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Metabolism and mechanism of action of 5-fluorodeoxycytidine

(dTMP synthetase/5-fluorodeoxyuridylate/S-49 cells/enzyme-deficient mutant)

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ABSTRACT **5-Fluoro-2'-deoxycytidine (FdCyd) is a potent inhibitor of growth of tissue culture cells. The major cytotoxic event appears to be inhibition of thymidylate synthetase as evidenced by reversal of the cytotoxicity with thymidine but not deoxycytidine and by the effect of FdCyd on nucleotide pools, which is characteristic of specific inhibition of this enzyme. The metabolism of FdCyd was established by using a method in which its cytotoxicity was compared in several S-49 mutant cell lines having defined single or double deficiencies of enzymes involved in nucleoside and nucleotide metabolism. Our results indicate that FdCyd is metabolized to 5-fluoro-2'-deoxyuridylate, a potent inhibitor of thymidylate synthetase by two pathways: (*i*) sequential reactions catalyzed by deoxycytidine kinase and deoxycytidylate deaminase and (*ii*) sequential reactions catalyzed by cytidine deaminase and thymidine kinase. We have shown that metabolism of FdCyd can be directed through the former pathway by inhibition of cytidine deaminase with tetrahydrouridine. Since cytidine deaminase appears to be responsible for catabolism of FdCyd in animals, our results suggest that the antineoplastic effects of FdCyd should be examined in combination with inhibitors of cytidine deaminase.**

5-Fluorodeoxycytidine (FdCyd) is a potent cytotoxic agent in tissue culture systems (1–3). Because the effects of FdCyd are so similar to those of 5-fluorodeoxyuridine (FdUrd) and are reversed by thymidine, it has been postulated that its mechanism of action involves deamination to FdUrd by cytidine deaminase, conversion to 5-fluorouridylic acid (FdUMP) by thymidine kinase, and subsequent inhibition of dTMP synthetase (4, 5). FdCyd has also shown activity as an antineoplastic agent in a number of experimental animal tumor models (6). However, since the spectrum of activity and *in vivo* efficacy of FdCyd was, with few exceptions, similar to that of FdUrd and 5-fluorouracil (FUra), studies of its mechanism of action and antineoplastic effects have been largely abandoned.

Our interest in FdCyd was stimulated by reports suggesting that the proposed mechanism of action of FdCyd might be incorrect or, at least, incomplete. First, FdCyd is a potent cytotoxic agent toward FdUrd-resistant sublines of P815Y (7) and L5178Y (3) that lack thymidine kinase. Second, the toxicity of FdCyd in mice was increased by administration of tetrahydrouridine (H_4Urd), a potent inhibitor of cytidine deaminase (8); if conversion to FdUrd were necessary for activation of FdCyd, the opposite effect would have occurred.

In this paper, we describe experiments to elucidate the mechanism of FdCyd metabolism and action in S-49 mouse lymphoma cells. The approach used relies mainly on use of the mutant cell lines deficient in one or more of the enzymes involved in nucleoside and nucleotide metabolism that have been developed by Martin and co-workers (9–11). By simply testing the effects of a nucleoside analog on such mutants, it is possible to determine which enzyme activities are necessary for conversion to the cytotoxic metabolite. As suspected, we find that the metabolism and action of FdCyd are more complex than originally proposed, and we suggest that, in combination with H_4Urd, its mode of metabolism and cytotoxicity may be unique among currently available antimetabolites.

MATERIALS AND METHODS

B. Ullman and D. W. Martin, Jr., generously provided wild-type S-49 mouse lymphoma cells (12) as well as the mutant strains used in this study. The deoxycytidine kinase-deficient (dCK^-), thymidine kinase-deficient (TK^-), and dCMP deaminase-deficient ($dCMPD^-$) cell lines are described elsewhere (9–11). Starting with the $dCMPD^-$ and the dCK^- cells, $dCMPD^-/TK^-$ and dCK^-/TK^- double mutants were selected for resistance to 5-bromodeoxyuridine as described for the TK^- line (10).

