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Metabolic Channeling of 5-Fluoro-2'-deoxycytidine Utilizing Inhibitors of Its Deamination in Cell Culture

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SUMMARY

The metabolism of 5-fluoro-2'-deoxycytidine (FdC) with and without tetrahydrouridine (H_4U) or 2'-deoxytetrahydrouridine (dH_4U) was examined in log phase HEp-2 cells using HPLC and TLC methods which quantified: (a) the incorporation of FdC-related antimetabolites into RNA and DNA and (b) pool size levels of FdC-related antimetabolites. [^{3}H]-FdC administered to log phase HEp-2 cells at a concentration of 0.01 μM for 24 hr resulted in the incorporation of 5.22×10^{-8} mol of FdC/mol of DNA phosphate, a 0.021% substitution of FdC for dC. Coadministration of 1.0 mM H_4U or dH_4U resulted in 2- and 25-fold increases in the incorporation of FdC, respectively. No detectable incorporation of 5-fluoro-2'-deoxyuridine (FdU) into HEp-2 DNA resulted (detection limit, approximately 5 fmol). In contrast, treatment of HEp-2 cells with 0.1 μM FdU resulted in the incorporation of 1.83×10^{-9} mol of FdU (74.7 fmol detected)/mol of DNA phosphate. A linear incorporation of FdC into the DNA of HEp-2 cells was found with increasing concentrations of FdC and 1.0 mM dH_4U. 0.1 μM FdC resulted in the incorporation of 2.39×10^{-6} mol of FUMP/mol of cytoplasmic RNA phosphate and 2.23×10^{-5} mol of FUMP/mol of nuclear RNA phosphate. Similarly, HEp-2 cells treated with 0.1 μM FdU resulted in the incorporation of 1.10×10^{-5} mol of FUMP/mol of nuclear RNA phosphate and 9.44×10^{-7} mol of FUMP/mol of cytoplasmic RNA phosphate. In contrast, no detectable FUMP incorporation into either nuclear or cytoplasmic RNAs of HEp-2 cells resulted when H_4U or dH_4U was coadministered with 0.1 μM FdC. Pool size analyses of log phase HEp-2 cells following a 30-min exposure to FdU or FdC with and without H_4U or dH_4U were also performed; 0.1 μM FdC treatment resulted in the formation of 169 fmol of FUMP/1.0×10^6 viable HEp-2 cells. Treatment with 0.1 μM FdU produced 253 fmol of FUMP/1.0×10^6 viable HEp-2 cells. In contrast, no detectable FUMP pools were formed when H_4U or dH_4U was coadministered with 0.1 μM FdC (detection limit, approximately 5 fmol). Pool levels of FdUMP, the inhibitor of thymidylate synthetase, were also assayed; 36.9 fmol of FdUMP/1.0×10^6 viable HEp-2 cells were detected upon administration of 0.1 μM FdC. Coadministration of 1.0 mM H_4U with 0.1 μM FdC increased FdUMP pools 2.3-fold, while 1.0 mM dH_4U resulted in a 3.2-fold decrease; 0.1 μM FdU resulted in the formation of 55.2 fmol of FdUMP/1.0×10^6 viable HEp-2 cells, 34.3% less than that formed when 1.0 mM H_4U was coadministered with 0.1 μM FdC. These studies demonstrate that the coadministration of H_4U effectively directs the metabolism of FdC in neoplastic cells through the deoxycytidine kinase-deoxycytidylate deaminase pathway to the formation of FdUMP without the incorporation of FUMP into RNA or the formation of RNA-level antimetabolite pools (i.e., FUra, FUrd, or FUMP); this metabolic restriction was not found to be due to the inhibition of thymidine phosphorylase by H_4U.

INTRODUCTION

Most antineoplastic agents, including the pyrimidine analogs FdU[1] and FUra, lack the selectivity necessary to inhibit neoplastic cells while at the same time causing minimal effects in host tissue. There are three major cytotoxic effects in neoplastic cells following FUra or

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FdU treatment: (a) the production of FdUMP, a potent inhibitor of thymidylate synthetase (EC 2.1.1.45) which results in the depletion of dTMP and the cessation of DNA synthesis (1, 2), (b) the misincorporation and subsequent repair of FdUTP into DNA (3, 4), and (c) the misincorporation of FUTP into RNA leading to the inhibition of RNA maturation (5). Although the major mechanism of action of FUra or FdU is thought to be via the production of FdUMP and the incorporation of FUMP into RNA is thought to contribute to "general" toxicity (6–8), the exact antitumor mechanism of action of these two agents remains controversial.

At the outset of our studies, we proposed that the utilization of FdC with H_4U would avoid the disadvantages of FUra or FdU treatment and lead to superior antitumor efficacy. Previous studies with FdC (9, 10) have pointed to two possible metabolic pathways available to cells for the production of FdUMP: (a) the dCK-dCMPD (EC 2.7.1.74, EC 3.5.4.12, respectively) and (b) the CD-dTK (EC 3.5.4.5, EC 2.7.1.21, respectively) pathways (Fig. 1). To circumvent systemic catabolism, which is responsible for the degradation of halogenated deoxyuridine analogs, and to exploit elevations of dCK and dCMPD present in many human malignant tumors (11), FdC was coadministered with H_4U, a potent inhibitor of cytidine deaminase. We hypothesized that this strategy would result in greater formation of FdUMP at the tumor site, thus affording a unique, target-directed chemotherapy. Consistent with this hypothesis, we have found that an optimal dose of FdC + H_4U resulted in greater antitumor efficacy than that of FUra, F_3dT, or FdU (10). These studies did not, however, establish the exact metabolic pathway utilized by FdC when coadministered with H_4U. Therefore, we sought direct pharmacokinetic evidence which would support our contention that the administration of H_4U would direct the metabolism of FdC and provide a DNA-directed production of FdUMP.

