RESEARCH ARTICLES

Studies on the Mechanism of Fluoropyrimidine Cytotoxicity in L1210 Cells: Correlation with Inhibition of Thymidylate Synthetase but Not with Incorporation into RNA[4]

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Abstract: The effects of 5-fluorouracil (FUra), 5-fluorouridine (FUrd), and 5-fluoro-2'-deoxyuridine (FdUrd) on L1210 cells were examined in an effort to determine whether the cytotoxicity of these fluoropyrimidines is more closely associated with incorporation of FUra residues into RNA or inhibition of thymidylate (dTMP) synthetase (5,10-methylenetetrahydrofolate: deoxyuridylate C-methyltransferase, EC 2.1.2.45) by 5-fluoro-2'-deoxyuridylate (FdUMP). In different batches of cells exposed to equitoxic (LD_{50}) doses of these drugs for 48 hr, the levels of free FdUMP, dUMP, and free dTMP synthetase were found to be very similar. However, the number of FUra residues incorporated into total cellular RNA were in the approximate ratio of 1:10:100 in cells treated with FdUrd, FUrd, and FUra, respectively. Although these results are consistent with a common DNA-directed mechanism of toxicity, thymidine (dThd), which should circumvent dTMP synthetase inhibition, did not rescue the cells from the effects of FUra. However, uridine (Urd), which should compete with FUra for incorporation into RNA, had no effect on the toxicity of FUra either. Urd at 10^{-5} M did not decrease the amount of incorporation of 10^{-7}M [^{3}H]FUra into total RNA, but a limited fractionation of polysomal RNA showed about a 4-fold decrease of incorporation of FUra into mRNA in the presence of Urd. Urd and dThd did effectively decrease the cytotoxicity of FUrd and FdUrd, respectively. These observations suggest that cell rescue experiments may not be reliable indicators of the mechanism of cytotoxicity of antimetabolites with complex mechanisms of action.

FUra has been extensively used in the treatment of various human tumors since its development in 1957 by Heidelberger (1). This drug is one of the few to elicit a response in patients suffering from advanced solid cancers, and is used as a curative therapy for certain epithelial neoplasms (2, 3). In addition, fluorinated pyrimidines derived from FUra have been very useful as biochemical tools of a variety of problems in molecular biology, cell biology, and enzymology (4). Although extensive and detailed investigations have resulted in a reasonably good understanding of the metabolism and the biochemistry of FUra (5, 6), the mechanism by which this drug exerts its primary cytotoxic (and, therefore, chemotherapeutic) effect has not yet been clearly established.

The early discoveries of Cohen and coworkers (7) that administration of FUra to cells produces a powerful inhibition of dTMP synthetase by the active metabolite FdUMP, resulting in inhibition of DNA synthesis while allowing RNA and protein to increase ("thymineless death"), have historically caused this to be considered the principal mechanism of cytotoxicity by most investigators. FdUMP has been shown to form a tightly-bound ternary covalent complex with dTMP synthetase in the presence of 5,10-CH_2H_4 folate (8). It has also long been known that, in addition to causing inhibition of DNA synthesis, FUra is also readily incorporated into RNA as a Ura analog (4). The major biochemical effect of the substitution of FUra for Ura seems to be an inhibition of the maturation of ribosomal RNA (5, 6). However, it has not been demonstrated that FUra incorporation into RNA leads to cell death, especially at the levels of incorporation that might result from clinically administered doses. Several years ago it was discovered that dThd, which should circumvent a blockage of dTMP synthetase activity by providing an alternate source of TTP, actually seemed to enhance the anti-tumor activity of FUra in animals (9, 10). The apparent chemotherapeutic enhancement was correlated with increased incorporation of FUra into RNA (10), thereby elevating the question of the relative importance of RNA as the site of action of FUra compared to inhibition of DNA synthesis to the status of a controversy. However, in spite of an intensive re-examination of this problem, it still remains unresolved.