Lack of thymidine kinase activity in the latter two cell lines was confirmed by resistance of the cells to 1 μM FdUrd and by the inability of a cell-free sonicate to phosphorylate thymidine in the following assay. Approximately 10^8 cells were sonicated for 20 sec in 0.3 ml of buffer (10 mM mercaptoethanol/20 mM sodium phosphate, pH 7.4) using a Biosonik sonicator set at pI = 40. Cellular debris was removed by centrifugation for 4 min in an Eppendorf Microfuge. The supernatants were diluted in the sonication buffer to an approximate protein concentration of 10 mg/ml, based on the absorption at 280 nm (1 A_{280} unit $\approx$ 1 mg/ml). Each incubation mixture (250 μl) contained 2.5 mM ATP, 2.5 mM $MgCl_2$, 5 mM NaF, 50 mM Tris·HCl (pH 8.0), 10 μM [^{14}C]thymidine (0.125 μCi; 1 Ci = 3.7×10^{10} becquerels), and 100 μg of protein. Aliquots were spotted on Whatman DE 81 filters. The filters were washed four times by immersion in 500 ml of 1 mM ammonium formate and then the amount of radioactive nucleotide bound was determined by scintillation spectrometry. With wild-type cell extract, accumulation of [^{14}C]dTMP was linear for at least 1 hr and with the amount of extract added. Extracts of both the $dCMPD^-/TK^-$ and the dCK^-/TK^- double mutants converted <1% of the [^{14}C]thymidine to nucleotides as compared with the wild-type extract.

All cell lines were maintained as suspension cultures in antibiotic-free Dulbecco's modified Eagle's medium containing 1 g of glucose and 0.11 g of sodium pyruvate per liter. The medium was supplemented with 10% heat-inactivated (56°C, 30 min) horse serum. For growth curves, aliquots of duplicate

Abbreviations: araC, 1-β-D-arabinofuranosylcytosine; araCTP, araC triphosphate; dCK^-, deoxycytidine kinase deficient; $dCMPD^-$, dCMP deaminase deficient; FdCyd, 5-fluorodeoxycytidine; FdUrd, 5-fluorodeoxyuridine; FdUMP, 5-fluorodeoxyuridylic acid; FUra, 5-fluorouracil, H_4Urd, tetrahydrouridine; TK^-, thymidine kinase deficient; EC_{50}, 50% growth-inhibitory concentration.

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cultures for each growth condition were counted in a Coulter model ZBI cell counter at 24, 48, and 72 hr. The percentage of control growth is 100 times the slope of the plot of the logarithm of the number of cells versus time, divided by the slope of the plot for the untreated control culture (13). The 50% growth-inhibitory concentration (EC_{50}) was estimated from plots of the percentage of control growth as a function of the logarithm of the inhibitor concentration. All additions were made at the beginning of the experiments.

The ribonucleoside and deoxyribonucleoside triphosphate content of cell extracts was assessed by HPLC techniques as reported (14, 15). FdCyd was a gift from M. J. Robins. FdUrd and H_4Urd were obtained from the Drug Synthesis and Development Branch of the National Cancer Institute. 1-β-D-Arabinofuranosylcytosine (araC) was purchased from Sigma and [^{14}C]thymidine was from Moravek Biochemicals (Brea, CA).

RESULTS AND DISCUSSION

Despite the antineoplastic activity of FdCyd against both cultured cells and transplanted tumors, details of the biochemical mode of action of FdCyd have not been established. Our interest in this compound arose from the possibility that its mode of metabolism and cytotoxic action might be unique among currently available antimetabolites.