The present study supplies direct evidence to support the channeling of FdC via H_4U in cell culture as determined by HPLC quantitation of FdC-related antimetabolite pools. This metabolic channeling was compared to the normal metabolism of FdC and FdU in log phase HEp-2 cells. Additionally, the incorporation of antimetabolites into both RNA and DNA of HEp-2 cells was assayed following treatments with FdU or FdC with and without H_4U or dH_4U, an inhibitor of both cytidine and deoxycytidylate deaminases.

[1] The abbreviations used are: FdU, 5-fluoro-2′-deoxyuridine; HPLC, high performance liquid chromatography; H_4U, tetrahydrouridine; dH_4U, 2′-deoxytetrahydrouridine; FdC, 5-fluoro-2′-deoxycytidine; FUMP, 5-fluorouracil-5′-monophosphate; dCMPD, deoxycytidylate deaminase; dCK, deoxycytidine kinase; CD, cytidine-deoxycytidine deaminase; dTK, thymidine kinase; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; dCMPK, deoxycytidylate kinase; HEp-2 cells, human epidermoid laryngeal carcinoma cells; FUra, 5-fluorouracil; FUrd, 5-fluorouridine; TS, thymidylate synthetase; EMEM, Eagle's minimal essential medium; PBS, phosphate-buffered saline; FdCTP, 5-fluoro-2′-deoxycytidine-5′-triphosphate; FdCMP, 5-fluoro-2′-deoxycytidine-5′-monophosphate; F_3dT, 5-trifluorothymidine; FUTP, 5-fluorouridine-5′-triphosphate; TLC, thin layer chromatography; PCA, perchloric acid; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Cell culture. HEp-2 cells were obtained from the American Type Culture Collection and were grown in EMEM with a 95% air/5% CO_2 humidified atmosphere. EMEM was supplemented with 10% fetal calf serum, 0.25 μg/ml amphotericin B, 100 units/ml penicillin, and 100 units/ml streptomycin, and sodium bicarbonate was increased to 0.1%. Media, sera, glutamine, trypsin, and antibiotics were purchased from Grand Island Biological Co., (Grand Island, NY), Flow Laboratories (McLean, VA), and K-C Biologicals (Lenexa, KS). HEp-2 cells were maintained and passed at confluency (about 3 days) as described previously (10). HEp-2 cells, which were previously shown to have elevated levels of dCK, dCMPD, and CD (10), were stored in liquid nitrogen with 10% glycerol. All cell lines were analyzed routinely and found to be free of *Mycoplasma* contamination (12). Cytotoxicity assays measuring per cent survival as colony-forming ability were performed as previously described (10).

Chemicals. Pyrimidine base and nucleoside and nucleotide standards were purchased from Sigma Chemical Company (St. Louis, MO), Calbiochem-Behring Corp. (La Jolla, CA), and P-L Biochemicals, Inc. (Milwaukee, WI) with the following exceptions. 2′-Deoxytetrahydrouridine was supplied and 5-fluoro-2′-deoxycytidine was custom synthesized by Calbiochem-Behring Corp. FdCMP was synthesized according to the method of Tanaka *et al.* (13). Tetrahydrouridine was a gift of Dr. Leonard Kedda of the National Cancer Institute (Bethesda, MD). Tetrabutylammonium phosphate was purchased from Eastman Kodak Co. (Rochester, NY).

Radiochemicals. Generally tritiated 5-fluoro-2′-deoxycytidine was custom synthesized by New England Nuclear (Boston, MA). Following HPLC analysis, [^{3}H]FdC (specific activity, 18.0 Ci/mmol) was found to be 99.9% pure and devoid of any measurable [^{3}H]FdU, its deaminated counterpart; no [^{3}H]dC was detected. [^{3}H]FdU was synthesized from [^{3}H]FdC by heating for 5 min at 100°, pH 5.0. The product was analyzed by reverse phase HPLC and found to be 100% FdU, with a specific activity of 14.7 Ci/mmol.

Enzymes. Pancreatic DNase I, snake venom phosphodiesterase I, protease K, RNase A, and bacterial alkaline phosphatase were purchased from Sigma Chemical Co.

Incorporation of FdC- and FdU-related antimetabolites into nucleic acids. Fifteen 100-mm tissue culture dishes were each seeded with 5.0×10^5 HEp-2 cells and incubated for 24 hr in a CO_2-humidified atmosphere at 37° to ensure log phase growth. EMEM containing [^{3}H]FdC with and without H_4U or dH_4U (or [^{3}H]FdU) was then applied and the cells were again incubated for 24 hr under the same conditions. At the end of this incubation period, media were aspirated and HEp-2 cells were washed three times with PBS (0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4, and 0.15% Na_2HPO_4) at 4° and gently scraped off with sterile rubber policemen. Cells were centrifuged for 15 min at 500 × *g* and 4°. Viability was determined via trypan blue exclusion (14) and cell pellets were divided into cytoplasmic and nuclear fractions with TEN buffer (10 mM Tris, pH 8.0, 100 mM NaCl, and 1.0 mM Na_2-EDTA) and centrifugation at 1000 × *g* for 10–15 min at 4°. The nuclear pellet was digested with 1% SDS and 100 μg/ml protease K for 18 hr at 37°; the cytoplasmic fraction was decanted and saved for further RNA analysis. The nuclear fraction was then extracted with phenol and chloroform:isoamyl alcohol (24:1 by volume), and total nucleic acid was precipitated in 70% ethanol and 0.25 M NaCl. After dialysis against three changes of TEA buffer (10 mM Tris buffer, pH 8.0, 1.0 mM Na_2-EDTA) at 4°, the concentration of total nucleic acid was determined by UV absorbance at 260 nm. Samples were then divided for the purifications of RNA and DNA.