In an effort to discover whether generation of FdUMP or incorporation into RNA is most closely associated with antitumor activity of fluoropyrimidines, we exposed cells in culture to equitoxic doses of the fluoropyrimidine derivatives FUra, FUrd, and FdUrd, each of which are converted to the active nucleotide forms by a different pathway. Then, the relative amounts of dUMP, free FdUMP, the ratio of FdUMP-bound to free dTMP synthetase, and the extent of incorporation into RNA generated from each drug were measured. The effects of dThd and Urd on the cytostatic activity of FUra and on its incorporation into RNA were also examined.

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Materials and Methods

Materials. L-1-(+)CH_2H_4folate was synthesized as described (11). All radiolabeled nucleosides and nucleotides were obtained from Moravek Biochemicals (City of Industry, CA). FUra, FdUrd, FdUMP and poly(U)Sepharose were obtained from Sigma Chemical Co. FUrd was a gift of Hoffman-LaRoche Inc., Nutley, N. J. All media and sera were obtained from the Grand Island Biological Co., Santa Clara, CA.

Cell culture techniques. L1210 and CCRF-CEM murine leukemia cells were grown in suspension culture RPMI 1640 medium supplemented with 10% dialysed fetal calf serum. Fresh cells were thawed from mycoplasma-free stocks at 2 month intervals to ensure genetic stability.

Determination of drug toxicity. As a measure of toxicity, the ability of cells treated with drugs to form colonies in soft agarose was measured according to the method described by Mulkins and Heidelberger (12).

Determination of growth inhibition. The fluoropyrimidines used in this study were tested for ability to inhibit the growth of cells by the technique of Grindey and Nichol (13) as adapted by Mulkins and Heidelberger (12).

Preparation of cell cytosol for determinations of metabolite and enzyme levels. L1210 cells (5 x 10^5/ml) were suspended in 250 ml of the standard growth medium containing the appropriate concentrations of FUra, FUrd, or FdUrd. The mixture was incubated for 48 hr, and the cells removed by centrifugation at 2000 rpm for 10 min. The cell pellet was washed twice with 5 ml of phosphate buffered saline. The pellet was suspended in 4.5 ml of 100 mM Tris buffer, pH 7.6, containing 20 mM 2-mercaptoethanol and sonicated at 4°C with three 10 sec bursts of 100 watts with the microtip of a Bronsonic ultrasonicator. The sonicate was centrifuged at 105,000 g for 60 min.

Determination of free dTMP synthetase. To aliquots of the cytosol (1.0 ml) were added [^{3}H]FdUMP (0.1 μM) and 5,10-CH_2H_4folate (0.1 mM) along with more of the sonication buffer to bring the volume to 2 ml. The amount of [^{3}H]FdUMP bound to dTMP synthetase was measured by treatment of the supernatants with a charcoal suspension containing dextran and bovine serum albumin to remove unbound [^{3}H]FdUMP (14).

Determination of free FdUMP levels. The procedure for preparation of the cytosol was the same except that the radiolabeled fluoropyrimidines were used. After aliquots of the cytosol were frozen and lyophilized to dryness, the residue was dissolved in water and applied to a mini-column of DEAE-cellulose. Elution of [^{3}H]FdUMP was carried out with 300 mM ammonium bicarbonate, as described by Moran et al. (14). This column fraction was further treated with excess bacterial dTMP synthetase in the presence of 0.1 mM 5,10-CH_2H_4 folate for 30 min. The amount of enzyme-bound [^{3}H]FdUMP resulting was measured by the charcoal absorption method (14).

Determination of dUMP levels. The concentration of dUMP in the cytosol was measured by converting the dUMP to [^{14}C]dTMP by bacterial dTMP synthetase in the presence of [^{14}C]CH_2H_4 folate formed with [^{14}C]CH_2O as described by Moran et al. (14).

Determination of incorporation into total RNA. The total amount of radiolabeled [^{3}H]FUMP incorporated into RNA was determined by precipitation of the RNA with trichloracetic acid after dissociation of the dTMP synthetase-[^{3}H]FdUMP complex as described by Washtien and Santi (15).

Isolation of polysomal RNA. Polysomes from L1210 cells were isolated by the magnesium precipitation method of Palmiter (16), and the total polysomal RNA was extracted according to Lee et al. (17).