In principle, the cytotoxic effects of FdCyd would result from one or more of three possible metabolites: FUra, FdUMP, or FdCTP (Fig. 1). Since FdCyd ($EC_{50} \approx 2$ nM) is some 10^3-fold more cytotoxic toward tissue culture cells than is FUra, we may rule out the possibility that the latter is responsible for the effects of FdCyd *in vitro*. However, as discussed below, FUra is probably the catabolite of FdCyd that is responsible for its *in vivo* activity. Conversion of FdCyd to FdUMP would result in potent inhibition of dTMP synthetase whereas conversion to FdCTP might be expected to result in effects on DNA synthesis in a manner similar to that of 1-β-D-arabinofuranosylcytosine 5'-triphosphate (araCTP) (16) and 1-β-D-arabinofuranosyl-5-fluorocytosine 5'-triphosphate (17). Distinguishing between these possible modes of action is most easily accomplished by demonstrating the reversal of cytotoxicity by agents that circumvent or abolish the specific blockade. That is, the effect of inhibition of dTMP synthetase can be effectively reversed by thymidine but only poorly reversed by deoxycytidine. The rationale for these effects is that thymidine provides a source of thymidine nucleotides and thus circumvents the block whereas deoxycytidine must be converted to dUMP, which must compete with the inhibitor for dTMP synthetase; in contrast, the effects of araC and 1-β-D-arabinofuranosyl-5-fluorocytosine are reversed by deoxycytidine but not thymidine (17). Cheong *et al.* (2) have reported that thymidine was much more effective than deoxycytidine at protecting H.EP 1 cells from the inhibitory effects of FdCyd (2), indicating that the toxic effects of this drug toward this cell line were due to inhibition of dTMP synthetase. Similarly, we have shown that, when S-49 cells are treated with a high concentration (10 times the EC_{50}) of FdCyd, coadministration of 3 μM thymidine permitted 67% of control growth, whereas deoxycytidine was much less effective, with only 40% of control growth observed at 50 μM (Fig. 2*A*). Parallel studies on reversal of the effect of FdUrd—a specific inhibitor of dTMP synthetase in S-49 cells (18)—yielded a nearly identical pattern; at 10 times the EC_{50} of FdUrd, 1 μM thymidine afforded 51% reversal of inhibition whereas only a 17% reversal occurred at 50 μM deoxycytidine (Fig. 2*B*). The pattern of reversal of araC cytotoxicity of S-49 cells by these nucleosides is clearly distinct from those described for FdCyd and FdUrd: at 10 times the EC_{50} of araC, 30 μM thymidine had no effect on growth inhibition whereas 30 μM deoxycytidine restored growth to 55% of control (Fig. 2*C*).

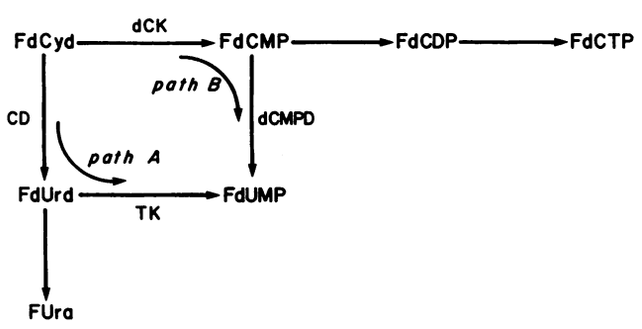

FIG. 1. Intracellular metabolism of FdCyd. CD, cytidine deaminase; dCK, deoxycytidine kinase; TK, thymidine kinase; dCMPD, dCMP deaminase.