Purification of nuclear RNA. RNA samples were digested with 300 units of DNase I for 12 hr and dialyzed against TEA buffer at 4°. Samples were then extracted with chloroform (3×) and the concentration of RNA was determined via UV absorbance. RNA samples were digested with 0.05 units of snake venom phosphodiesterase for 24 hr at 37° and the RNA was analyzed for the presence of FUMP by HPLC

using a C_{18} reverse phase column (15); this then allowed us to quantitate the amount of antimetabolites incorporated into RNA.

Purification of nuclear DNA. DNA samples were digested with 0.5 μg/ml ribonuclease A (5 min preboiled) for 12 hr at 37° and dialyzed against three changes of TEA buffer at 4°. After extraction with chloroform (3×) and determination of DNA concentration via UV absorbance, samples were sheared several times via passage through a 26-gauge needle and digested with 300 units of DNase I for 24 hr at 37°. The pH was adjusted to 8.5 and the samples were digested with 0.05 unit of snake venom phosphodiesterase and 500 μg of bacterial alkaline phosphatase for 24 hr at 37°. The deoxynucleoside components of DNA were separated from FdC and FdU by HPLC analysis using a C_{18} reverse phase column according to the method of Briggle *et al.* (15).

Purification of cytoplasmic RNA. The cytoplasmic fractions (supernatant) were digested with 1% SDS and 100 μg/ml protease K for 12 hr at 37°. Samples were deproteinized via chloroform extractions (3×), dialyzed against three changes of TEA buffer, pH 8.0, and digested with 300 units of DNase I for 12 hr at 37°. Samples were then dialyzed as previously described and the concentration of RNA was determined via UV absorbance. Samples were digested for 24–48 hr at 37° with 0.05 unit of snake venom phosphodiesterase, pH 8.5, extracted with 6% perchloric acid, and neutralized with KOH, and then the RNA was analyzed for the presence of FUMP by HPLC using a reverse phase C_{18} column (15).

Quantitation of antimetabolite pool size levels. Ten 100-mm tissue culture dishes were each seeded with 5.0×10^5 HEp-2 cells and incubated for 24 hr under conditions previously described to ensure log phase growth. EMEM was aspirated, replaced with EMEM containing various drug combinations, and incubated for 30 min under conditions described previously. After the 30-min exposure, HEp-2 cells were washed twice with PBS at 4°, placed on ice, and gently detached by scraping. Cells were centrifuged at 500 × *g* and 4° for 1 min. Viability was determined by trypan blue exclusion (14). Cell pellets were washed once with PBS at 4° and cell washings were analyzed for liberated antimetabolite nucleosides, nucleotides, and free bases. The final cell pellet was resuspended in 1.0 ml of distilled water containing 10 mM NaF (to inhibit liberated phosphorylase activity) and 4.5 mM *p*-nitrophenyl phosphate (to inhibit liberated phosphatases) and then sonicated (15-sec pulses at 20 kHz) at 4°. Samples were then heated (or left unheated for unbound FdUMP quantitation) at 65° for 15 min to dissociate FdUMP from the ternary complex, FdUMP-TS-5,10-methylene tetrahydrofolate (16). Samples were cooled to 4° and extracted for 1.0 hr with 6% $HClO_4$. KOH (5.0 M) was added to neutralize the samples and, following deproteination with chloroform, samples were analyzed by reverse phase HPLC (15) and various antimetabolite pool size levels were quantitated. No general tritium exchange was detected between FdC-related antimetabolites and either buffer systems or RNA, DNA, or pool constituents which would have complicated the quantitation of FdC-related antimetabolites; no radioactivity coeluted with normal DNA or RNA bases or normal nucleoside or deoxynucleoside pool constituents, as measured at $A_{260\,nm}$ and by liquid scintillation.

HPLC techniques. Quantitation of the incorporation of antimetabolites into RNA and DNA and the quantitation of antimetabolite pools were accomplished by reverse phase HPLC (Waters Associates, Milford, MA) using tetrabutylammonium phosphate and methanol on a C_{18} column (Alltech, Deerfield, IL) (15). Antimetabolites eluting from HPLC columns were collected in 0.7- or 1.0-ml fractions and the radioactivity in each fraction was determined with a Packard Tri-Carb Liquid Scintillometer (model 3320) with Aquasol scintillant (New England Nuclear). Quenching was not detected in any of the samples tested.

TLC techniques. FdC and FdU were separated by TLC using Baker-Flex cellulose sheets (J. T. Baker, Atlanta, GA) and an isopropanol:HCl:water (4:1:1 by volume) solvent system. R_f values were 0.58 and 0.78 for FdC and FdU, respectively. Appropriate spots were visualized via UV absorbance, excised, and counted as previously described using a nonaqueous scintillant (3.8 liters of toluene/15 g of 2,5-diphenyloxazole/0.38 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene).

RESULTS

Incorporation of FdC into DNA. Since it was previously shown that FdC can be anabolized to FdCTP and ultimately become an efficient substrate for DNA polymerase (13, 17), the incorporation of FdC in HEp-2 cell DNA and the effects of H_4U or dH_4U on such incorporation were examined. The addition of 0.01 μM FdC to log phase HEp-2 cells for 24 hr resulted in the incorporation of 5.22×10^{-8} mol of FdC/mol of DNA phosphate as determined by HPLC; this corresponds to a 0.021% substitution of FdC for dC. The coadministration of 1.0 mM H_4U, which was previously shown to result in 93% inhibition of cytidine deaminase (10), resulted in a 2-fold increase in the incorporation of FdC into HEp-2 DNA. dH_4U (1.0 mM), which leads to 92 and 95% inhibitions of cytidine and deoxycytidylate deaminases (10), respectively, resulted in a 25-fold increase in the incorporation of FdC (0.53% substitution of FdC for dC) into HEp-2 DNA; dH_4UMP, which has been shown to be formed from dH_4U *in vivo* (18), inhibits dCMPD (Fig. 1).