Fractionation of polysomal RNA. The RNA isolated from polysomes was fractionated by the method of Eiden and Nichols (18), involving preferential absorption of poly-(A)RNA to a poly (U) Sepharose column.

Determination of total RNA. The concentration of RNA in the cell cytosols was estimated by the method described by Wilkinson and Crumley (19).

Results

Correlation of FdUMP, dUMP, free dTMP synthetase and RNA incorporation with toxicity. The doses at which each fluoropyrimidine inhibits cell growth by 50% (IC_{50}) were almost identical to the concentrations required to kill 50% of the cells (LD_{50}) as determined by a soft agar cloning assay (Table 1). For convenience, we therefore used cell-growth inhibition assays in the rescue experiments. L1210 cells in culture were exposed to three fluorinated pyrimidine derivatives FUra, FUrd, and FdUrd at concentrations of 3 x 10^{-7} M, 5 x 10^{-9} M, and 3 x 10^{-10} M, respectively. Table 2 shows the

Table 1. Concentrations of the fluoropyrimidines in cultures of L1210 cells required for 50% lethality (LD_{50}) and 50% inhibition of cell growth (IC_{50}).*

Drug	LD_{50}	IC_{50}
FUra	3 ± 1 x 10^{-7} M	3.6 ± 0.7 x 10^{-7} M
FdUrd	3 ± 1 x 10^{-10} M	5.3 ± 1.1 x 10^{-10} M
FUrd	5 ± 1 x 10^{-9} M	4.1 ± 1.2 x 10^{-9} M

* Cells were grown for 48 hr (4–5 doublings) in the presence of the drugs.

Table 2. Comparison of free FdUMP, dUMP, incorporation of FUra into total RNA, and the amount of free dTMP synthetase following exposure of L1210 cells in culture to equitoxic (LD_{50}) doses of the fluoropyrimidines.*

Drug	Free FdUMP	dUMP	FUra residues in total RNA	Ratio of free dTMP synthetase concentration in drug-treated and control cells
	(pmoles/10^9 cells)			
FUra	4.2±1.1	376±20	12.9±2.2×10^3	0.48±0.04
FdUrd	3.4±0.5	322±24	0.12±0.02×10^3	0.45±0.05
FUrd	3.2±0.6	337±30	1.9±0.4×10^3	0.50±0.05

* The values are the means ± SD of 6-12 different experiments. The free FdUMP, dUMP, and radiolabeled RNA were determined using different aliquots of the same cytosol of cells treated with the radiolabeled drugs. The free dTMP synthetase levels were determined in separate experiments using the unlabeled drugs.

quantitites of free FdUMP, free dTMP synthetase, dUMP and amount of incorporation into total RNA after a 48 hr exposure to these conditions. At equitoxic doses the levels of FdUMP, dUMP and free dTMP synthetase originating from all three forms of the drug are very similar, whereas the amount of incorporation of FUra into total RNA varies widely, over a range of about 100-fold.

Cell protection experiments. dThd at a concentration of 10^{-6} M increased the IC_{50} value of FdUrd by about 20-fold (Fig. 1) but did not alter the inhibitory effect of FUra (Fig. 2). Urd at a concentration of 10^{-4} M competed effectively with FUrd, increasing the IC_{50} value for this drug from 5×10^{-9} M to 1×10^{-7} M (Fig. 3). However, 10^{-4} M Urd did not appreciably change the IC_{50} value of FUra (Fig. 4).

Competition between FUra and Urd for incorporation into RNA. Although dThd did not appreciably rescue the cells from the inhibitory effect of FUra, neither did Urd at a fairly high concentration fo 10^{-4} M. Rescue of the cells from FUra by Urd would be reasonable to expect if incorporation of FUra into RNA *via* its ribonucleotides (FUMP, FUDP, and FUTP) were the major mechanism of cytotoxicity, since Urd is converted to the analogous uridine nucleotides UMP, UDP, and UTP. In order to determine the extent of competition for incorporation, we incubated 10^{-7} M [^{3}H]FUra with the L1210 cells in the absence and presence of 10^{-5} M Urd. The Urd had no effect on the extent of incorporation of [^{3}H]FUra into the total RNA fraction as well as the RNA content of the cells (Table 3).