We have found that specific inhibition of dTMP synthetase in logarithmically growing cells produces a characteristic, and perhaps unique, pattern of changes in NTP pool sizes (unpublished results). A primary effect of inhibition of dTMP synthesis in tissue culture cells results in a decline in dTTP levels that, through direct and indirect effects on ribonucleotide reductase, causes changes in the levels of other dNTPs that are in good agreement with reported models of allosteric effects on this enzyme (19, 20). A decrease in dTTP is accompanied by a decline in dGTP levels because the former is an allosteric activator of GDP reduction. There is often a smaller increase in dATP that presumably results from the release of allosteric inhibition of ADP reduction by dTTP even in the face of decreased ADP reduction resulting from depletion of the positive activator, dGTP. If the blockade of dTMP synthetase persists, a decrease in the allosteric inhibition of CDP reduction by dTTP can also be manifested by increases in dCTP. However, we usually do not observe increased dCTP until after the aforementioned changes have occurred. Changes in pool sizes of ribonucleotides have been found to be insignificant if the blockade of dTMP synthetase is truly specific. This pattern of characteristic changes that results from intracellular depletion of dTMP and dTTP is illustrated in Table 1, which shows the deoxyribonucleoside triphosphate levels in cells treated with FdUrd for 3 hr, which under the conditions used causes specific inhibition of dTMP synthetase (18). Note that, as described above, there is a significant depletion of dTTP and dGTP, and a slight increase in dATP; longer periods of incubation result in an increase in dCTP (not shown). Ribonucleotide pools are slightly increased after 3 hr of incubation (Table 2). Treatment of S-49 cells with araC produced an entirely different profile of intracellular dNTP pool sizes: there is a slight depletion of dCTP, a moderate increase in dTTP, and very large (*ca.* 3-fold) increases in both dGTP and dATP. As with inhibitors of dTMP synthetase, araC did not deplete the pool sizes of NTPs. When S-49 cells were treated with FdCyd, there were no significant changes observed in the NTP pool sizes; however, there were large decreases in dTTP and dGTP and an increase in dATP—characteristic of specific inhibition of dTMP synthetase as is observed with FdUrd.

The above results thus show that the cytotoxic effect of FdCyd on S-49 cells is primarily, probably exclusively, a result of dTMP synthetase inhibition. Nucleotides of FdCyd (e.g., FdCMP) are not themselves potent inhibitors of this enzyme (21), and the most logical candidate for the cytotoxic metabolite is FdUMP. As shown in Fig. 1, there are two major metabolic routes by which FdCyd can be converted to FdUMP. The first path (path A) involves cytidine deaminase-catalyzed deamination to FdUrd and its subsequent phosphorylation to FdUMP