To demonstrate that FdC was indeed the antimetabolite incorporated into the DNA of HEp-2 cells, samples were analyzed by HPLC and appropriate peaks of FdC and FdU were collected and counted. FdC and FdU peaks from HPLC analyses were then reanalyzed using TLC (Table 1). TLC analysis of FdU peaks (determined via HPLC analysis of 12 pmol of FdU standard) showed no counts associated with FdU (R_f 0.78) or FdC (R_f 0.58). FdC peaks generated via HPLC (12 pmol of FdC standard included in all samples) were then reanalyzed by TLC (Table 1) and counts associated solely with FdC were detected; no detectable counts associated with FdU were found. Levels of FdC incorporated, as determined by TLC (Table 1), were consistent with those found using HPLC. Further confirmation that FdC was the antimetabolite incorporated into HEp-2 DNA came from the fact that FdC, when boiled for 5 min at pH 5.0, was shown by standard analysis to be converted 100% to FdU. When samples were heated in this way, all counts

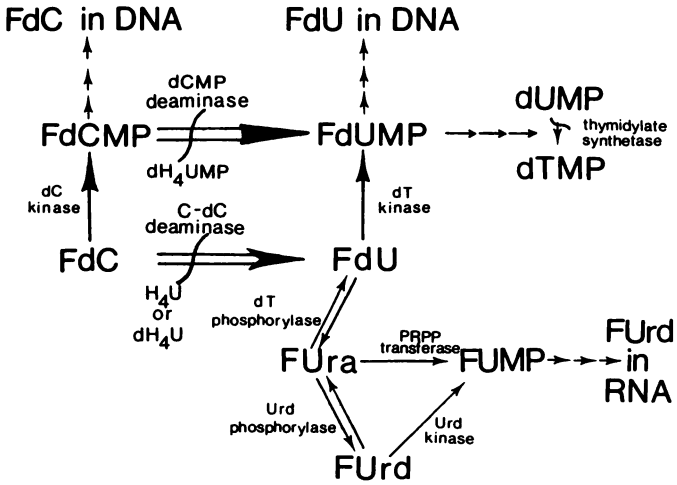

FIG. 1. *H_4U leads to the inhibition of CD, while dH_4U leads to the inhibitions of both CD and dCMPD*

TABLE 1

Confirmatory evidence via TLC that FdC is the antimetabolite incorporated into the DNA of log phase HEp-2 cells treated with FdC for 24 hr

1.0×10^5 HEp-2 cells were plated on each of 10 100-mm tissue culture dishes. At the end of a 24-hr incubation period to ensure log phase growth, Eagle's minimal essential medium containing drugs was added and exposure was allowed to continue for 24 hr. DNA from these cells was then purified and digested with DNase I, alkaline phosphatase and snake venom phosphodiesterase. Deoxyribonucleoside components of DNA were analyzed by HPLC with a reverse phase C_{18} column and a detection limit of 5 fmol.

Condition	FdC[a] (R_f 0.58)	FdU[a] (R_f 0.78)	DNA[b] concentration	FdC incorporated in DNA phosphate[c]
	cpm		*μg/ml*	*mol/mol*
A. FdC (0.01 μM)	633	142	21.5	6.38×10^{-8}
B. FdC (0.01 μM) + H_4U (1.0 mM)	1433	105	21.3	1.26×10^{-7}
C. FdC (0.01 μM) + dH_4U (1.0 mM)	2554	86	19.3	1.54×10^{-6}
D. FdC (1.0 μM) + dH_4U (1.0 mM)	4516	126	13.4	2.79×10^{-5}
D.[d] FdC (1.0 μM) + dH_4U (1.0 mM)	241	2461	13.4	2.96×10^{-5}[e]
D.[d] FdC (1.0 μM) + dH_4U* (1.0 mM)	2284	259	13.4	2.88×10^{-5}

[a] Following the injection of 25 μl of each sample into the HPLC, fractions were collected and analyzed via liquid scintillation counting of 10 μl/fraction. Once FdC and FdU peaks were determined via counting and HPLC standard analysis, the corresponding fractions were combined and 48 μl was run on a thin layer chromatography cellulose sheet with FdC and FdU standards; only 8 μl was analyzed with samples C and D. FdC and FdU spots were analyzed as described in Materials and Methods.

[b] The concentration of DNA determined by absorbance at 260 nm from untreated HEp-2 cells was 15.7 μg/ml.

[c] Determined by TLC.

[d] A portion of sample D was heated for 5 min at 100° or left untreated (*). The samples were then reanalyzed by HPLC and TLC as in Footnote a.

[e] Moles listed are moles of FdU incorporated/mol of DNA phosphate; only counts associated with FdU were detected.

were associated by retention time with HPLC analysis of standard FdU, and no FdC was detected. TLC analysis of heated and unheated samples (sample D, Table 1) showed that, when heated, the amount of FdU derived from FdC was essentially equivalent to the amount of FdC from an unheated portion of the same sample.

The exogenous conversion of FdC to FdU via fetal calf serum-derived cytidine deaminase, which could result in the treatment of log phase HEp-2 cells with FdU and thereby make the interpretation of data extremely complex, was then examined. No conversion of FdC to FdU occurred with normal EMEM containing 10% fetal calf serum under conditions identical to those described for incorporation or pool size experiments as assayed by HPLC (data not shown).