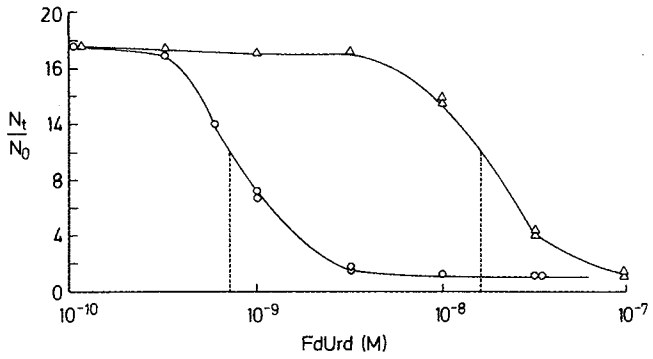

Fig. 1 Effect of Thd on the cytotoxicity of FdUrd to L1210 cells as determined by measuring cell growth inhibition. Cells were exposed for 48 hr to various concentrations of FdUrd alone (O) and FdUrd + 10^{-6} M dThd (△). The dotted lines indicate the IC_{50} values.

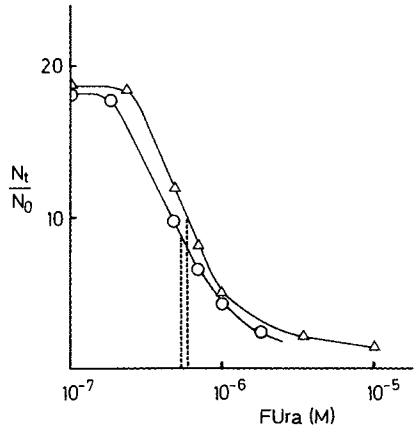

Fig. 2 Effect of dThd on the cytotoxicity of FUra to L1210 cells as determined by measuring cell growth inhibition. Cells were exposed for 48 hr to FUra alone (O) and FUra + 10^{-6} M dThd (△).

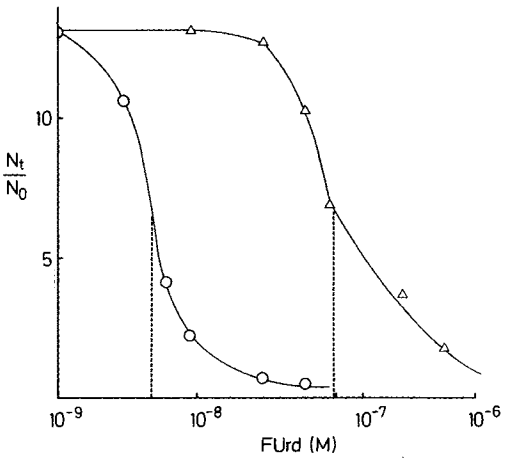

Fig. 3 Effect of Urd on the cytotoxicity of FUrd to L1210 cells as determined by measuring cell growth inhibition. Cells were exposed for 48 hr to FUrd alone (O) and FUrd + 10^{-4} M Urd (△).

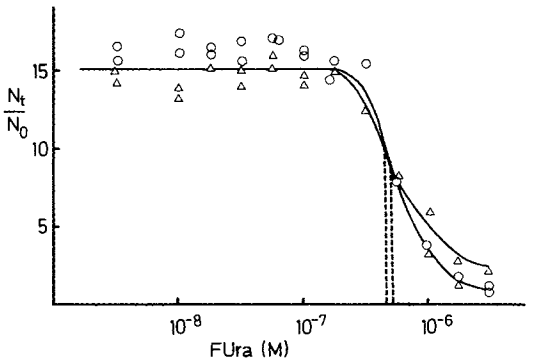

Fig. 4 Effect of Urd on the cytotoxicity of FUra as determined by measuring cell growth inhibition. Cells were exposed for 48 hr to FUra alone (O) and FUra + 10^{-4} M Urd (△).

Fractionation of polysomal RNA. We examined the incorporation of FUra into RNA more closely by performing a limited fractionation of polysomal RNA. The use of poly(U)sepharose affinity columns allows the separation of poly(A)RNA (mRNA) from rRNA and tRNA found in polysomes (18).