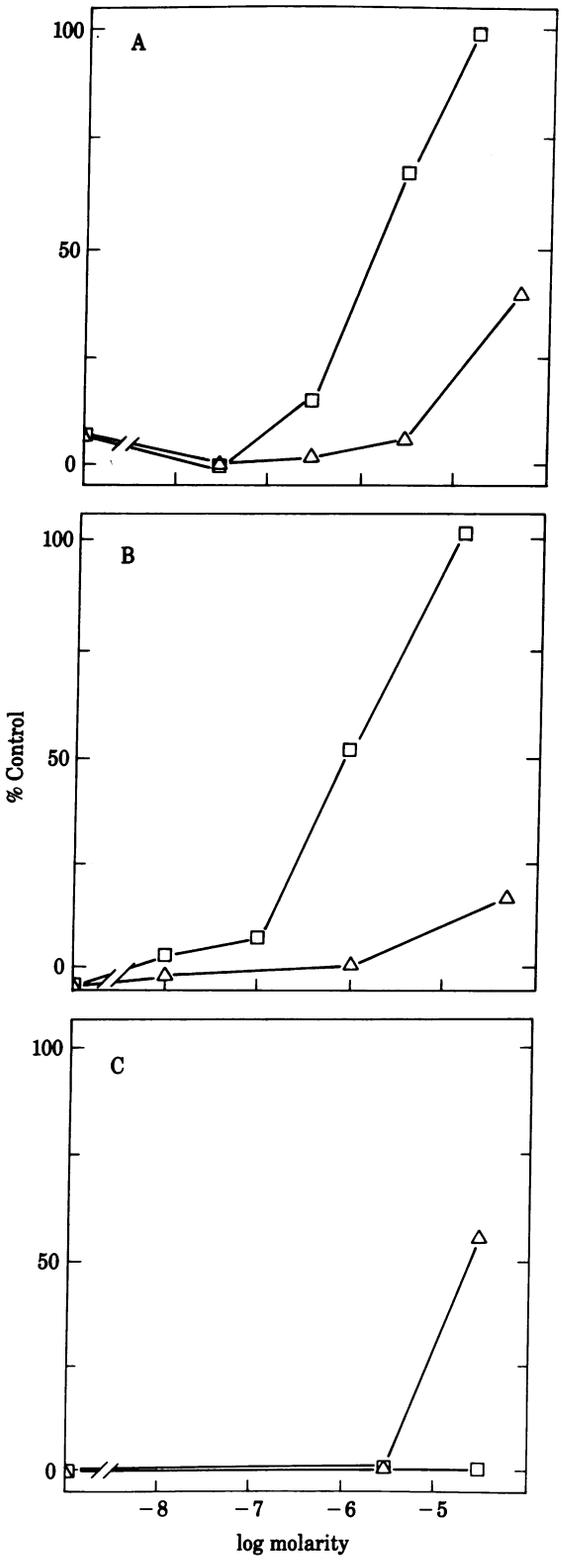

FIG. 2. Protection from the cytotoxicity of FdCyd (*A*), FdUrd (*B*), and araC (*C*) by thymidine and deoxycytidine. Concentrations of the cytotoxic agents were held constant at approximately 10 times their EC_{50} values, while those of thymidine (□) and deoxycytidine (△) were varied. The concentrations and percent of control growth of the cytotoxic compounds alone were FdCyd, 30 nM, 7%; FdUrd, 10 nM, −6%; araC, 3 μM, 1%.

by deoxycytidine kinase. Path B involves initial phosphorylation of FdCyd by deoxycytidine kinase to form FdCMP followed by dCMP deaminase-catalyzed deamination to FdUMP (22, 23). It is noted that the first enzyme in path B, deoxycytidine kinase, is required for initial activation of araC, while thymidine kinase is required for activation of FdUrd, but none of the enzymes depicted are essential in the metabolic activation of FUra (24). In the discussion below, we describe results that elucidate the pathway(s) of metabolic conversion of FdCyd to FdUMP in S-49 cells. The approach used was to determine the cytotoxicity of FdCyd in mutant lines of S-49 cells that were deficient in one or more of the enzyme activities presumed to be important in the activation of FdCyd (Fig. 1). The only mutant not available to us was one deficient in cytidine deaminase, but this activity can be effectively inhibited by H_4Urd. If a specific deficiency greatly reduced the cytotoxicity of FdCyd, we concluded that the pathway involved was important in metabolism to FdUMP. Although it cannot be assumed that other metabolic processes in specific mutants remain unaffected, we were able to formulate a consistent interpretation of the results that primarily relies on the effects of the defined enzyme deficiencies.

The EC_{50} values for FdCyd in each of the cell lines used and, where pertinent, the EC_{50} values for FdUrd, araC, and H_4Urd are summarized in Table 3. The authenticity of the mutant lines was verified by showing that cell extracts possessed <1% of the relevant activity as compared with wild-type S-49. Further, the TK^- mutant is 1/10,000th as sensitive as the wild-type FdUrd because thymidine kinase is required for phosphorylation to the cytotoxic metabolite FdUMP. Likewise, the dCK^- line is 1/1,000th as sensitive to araC, which requires this enzyme in the initial step leading to formation of the cytotoxic metabolite araCTP.