Fig. 2 summarizes a series of experiments in which log phase HEp-2 cells were treated with various concentrations of FdC and 1.0 mM dH_4U. The extent of incorporation of FdC into the DNA of treated HEp-2 cells was then monitored. A linear relationship between the incorporation of FdC and the concentration of exogenously supplied FdC was found with a correlation coefficient of 0.983.

Since FdC was thought to incorporate in place of deoxycytidine, 0.1 mM dC was coadministered with 0.1 μM FdC and 1.0 mM dH_4U. The administration of 0.1 mM dC led to a 5.3-fold decrease in the incorporation of FdC into the DNA of log phase HEp-2 cells (Table 2, experiment 2). The administration of 0.1 mM dC to 0.1 μM FdC + 1.0 mM H_4U resulted in a 2.1-fold decrease in the incorporation of FdC into the DNA of HEp-2 cells (Table 2, experiment 1). No detectable incorporation of FdU into the DNA of log phase HEp-2 cells was found following the administrations of FdC with and without H_4U or dH_4U. Since the possibility existed that FdU, derived from FdC, was not detected in DNA due to the combined actions of the dUTPase and uracil-*N*-glycosylase (19–21), we investigated whether or not FdU treatment at concentrations comparable to those of FdC (above) would result in the incorporation of FdU into HEp-2 DNA over a 24-hr period (Table 2). In comparison to a treatment of log phase HEp-2 cells with 0.1 μM FdU which resulted in the incorporation of 1.84×10^{-8} mol of FdU/mol of DNA phosphate, administrations of FdC with or without H_4U and dH_4U resulted in no detectable FdU incorporated into DNA (detection limit, 1.0×10^{-9} mol of FdU or FdC/mol of DNA phosphate). It is interesting that the extent of FdU incorporation with 0.1 and 1.0 μM FdU treatments was essentially equivalent. Whether this reflects extensive inhibition of growth (reflected in lower DNA concentration) with 1.0 μM FdU or extensive and constant repair processes remains undetermined.

Incorporation into RNA. After determining that FdC was incorporated into the DNA of HEp-2 cells, we asked whether or not FdC-related antimetabolites were incorporated into the RNA of log phase HEp-2 cells. Therefore, we set out to quantitate the extent of conversion of FdC-related antimetabolites to the RNA level (i.e., production of FUra, FUrd, and FUMP). Since the incorporation of FUMP into RNA may have been below our detection limit, we designed our initial experiments so

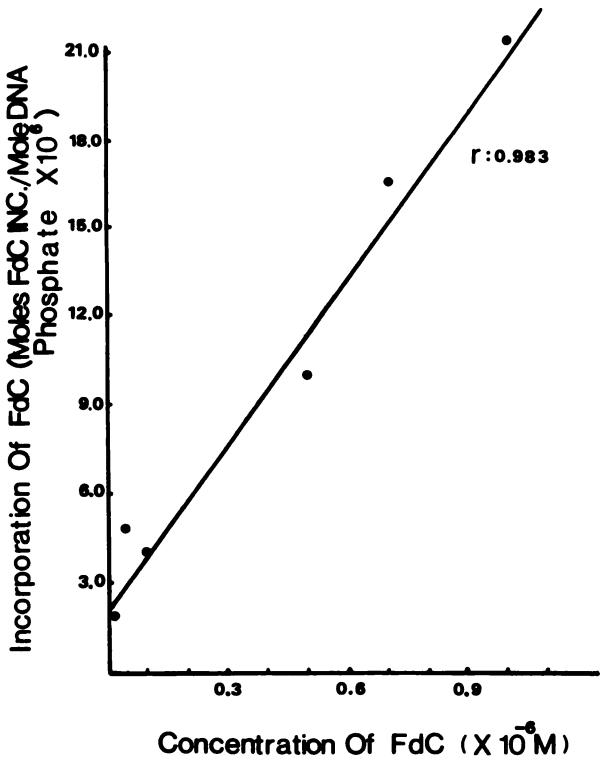

FIG. 2. *Incorporation of FdC into the DNA of HEp-2 cells as a function of concentration during a 24-hr incubation period*

1.0 mM dH_4U was coadministered with each concentration of FdC used.

that both the formation and incorporation of FUMP could be assayed (see legend to Table 3). When FdC was administered alone without H_4U or dH_4U, extensive formation and incorporation of FUMP into both the nuclear and cytoplasmic RNAs occurred in 24 hr (Table 3). However, when H_4U or dH_4U was coadministered, no FUMP (a combination of FUMP pools and FUMP incorporated into RNA) was detected in either the cytoplasmic or nuclear RNA fractions. When the concentration of FdC was increased to 0.7 μM with 1.0 mM dH_4U, a small amount of FUMP was detected in the cytoplasmic RNA fraction; however, no FUMP was detected in the nuclear RNA fraction.

The incorporation of FUMP (without FUMP pools) into both the cytoplasmic and nuclear RNAs of HEp-2 cells following various drug treatments was then investigated (Table 2). When coadministered with H_4U or dH_4U, 0.1 μM FdC resulted in no detectable incorporation of FUMP (derived from [^{3}H]FdC) into either the cytoplasmic or nuclear RNAs of HEp-2 cells. In contrast, treatment of log phase HEp-2 cells solely with 0.1 μM FdC resulted in considerable incorporation of FUMP into both cytoplasmic and nuclear RNAs. When the concentration of FdC was raised from 0.1 to 0.7 μM with 1.0 mM H_4U (Table 2, experiment 2), FUMP was found to be incorporated into both cytoplasmic and nuclear RNAs. This is similar to the results obtained with FdC (0.7 μM) + dH_4U (1.0 mM) (Table 3). Apparently, when the concentration of FdC was increased, it effectively competed with either H_4U or dH_4U for cytidine deaminase, leading to its conversion to RNA-level antimetabolites.

