arms) containing 10 ml of medium and additions as described. Incubation was at 37°C in a New Brunswick G-77 water bath shaker. The media were inoculated with an overnight culture such that the absorption at 420 nm was 0.07 to 0.15. An absorption reading of 0.1 was equal to 4.2×10^7 colony-forming units per ml. Thereafter, absorption readings were taken every 30 min at 420 nm with a Bausch and Lomb Spectronic 20 colorimeter.

Isolation of drug-resistant strains. Two sublines of *E. coli* B3, one capable of full growth in 10 μ g of

FU per ml of medium and a second exhibiting full growth in 200 μ g of Ftorafur per ml, were obtained by serial cultivation of the parent strain in increasing concentrations of the respective drugs. Pure colonies were isolated by streaking onto nutrient agar. The stability of the phenotype was tested by carrying each strain for several transfers in the absence of drug. The susceptibility of each strain to varying concentrations of the agent was then tested as described above.

RESULTS AND DISCUSSION

For simplicity, *E. coli* B3 was chosen for these experiments to assure that thymidylate synthetase would not, in this case, be a primary target. (The organism requires thymidine.) Thus, any observed inhibition by FU would presumably reflect formation of FU ribonucleotides and incorporation into ribonucleic acid. In these forms, FU has been demonstrated to interfere with several cell functions in *E. coli*, including cell wall synthesis (8), ribosome maturation (1), and the fidelity of translation (6).

The growth curves of Fig. 1 and 2 show the comparative inhibitory activities of FU and Ftorafur toward *E. coli* B3. Very low concentrations of FU, 0.05 to 0.2 μ g/ml, dramatically inhibited the cultures. Ftorafur, on the other hand, was active only at very high levels (25 to 200 μ g/ml). It is possible that this inhibition arose from spontaneous cleavage of Ftorafur, resulting in the formation of FU, especially since very low levels of FU (0.05 μ g/ml) can produce significant inhibition. The following experiments were performed to clarify this point.

Since the synthesis of deoxyribonucleic acid should not be affected by FU in this system, the inhibition produced should be reversible by uracil. When a culture of strain B3 was exposed

FIG. 1. Inhibition of growth of *E. coli* B3 by FU. Cultures were grown as described in minimal medium supplemented with thymidine and varying concentrations of FU. Symbols: ●, control; ○, ▲, and △, 0.05, 0.08, and 0.20 µg of FU per ml in the culture medium, respectively.

FIG. 2. Inhibition of growth *E. coli* B3 by Ftorafur. The experiments were executed as described for Fig. 1. Symbols: ○, control; ●, □, and △, 25, 75, and 200 µg of Ftorafur per ml in the culture medium.

to a highly inhibitory concentration of FU (10 µg/ml), the presence of 20 µg of uracil per ml almost entirely abolished the effect (Fig. 3A). The fact that a nearly complete reversal occurred suggests that uracil was preferentially

used by the cell. In a comparable experiment with Ftorafur (Fig. 3B), uracil again effected a complete reversal of the inhibition, suggesting similarities in the action of the two agents.

If the action of Ftorafur arises from a spontaneous cleavage that produces FU, it should be possible to enhance the inhibitory activity of the compound by preincubation. Figure 4 shows

FIG. 3. Reversal of FU (A) and Ftorafur (B) inhibition of growth by uracil. Cultures were grown as for Fig. 1 with the following additions: ●—●, control (no additions); ●—●, 10 µg of ml FU per ml (A) or 100 µg of Ftorafur per ml (B); ●—●, 10 µg of FU per ml plus 20 µg of uracil per ml (A) or 100 µg of Ftorafur per ml plus 20 µg of uracil per ml (B).

FIG. 4. Effect of preincubation on growth inhibitory properties of FU and Ftorafur. Portions of media containing Ftorafur (160 µg/ml) were preincubated for 18 h at 37 and 0°C. Each was then diluted 1:1 with minimal medium containing the inoculum. Cultures were grown and monitored as described for Fig. 1. Symbols: ○, control; ●, Ftorafur at 0°C; ▲, Ftorafur at 37°C.

FIG. 5. Resistance of *E. coli* B3 (A), an *Ft*-resistant strain (B), and an *FU*-resistant strain (C) to *FU* and *Ftorafur*. Cultures were grown as for Fig. 1. Symbols: Control cultures (○) contained no drug; *FU* concentration in each case was 10 $\mu\text{g/ml}$ (△); *Ftorafur* concentration was 200 $\mu\text{g/ml}$ (●).

the results of preincubation of *Ftorafur* in medium without cells. After preincubation for 18 h and subsequent incubation with cells, the inhibitory activity of *Ftorafur* was greatly enhanced. When a similar experiment was carried out with *FU* (not shown), there was no change in drug activity. Although this observation has no bearing on other possible modes of action of *Ftorafur*, it does substantiate the spontaneous cleavage hypothesis.

Another means of detecting similarities or dissimilarities in the mode of action of drugs is to isolate drug-resistant strains and determine the degrees of cross-resistance to potentially related agents. An *FU*-resistant strain was isolated from *E. coli* B3 as described in Materials and Methods. This strain could grow normally in the presence of at least 10 μg of *FU* per ml (Fig. 5C), a level which is extremely toxic to the parent strain, *E. coli* B3 (Fig. 5A). When the *FU*-resistant strain was challenged with a toxic concentration of *Ftorafur* (200 $\mu\text{g/ml}$) the strain was found to be equally resistant (Fig. 5C). Likewise, a *Ftorafur*-resistant strain was cross-resistant to a high concentration of *FU* (Fig. 5B). Although the mechanisms of the resistance demonstrated by these strains are not known, the observation that they are indeed cross-resistant suggests again that *FU* and *Ftorafur* share similar mechanisms of action in *E. coli*.

The comparative experiments described here indicate that *Ftorafur* acts only as a release form of 5-fluorouracil in this system. All attempts to observe inhibitory activity unattributable to *FU* have been negative. Repeated

attempts were made to detect enzymatic cleavage of *Ftorafur*, both with crude cell-free preparations of bacteria and purified pyrimidine nucleoside phosphorylase; all were unsuccessful. Since the spontaneous cleavage of *Ftorafur* to form *FU* can occur at a very slow rate in solution (unpublished observation), the inhibition observed toward *E. coli* probably reflects the formation of *FU* in this way.

The antitumor activity and toxicity of *Ftorafur* in man may be attributable, in part, to the spontaneous cleavage reaction. It is, however, probable that the agent is metabolized in the liver. Definition of the products of this metabolism, their rates of formation, and their cytotoxic activity, if any, will clarify this question.

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