Table 3. Effect of Urd on the incorporation of [^{3}H]FUra into total RNA of L1210 cells.*

Reaction mixture contains	[^{3}H]FUra in total RNA (pmol/10^6 cells)	Concentration of RNA (μg/10^6 cells)
10^{-7}M[^{3}H]FUra	3.2 ± 0.1	115 ± 10
10^{-7}M [^{3}H]FUra + 10^{-5}M Urd	3.1 ± 0.1	117 ± 15

* L1210 cells (5×10^4/ml) were incubated with the indicated components for 48 hr under the standard cell culture conditions described in Materials and Methods. The values represent the mean ± SE of 3 determinations.

When the L1210 cells were treated with 10^{-8} M [^{3}H] Urd for 48 hr (about 4 doublings of the cells), the mRNA fraction contained only about 1 % of the radiolabel found in total polysomal RNA (Fig. 5). However, in the presence of actinomycin D, which specifically inhibits the synthesis of ribosomal RNA (20), the amount of [^{3}H]Urd incorporated into poly(A)RNA rose to 25 % of the total incorporation (Fig. 5). Results qualitatively similar to these were obtained by Lindberg and Persson (21) upon exposure of KB cells to [^{3}H] Urd for 2 hr.

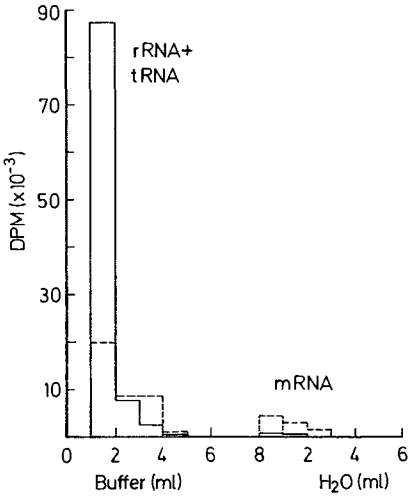

Fig. 5 Fractionation of polysomal RNA from L1210 cells following 48 hr exposure to 10^{-8} M [^{3}H]Urd(-) and 10^{-8}M [^{3}H]Urd plus 0.04 μg/ml of actinomycin D (---).

When the cells were exposed to 10^{-7} M [^{3}H]FUra for 48 hr, 67 % of the radioactivity incorporated into polysomal RNA was found in mRNA (Fig. 6). In the presence of 10^{-5} FUrd, the amount of FUra found in mRNA decreased to 28 % of the total incorporation, corresponding to a 4-fold decrease in the amount of radioactivity in the mRNA fraction (Fig. 6). However, the amount of [^{3}H]FUra in the rRNA plus tRNA fraction increased by 25 % upon exposure of the cells to 10^{-5} FUrd.

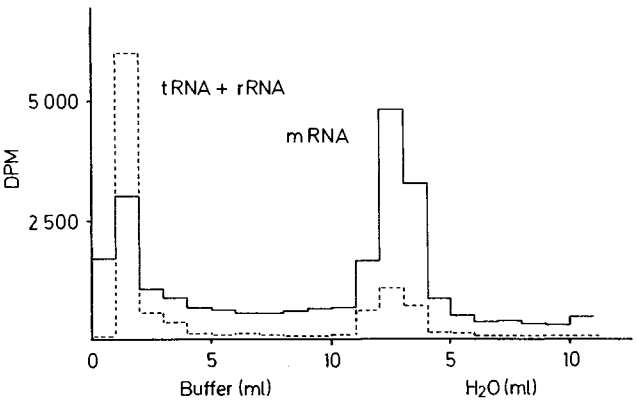

Fig. 6 Fractionation of polysomal RNA from L1210 cells following exposure to 10^{-7} M [^{3}H]FUra alone (-) and in the presence of 10^{-5} M Urd (---) for 48 hr.