The extent of FUMP incorporation into the RNA of HEp-2 cells derived from FdU treatments as compared to that of FdC with and without H_4U or dH_4U was also examined (Table 2, experiment 1). FUMP was found to be incorporated into the RNA of HEp-2 cells treated with FdU or FdC. Comparable levels of FUMP were found with 0.1 μM FdC or FdU. No FUMP was found incorporated into the RNA of HEp-2 cells treated with FdC and a deaminase inhibitor, H_4U or dH_4U.

Pool size studies. We initially performed pool size studies without inhibitors of liberated phosphorylase and phosphatase enzymes (data not shown). However, the presence of a significant amount of FdU following FdC + H_4U or dH_4U treatments (data not shown) to log phase HEp-2 cells was inconsistent with previous RNA incorporation data shown in Table 2. Therefore, we investigated the possibility that liberated phosphatases (via sonication in our extraction protocol) converted FdUMP to FdU. When NaF and *p*-nitrophenyl phosphate were administered to inhibit liberated phosphorylase and phosphatase enzymes, respectively, a significant decrease in the formation of FdU resulted; essentially no FdU, FUra, FUrd, or FUMP levels were found when either H_4U or dH_4U was coadministered with FdC (Table 4). Thus, it seems that liberated phosphatases may convert FdUMP to FdU and alter the quantitation of actual antimetabolite pools. Similarly, liberated phosphorylases may alter quantitation of antimetabolite pools by allowing the conversion of FdU to FUra, thus leading to the production of FUrd and FUMP (see Fig. 1).

Using this protocol, the metabolism of FdU was compared to that of FdC with and without H_4U or dH_4U (Table 4). Treatment of log phase HEp-2 cells with 0.1 μM FdC resulted in an essentially equal distribution of antimetabolite pools (Table 4), including all RNA-level antimetabolites (FUra, FUrd, and FUMP). However, when coadministered with either H_4U or dH_4U, FdC-treated log phase HEp-2 cells showed little or no formation of RNA-level antimetabolites or FdU.

The extent of FdCMP formation in 30 min was also assayed (Table 4). Coadministration of dH_4U with FdC resulted in the greatest levels of FdCMP; thus, the administration of dH_4U directs the metabolism of FdC towards its incorporation into DNA. H_4U coadministered with FdC resulted in the formation of FdCMP, but less than that formed with dH_4U. Administration of FdC alone resulted in the least amount of FdCMP. These data are consistent with data concerning RNA and DNA antimetabolite incorporation shown in Tables 1–3.

The formation of FdCMP in 24 hr was also quantitated (Table 3). Levels of FdCMP pools produced in 24 hr were consistent with, and reflected the incorporation of, FdC into the DNA of log phase HEp-2 cells. In experiment 1 (Table 3), 12.7 fmol of FdCMP were formed when HEp-2 cells were treated with 0.01 μM FdC. When 1.0 mM H_4U or 1.0 mM dH_4U was coadministered with 0.01 μM FdC, 4.2- and 10.4-fold increases, respectively, in the formation of FdCMP resulted. In experiment 2, Table 3, 1.3- and 2.8-fold respective increases in the formation of

TABLE 2

The extent of incorporation of FdU-related antimetabolites into the RNA and DNA of HEp-2 cells compared to FdC with and without H_4U or dH_4U

Log phase HEp-2 cells were exposed to various FdC regimens and nuclei were separated from the cytoplasm. From the nuclear fraction, total nucleic acid was isolated and digested with either RNase (B) or DNase I (A). Cytoplasmic DNA was degraded with DNase I and eliminated via dialysis and the RNA was digested with snake venom phosphodiesterase as described for the nuclear RNA fraction. 5′-Monophosphate components of RNA were analyzed and the amount of FUMP present was quantitated by HPLC as described in Materials and Methods. DNA was purified and analyzed by HPLC as described in Materials and Methods. ND, not done.

Expt. and condition	A. RNA incorporation				B. DNA incorporation			No. viable cells[d]	Survival[e] (±SE)
	Nuclear		Cytoplasmic		DNA concentration[c]	FdU	FdC		
	RNA concentration[a]	Incorporation	RNA concentration[b]	Incorporation					
	μg/ml	*mol FUMP/mol RNA phosphate*	*μg/ml*	*mol FUMP/mol RNA phosphate*	*μg/ml*	*mol/mol DNA phosphate*		*×10⁶*	%
1. FdC (0.1 μM)	12	2.23×10^{-5}	92	2.39×10^{-6}	104	0[f]	2.11×10^{-7}	9.70	36.7 ± 4.81
1. FdC (0.1 μM) + H_4U (1.0 mM)	12	0[f]	47	0[f]	125	0[f]	3.01×10^{-7}	11.3	47.6 ± 2.37
1. FdC (0.1 μM) + H_4U (1.0 mM) + dC (0.1 mM)	9	0[f]	83	0[f]	157	0[f]	1.41×10^{-7}	17.4	49.8 ± 3.85
1. FdC (0.1 μM) + dH_4U (1.0 mM)	12	0[f]	38	0[f]	126	0[f]	5.06×10^{-6}	14.0	ND
1. FdU (0.1 μM)	11	1.10×10^{-5}	90	9.44×10^{-7}	147	1.84×10^{-6}	0[f]	10.3	43.5 ± 4.67
1. FdU (1.0 μM)	7	1.22×10^{-5}	35	5.55×10^{-7}	139	1.04×10^{-6}	0[f]	4.65	1.20 ± 0.02
2. FdC (0.7 μM) + H_4U (1.0 mM)	1.2	4.40×10^{-7}	21	1.41×10^{-7}	59	0[f]	3.42×10^{-6}	4.59	6.9 ± 4.00
2. FdC (0.1 μM) + dH_4U (1.0 mM)	1.1	0[f]	24	0[f]	57	0[f]	3.39×10^{-6}	12.9	ND
2. FdC (0.1 μM) + dH_4U (1.0 mM) + dC (0.1 mM)	1.2	0[f]	27	0[f]	58	0[f]	6.35×10^{-7}	ND	ND

[a] The concentration of nuclear RNA from untreated HEp-2 cells was 9.60 μg/ml.