Table 3 shows that elimination of any single activity involved in the metabolism of FdCyd to FdUMP has little effect on its cytotoxicity. Thus, individual blockade of metabolism via path A (H_4Urd or TK^-) or via path B (dCK^- or $dCMPD^-$) may reduce but does not prevent conversion of FdCyd to FdUMP. The log-

Table 1. Deoxyribonucleoside triphosphate pools

	% control			
	FdUrd	araC	FdCyd	FdCyd/ H_4Urd
dCTP	92	83	92	79
dTTP	18	145	14	9
dATP	119	320	165	135
dGTP	35	278	22	11

Wild-type S-49 cells were grown to a density of 2.6×10^5/ml and divided into 75-ml aliquots. The cells were then incubated for 3 hr at 37°C with no addition (control), 10 nM FdUrd, 3 μM araC, 30 nM FdCyd, or 30 nM FdCyd/0.1 mM H_4Urd. The dNTP pools were quantitated by HPLC (14). Control culture levels were dCTP, 24 pmol per 10^6 cells; dTTP, 22 pmol per 10^6 cells; dATP, 20 pmol per 10^6 cells; dGTP, 9 pmol per 10^6 cells.

Table 2. Ribonucleoside triphosphate pools

	% control			
	FdUrd	araC	FdCyd	FdCyd/ H_4Urd
CTP	116	119	110	98
UTP	110	105	112	94
ATP	113	107	118	105
GTP	106	121	135	115

Aliquots of the neutralized trichloroacetic acid extracts used in Table 1 were analyzed for NTP pools by HPLC (15). Control culture levels were CTP, 230 pmol per 10^6 cells; UTP, 420 pmol per 10^6 cells; ATP, 1,250 pmol per 10^6 cells; GTP, 190 pmol per 10^6 cells.

Table 3. EC_{50} values (molar) of FdCyd and related compounds in wild-type (wt) and enzyme-deficient S-49 cells

Compound	Cell type					
	wt	TK^-	dCK^-	$dCMPD^-$	dCK^-/TK^-	$dCMPD^-/TK^-$
FdCyd	2×10^{-9}	1×10^{-8}	2×10^{-8}	3×10^{-9}	$>3 \times 10^{-5}$	1×10^{-6}*
FdCyd/H_4Urd†	2×10^{-9}	$<3 \times 10^{-8}$	8×10^{-6}			
H_4Urd	$>3 \times 10^{-3}$					
FdUrd	9×10^{-10}	1×10^{-5}				
araC	3×10^{-7}		2×10^{-4}			

Stock cell cultures in logarithmic growth were diluted to 5×10^4 cells per ml and 1-ml samples were dispensed into the wells of 24-well plates containing the cytotoxic agents. Inhibition of growth by several (four–six) concentrations of each agent was determined, and EC_{50} values were estimated from the data by interpolation. Values reported are means of two or three determinations that did not vary by more than 50% of the mean.

*Minimum concentration that inhibited 50%; higher concentrations were not more inhibitory.

†H_4Urd was constant at 0.1 mM.

ical interpretation of these findings is that conversion of FdCyd to FdUMP may occur through either of the two metabolic pathways. If this is correct, then blockage of both pathways should prevent the metabolism of FdCyd to FdUMP and significantly decrease the cytotoxicity of FdCyd. By using the double mutants and H_4Urd, a number of different double blocks can be established. First, the dCK^-/TK^- mutant should prevent formation of FdCMP and conversion of FdUrd to FdUMP, permitting only accumulation of FdUrd as a nontoxic metabolite. This double mutant was found to be extremely resistant to FdCyd, having a EC_{50} >10,000 times that observed in the wild type. Second, the $dCMPD^-/TK^-$ double mutant should prevent both phosphorylation of FdUrd and deamination of FdCMP; one might predict an accumulation of nontoxic FdUrd and nucleotides of FdCyd in this cell line. This cell line was found to be 500 times more resistant to FdCyd than the wild-type cells. The fact that the $dCMPD^-/TK^-$ cell line is not quite as resistant to FdCyd as the dCK^-/TK^- mutant may be due to some residual enzyme activity in the former or possibly to another cytotoxic mechanism of FdCyd that is manifested at high concentrations of the drug; in this regard, FdCTP formation should not be prevented in the $dCMPD^-/TK^-$ cells and, at high intracellular concentration, might be responsible for the cytotoxicity observed. Third, inhibition of cytidine deaminase in the dCK^- mutant by H_4Urd should block both possible initial conversions of FdCyd and completely prevent its metabolism. In the presence of 0.1 mM H_4Urd, the cytotoxicity of FdCyd to the dCK^- cells is 1/400th of that of the untreated dCK^- line and 1/4,000th of that of the wild-type cells in the presence or absence of H_4Urd. In addition to demonstrating the requirement for at least one of the two pathways shown in Fig. 1, this result demonstrates the efficacy of H_4Urd as an intracellular inhibitor of cytidine deaminase-catalyzed deamination of FdCyd. Further, the effect of the FdCyd/H_4Urd combination on nucleotide pool sizes (Tables 1 and 2) shows the pattern characteristic of specific dTMP synthetase inhibition.