Discussion

Efforts to establish the relative importance of DNA- and RNA-directed toxicities of the fluoropyrimidines have generated an extensive literature, but one which is often confusing and contradictory (5, 6). Not the least of the questions that remain unanswered is the precise manner in which incorporation of FUra into RNA causes cytotoxicity. The effects on the functioning of tRNA and mRNA are often slight or nonexistent even at massive degrees of substitution (4, 5, 6). While the most FUra-sensitive process seems to be the maturation of rRNA, it has not really been established at what percentage of substitution of FUra residues for Ura sufficient damage is done to cause cell death. Nevertheless, it has been assumed that at least a portion of the cytotoxic effect of the fluoropyrimidines is derived from inhibition of RNA function if only because it seems unlikely that the combined effects of incorporation of FUra, *viz.*, decreased content of modified bases in tRNA, altered post-transcriptional modification of nuclear RNA, and inhibited maturation of rRNA, would be without some deleterious effect on the vitality of cells (5).

In this study, we approached the problem of correlating the toxicity of the fluoropyrimidines with either inhibition of dTMP synthetase or incorporation into RNA by determining these parameters in cells treated with *equitoxic* doses of three widely studied fluoropyrimidines – FUra, FUrd, and FdUrd. These compounds are converted to common active metabolites, as shown in Fig. 7, by different enzymatic pathways. Thus, there is the possibility of generating variable ratios of FdUMP to F-RNA in the cell depending on which fluoropyrimidine is used. The rationale was simply that at equitoxic doses of each drug, if inhibition of dTMP synthetase by FdUMP is responsible for toxicity, then FdUMP levels, or actually the ratio of free to FdUMP-bound dTMP synthetase, should be the same in cells treated with all three agents, whereas if RNA incorporation were the determining factor for toxicity, then similar levels of incorporation (or at least a lack of correlation with FdUMP levels and TS inhibition) should be observed. Using the same reasoning, Houghton *et al.* (22) performed a study in which they found that the amount of incorporation into RNA of gastrointestinal tissue was similar with all three fluoropyrimidines at doses producing equal weight loss in mice. A cell culture system, however, allows a direct measurement of drug toxicity to the target cells. As shown in Table 2, at equitoxic (and, as shown in Table 1, equicytostatic) concentrations of all three fluoropyrimidines, the free FdUMP levels and the ratios of free to bound dTMP synthetase are equal. In addition, the degree of inhibition of dTMP synthetase correlates quite closely with the degree of cell growth inhibition, which has been previously observed with other deoxyribonucleosides (e. g., CF_3dUrd and NO_2dUrd) having dTMP synthetase as their primary intracellular target (23). In contrast, the degree of incorporation of FUra into RNA varies over 100-fold at drug concentrations giving the same cytotoxicity. A similar lack of correlation has been found in a recent study showing that the dThd-FUra combination in human patients with colorectal cancer gave no improvement in clinical response over FUra alone in spite of a 4-fold increase in incorporation of FUra into total RNA (24). However, FdUMP levels in this tissues of these patients were found to be decreased (probably because of competion of dThd and FdUrd for binding to dThd kinase) (24).