[b] The concentration of cytoplasmic RNA from untreated HEp-2 cells was 52.2 μg/ml.

[c] The concentration of DNA from untreated HEp-2 cells was 98.8 μg/ml.

[d] Viable cell counts at the end of drug exposure were performed via trypan blue exclusion (14).

[e] In a separate experiment, 5.0×10^5 log phase HEp-2 cells were exposed to various drug treatments for 24 hr and then colony-forming ability was assayed as previously described (10).

[f] <5.0 fmol detected which corresponds to $<1.0 \times 10^{-9}$ mol incorporated/mol of RNA or DNA.

TABLE 3

HPLC quantitation of the formation of FdCMP and FUMP antimetabolite pools and the incorporation of FUMP into log phase HEp-2 cells

Log phase HEp-2 cells were exposed to tritiated drug regimens for 24 hr as described in Materials and Methods. Following SDS-protease K digestion, DNA samples were treated with RNase. DNA was precipitated and separated from the RNA components at −20° with 0.25 M NaCl + 70% ethanol, extensively dialyzed, digested with DNase I, snake venom phosphodiesterase and bacterial alkaline phosphatase, and analyzed by HPLC as described in Materials and Methods. The results of DNA analyses are presented in Table 2 and Fig. 2. The 3′-monophosphate components of the RNA fractions were purged of 70% ethanol, extracted with chloroform and 6% perchloric acid at 4°, and analyzed via HPLC using a reverse phase C_{18} column. The cytoplasmic fraction was digested with RNase, extracted with chloroform and 6% perchloric acid, and analyzed via HPLC under conditions identical to that of the nuclear fraction analyses.

Expt.	Condition			FdCMP formed in HEp-2 cells	FUMP formed or incorporated into HEp-2 cells RNA	
	FdC	H_4U	dH_4U		Nuclear	Cytoplasmic
				fmol/1 × 10^6 cells	fmol/1 × 10^6 cells	
1	0.01			12.7	104.6	506.6
1	0.01	1.0		53.6	0[a]	0[a]
1	0.01		1.0	132.2	0[a]	0[a]
2	0.1			37.4	78.0	1351.5
2	0.1	1.0		47.4	0[a]	0[a]
2	0.1		1.0	105.6	0[a]	0[a]
2	0.1		0.1	115.3	0[a]	0[a]
2	0.1		0.01	84.0	0[a]	0[a]
2	0.7		1.0	265.3	0[a]	37.7

[a] <5.0 fmol were detected.

FdCMP resulted over that formed with 0.1 μM FdC, when 1.0 mM H_4U or dH_4U was coadministered with 0.1 μM FdC. We also determined the effect of lower dH_4U concentrations on the formation of FdCMP in log phase HEp-2 cells. Within the concentrations of dH_4U utilized (1.0–0.01 mM) with 0.1 μM FdC, no significant difference resulted. Evidently, within this concentration range, cytidine, and dCMP deaminases were effectively inhibited.

The administration of FdU to HEp-2 cells resulted in the extensive formation of RNA-level antimetabolites (FUra, FUrd, and FUMP) and FdU; formation of FUrd, FUra, FUMP, and FdUMP pools was similar to the case when only FdC was administered to log phase HEp-2 cells (Table 4).

Since the generation of FdUMP is thought to be the critical determinant of antineoplastic activity with fluoropyrimidines, we quantified its formation (Table 4). Free and bound FdUMP levels were assayed following exposure of log phase HEp-2 cells to FdC with and without H_4U or dH_4U as compared to equivalent concentrations of FdU (Table 4). We also analyzed the reproducibility of our pool size studies via triplicate drug exposure followed by HPLC analysis of one condition, FdC + H_4U (Table 4). Following statistical analysis, a standard error of less than 6% was found. Significantly greater amounts of both free and bound FdUMP were formed when H_4U was coadministered with FdC than when FdU was administered. Without a deaminase inhibitor, the administration of FdC resulted in a substantial amount of bound FdUMP; however, no free FdUMP levels were detected. When both cytidine and deoxycytidylate deaminases were inhibited, following dH_4U treatment, very little bound FdUMP and no free FdUMP levels were detected. It should be noted that no FdU, FUra, or FUrd along with any of the nucleotides derived from either [^{3}H]FdC or [^{3}H]FdU were detected in any cell pellet washings. Thus, the protocol developed in our studies apparently prevents the diffusion of nucleoside and nucleotide antimetabolites out of HEp-2 cells.

TABLE 4

Antimetabolite pool size analyses via HPLC of log phase HEp-2 cells over a 30-min period following various drug exposures

Log phase HEp-2 cells (combination of 15 100-mm tissue culture dishes each plated with 5.0 × 10^5 cells) were exposed to tritiated drugs for 30 min. Cells were washed with PBS at 4° and gently detached via rubber policemen. The resulting cell pellet was prepared and analyzed by HPLC as described in Materials and Methods.