In summary, our results show that the cytotoxicity of FdCyd in tissue culture cells is due to the inhibition of dTMP synthetase. Through the use of mutant cell lines and H_4Urd, we have shown that the metabolic activation of FdCyd to FdUMP may proceed by either (*i*) initial deamination to FdUrd followed by thymidine kinase-catalyzed phosphorylation, or (*ii*) initial conversion to FdCMP followed by deamination. Importantly, H_4Urd, which in itself is not cytotoxic, can prevent deamination of FdCyd to FdUrd but does not affect the alternative pathway to FdUMP or the cytotoxicity of FdCyd. The discussion below describes why these results suggest that FdCyd, in combination with H_4Urd, should be reconsidered as a potential anticancer agent.

In most tissue culture cells, the cytotoxic effect of FdUrd is a result of inhibition of dTMP synthetase by FdUMP, with little or no incorporation of metabolites into RNA. FUra is converted to FdUMP with resultant inhibition of dTMP synthetase but is also extensively incorporated into RNA (4, 18, 24). Which of these effects is primarily responsible for the action of FUra remains controversial. In spite of the controversy, it is clear that, in tissue culture systems, FdUrd and FUra may behave as quite different agents. *In vivo* FdUrd and FUra show the same spectrum of activity at approximately equimolar concentrations. FdUrd is an excellent substrate for both thymidine and uridine phosphorylases, and it is probable that *in vivo* FdUrd is converted to FUra more rapidly than it enters target cells to be directly converted to FdUMP. This has been recognized by workers attempting to develop agents that might block this catabolic process and thus reveal the direct effect FdUrd may have on dTMP synthesis without the RNA incorporation so prevalent with FUra. In animal tumor models, FdCyd also shows a similar spectrum of activity and potency as does FUra, which is in marked contrast to their differences in tissue culture cells. Thus, it is reasonable to surmise that FdCyd also serves as a source of FUra *in vivo*. Since mammalian cells do not possess enzymes that cleave the glycosidic bond of cytosine nucleosides (25), the metabolism must involve deamination by cytidine deaminase to give FdUrd followed by phosphorolysis to FUra. If cytidine deaminase could be blocked, the catabolism of FdCyd would be prevented and its metabolism would be directed through path B, involving phosphorylation to FdCMP and subsequent deamination by dCMP deaminase to the dTMP synthetase inhibitor, FdUMP. H_4Urd is an effective nontoxic inhibitor of cytidine deaminase both *in vitro* and *in vivo* (26). We believe that an *in vivo* trial of FdCyd in combination of an inhibitor of cytidine deaminase is warranted. Based on the discussion above, we expect that the antitumor effect of this regimen would be quite different than that observed for FdCyd alone. Indeed, H_4Urd has been shown to significantly increase the toxicity of FdCyd in mice (8). This combination might be particularly useful against neoplasms such as acute nonlymphocytic leukemia that are responsive to araC (27). Further, since the mechanisms of action of araC and FdCyd are so different but both drugs are targeted toward cells rich in deoxycytidine kinase, they could act synergistically providing that H_4Urd was used to prevent catabolism.

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