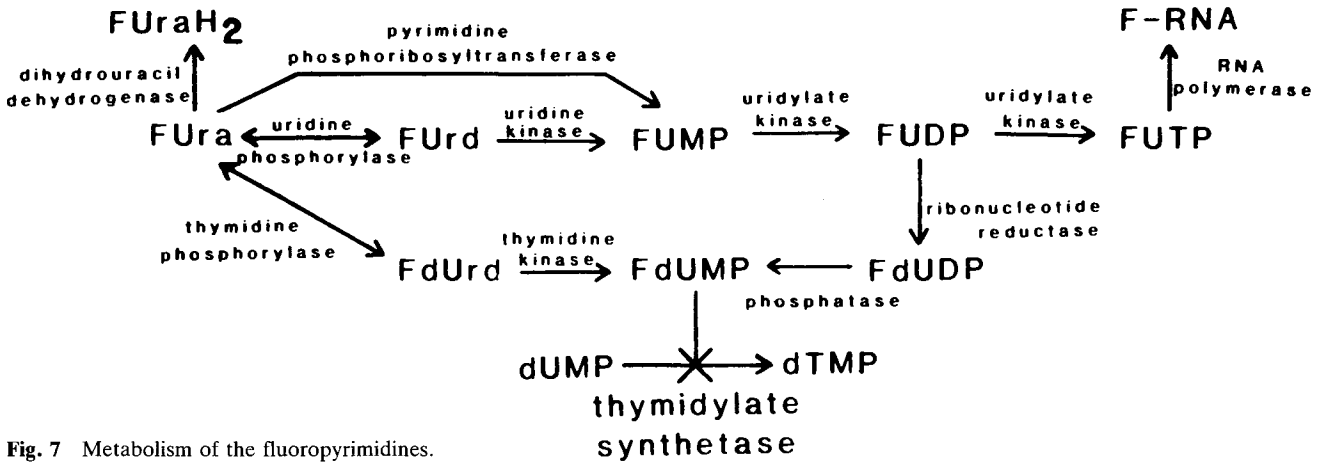

Fig. 7 Metabolism of the fluoropyrimidines.

The mechanism of action of an antimetabolite is often studied by determining an apparent cytotoxicity value in the presence of a natural analog or end-product metabolite. The degree to which cells are protected or rescued from the cytotoxic drug is then expected to provide clues as to the intracellular site(s) of action. For example, administering FUra leads to inhibition of dTMP synthetase, so dThd added to the system should circumvent this inhibition by providing an alternate source of dTMP for DNA synthesis. The "rescuability" of cells by dThd has often been used as a diagnostic criterion of the degree to which inhibition of dTMP synthetase contributes to cytotoxicity (5), and lack of or minimal rescue by dThd, is often considered as positive evidence for an RNA-directed effect. Because the ability of dThd to reverse FUra toxicity varies widely among cell lines (5), it is now believed that the mechanism of cytotoxicity of FUra could be either RNA-directed or DNA-directed, depending on the cell line.

Because of the heavy reliance on this type of data for mechanistic diagnoses, we considered it of interest to determine whether protection experiments would be in agreement with the data in Table 2, which clearly are consistent with a mechanism of cytotoxicity involving inhibition of dTMP synthetase. In fact, we found that dThd, although protecting the cells from FdUrd (Fig. 1), had little or no effect on the cytotoxicity of FUra (Fig. 2), which would normally be interpreted as evidence that dTMP synthetase inhibition had no connection with cytotoxicity. Following the same logic as in the dThd-protection experiments, one would assume that if the action of FUra were RNA-directed, then Urd should overcome the toxic effects of FUra, since these compounds give rise to analogous sets of metabolites, starting with UMP and FUMP, which would compete for the active sites of RNA synthesizing enzymes. However, Urd did not protect the cells from FUra either (Fig. 4), which is not consistent with an RNA-directed activity of FUra. Since these results appear to be contradictory, one is faced with the possibilities that FUra exerts cytotoxicity by yet a third mechanism (e. g., incorporation into DNA), or alternately, that the often simple logic underlying the predicted outcomes of many rescue or protection experiments is not always valid. In this discussion, we will focus on the latter alternative.

The literature contains numerous examples of complex effects which cause the rescuability of cells with the same agent to vary, not only with the particular fluoropyrimidine that is being used, but with duration of exposure to the drug, concentration during exposure, or the phase of the cell cycle (5, 6, 25). The biochemical processes responsible for such phenomena have been elucidated in only a few cases. A further complicating factor is illustrated by some of the results in this study, which suggest that the metabolic pathway followed by FUra into RNA is not identical to that of the ribonucleosides FUrd and Urd. For example, 1) the lack of appreciable competition between FUra and Urd for incorporation into RNA (Table 3); 2) the results in Figs. 5 and 6, which show substantial differences in the relative proportions of FUra and Urd being incorporated into mRNA compared to rRNA; 3) the greater amount of F-RNA generated from FUra compared to FUrd at equitoxic levels of these compounds, are *prima facie* indications that nucleotides derived from FUra are generated in a different cellular location and do not mix rapidly with those derived from FUrd and Urd (which do compete with each other, as shown in Fig. 3). Unequal intracellular distribution and utilization of FUTP had been previously offered as an explanation to account for the approximately 5-fold greater incorporation of FUra into mRNA than into rRNA (26). Other relevant observations from previous studies are that FUra in Ehrlich ascites cells undergoes a considerably higher incorporation into nuclear RNA compared to cytosolic RNA (5), suggesting that FUra may be preferentially converted to FUTP in the nucleus, whereas Urd (and presumably related compounds such as FUrd) enters largely into the cytosolic nucleotide pool in Novikoff hepatoma cells (27). Similar factors may affect the rescue of cells by dThd. The concept of two distinct TTP pools, one of which is rapidly utilized for DNA synthesis, has been proposed (28). A recent study has shown that dTMP synthetase during the S phase becomes localized in the nucleus as part of a "replitase complex", the environment of which may not be rapidly affected by precursors which enter first into the cytosolic nucleotide pool (e. g., dThd) (29). As pointed out by Myers (6), it is difficult at this time to fully appreciate what effects a compartmentalization of nucleotide pools would have on the types of rescue and protection experiments that have been performed extensively in the past. It is clear that this question needs to be studied further.

Although there was no discernible competition between FUra and Urd for incorporation into total RNA at relative concentrations ratios of 100, we did observe a substantial inhibition by Urd of the incorporation of FUra specifically into mRNA (Fig. 6). It has been suggested that substitution of FUra for Ura in mRNA could result in miscoding and translational errors, causing fraudulent protein synthesis and thereby

cytotoxicity (26). However, our results do not support this mechanism insofar as they show a lack of correlation between incorporation of FUra into mRNA, which decreased 4-fold in the presence of Urd, and the toxicity of FUra, which remained unchanged. On the other hand, the amount of [^{3}H]FUra incorporated into the rRNA + tRNA fraction appears to have *increased* by about 25 % in the presence of Urd. We believe the most likely cause of this phenomenon is the generation of a small amount of [^{3}H]FUrd from an enzymatically catalysed transribosylation reaction between [^{3}H]FUra and Urd. The resulting [^{3}H]FUrd should then become just as readily incorporated into rRNA, as is [^{3}H]Urd (Fig. 5). Analogous transribosylation reaction between FUra and deoxyribonucleosides have been demonstrated previously (30).

Abbreviations

FUra, 5-fluorouracil; FdUMP, 5-fluoro-2'-deoxyuridylate; FdUrd, 5-fluoro-2'-deoxyuridine; FUrd, 5-fluorouridine; FUMP, fluorouridine-5'-phosphate FUDP, fluorouridine-5'-diphosphate; FUTP, fluorouridine-5'-triphosphate; dThd, thymidine; dTMP, thymidine-5'-phosphate; TTP, thymidine-5'-triphosphate; dUrd, 2'-deoxyuridine; dUMP, 2'-deoxyuridine-5'-phosphate; Urd, uridine; 5,10-CH_2H_4folate, 5,10-methylenetetrahydrofolate; dTMP synthetase, thymidylate synthetase; F-RNA, fluorouracil-containing RNA.

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An Efficient In Vitro Assay for Acetylcholinesterase Reactivators Using Immobilized Enzyme

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Abstract: A new and efficient *in vitro* assay for evaluating reactivators of organophosphate-inhibited acetylcholinesterase has been developed. Low density polyethylene beads (4 mm) were functionalized to terminal aldehydes and used to immobilize acetylcholinesterase (AChE, *Electrophorus electricus*, E.C. 3.1.1.7) via a stable Schiff base link. AChE activity in columns containing immobilized enzyme could be continuously monitored spectrophotometrically in a closed loop flow system using acetylthiocholine and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Immobilized enzyme exhibited good esterase activity (0.5 units/bead), which could be retained on storage at −16°C for four months. The kinetics for substrate hydrolysis were flow-rate dependent below substrate saturation levels. This system allowed for independent inhibition and reactivation of the enzyme. Immobilized enzyme could be inhibited with diisopropylfluorophosphate (DFP) and 20–90 % of original activity restored with several oximes in less than 20 minutes. The extent of reactivation was dependent on the concentration of the reactivators. This system has advantages over previously reported procedures, because hydrolysis of substrate due to reactivator is minimized and inhibitor-reactivator interactions are eliminated, subsequently minimizing the need to correct experimental results.

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