Expt. and Condition	Formation in viable HEp-2 cells[a]							
	FUra (6, 7)	FdC (10, 11)	FUrd (12, 13)	FdU (14, 15)	FdCMP (30–33)	FUMP (39–41)	Free FdUMP[b] (9, 10)	Bound FdUMP[b] (9, 10)
	fmol/1 × 10^6 cells							
1. FdC (0.1 μM)	63.8	67.9	48.6	172	35.8	169	0[c]	36.9
1. FdC (0.1 μM) + H_4U[e] (1.0 mM)[d]	0[c]	54.9 (±2.87)	0[c]	0[c]	91.5 (±2.83)	0[c]	22.0 (±1.84)	62.0 (±1.04)
1. FdC (0.1 μM) + dH_4U (1.0 mM)	0[c]	54.2	0[c]	0[c]	206.5	0[c]	0[c]	11.4
1. FdU (0.1 μM)	181	0[c]	159.7	215.7	0[c]	253	11.3	43.9
2. FdU (0.01 μM)	46.9	0[c]	41.3	76.2	0[c]	89.2	0[c]	9.63

[a] Viability was measured via trypan blue exclusion (14). Samples were injected into the HPLC with appropriate standards and fractions were collected (1.0-ml fractions/min) as described previously (15); fraction numbers are in parentheses. For the analyses of FdUMP, a flow rate of 2.0 ml/min was utilized.

[b] Analysis of FdUMP was performed using 20% methanol. Free FdUMP represents that amount of FdUMP not bound to thymidylate synthetase.

[c] <5.0 fmol detected.

[d] Standard error is in parentheses.

[e] Numbers represent the mean value of triplicate drug exposures followed by three separate HPLC analyses.

We have not assayed for di- and triphosphate antimetabolites for three reasons: (a) less than 6% of the total radioactivity, as assayed in 30 min, was associated with di- and triphosphate antimetabolite pools (FdCDP, FdCTP, FdUDP, and FdUTP), which elute later than at 70 min using 20% methanol; (b) FdCDP, FdCTP, FdUDP, and FdUTP standards have not yet been synthesized; and (c) the incubation periods (30 min) used do not seem to result in di- and triphosphate antimetabolite pools indicative of regulatory events which may occur during actual drug treatments. Studies concerned with the kinetics of antimetabolite pool formation over various time periods are in progress.

It became apparent that the restriction of FdC-related antimetabolites to the DNA level afforded by H_4U coadministration (i.e., production of FdCMP and FdUMP only) may be the result of extensive inhibition of thymidine phosphorylase by H_4U. Therefore, we investigated the effect of H_4U upon the metabolism of FdU (Table 5), which, if H_4U inhibited thymidine phosphorylase, would be restricted entirely to FdUMP and FdU and no formation of FUrd, FUra, or FUMP would result. When 1.0 mM H_4U was coadministered with 0.1 μM FdU, the resulting antimetabolite pools formed in 30 min were no different from those produced from exposure solely with 0.1 μM FdU or with 0.01 mM H_4U and 0.1 μM FdU (Table 5A); when ratios of antimetabolite pools formed divided by the total amount of antimetabolites detected are compared, the aforementioned drug exposures result in similar ratios.

Also investigated in Table 5 was the possibility of antimetabolite pool size alterations caused by the extraction protocol using phosphatase and phosphorylase inhibitors (Table 4). When cells were extracted directly from tissue culture plates with 6% PCA, neutralized with 5.0 mM KOH, and analyzed by HPLC, the resulting antimetabolite pools formed were similar to those formed using the extraction protocol developed in this laboratory (compare A and B, Table 5). However, total FdUMP pools as assayed by acid extraction (specifically bound FdUMP levels) were not identical to those detected following extraction using inhibitors of thymidine phosphorylase and phosphatases. In all cases, the bound FdUMP levels were lower following acidic extraction than following extraction using phosphatase and phosphorylase inhibitors. This is most likely due to the inability of heating at 65° for 15 min in releasing bound FdUMP from its inhibitory complex with TS following KOH neutralization; heating of an unneutralized 6% PCA extraction mixture must be avoided when exposing cells to FdC to avoid the spontaneous deamination of FdCMP to FdUMP which would result in significant pool size aberrations. Additionally, we have investigated the possibility that acid extraction at 4° could result in: (a) conversion of one form of ribo- or deoxyribonucleotide to another nucleotide (e.g., deamination) or nucleoside; (b) the unlikely conversion of deoxyribunucleosides to ribonucleosides; and (c) conversion of deoxyribonucleosides or deoxyribonucleotides to free bases. PCA (6%) extraction under conditions used in this study did not: (a) convert [^{3}H]FdC or [^{3}H]FdU to any free base or ribonucleoside, (b) convert FdUMP or FdCMP to nucleosides or free bases, or (c) alter any 5′-triphosphate deoxyribonucleoside standard tested.

DISCUSSION

The data presented in this paper have demonstrated that the metabolism of FdC can be directed using inhib-

TABLE 5

The effect of H_4U upon the formation of FdU-related antimetabolite pools in 30 min

Experimental conditions were identical to those described in Table 4 and in Materials and Methods.

Condition			Formation in viable HEp-2 cells[a]							
FdC	FdU	H_4U	FUra (6, 7)	FdC (10, 11)	FUrd (12, 13)	FdU (14, 15)	FdCMP (30–33)	FUMP (39–41)	Free FdUMP	Bound FdUMP
μM	μM	mM	*fmol/1 × 10^6 cells*							
A. Inhibitor extraction[b]										
0.1		1.0	0[c]	79.8	0[c]	0[c]	75.6	0[c]	31.0	54.2
	0.1	1.0	109	0[c]	76	114	0[c]	41.6	12.3	24.6
	0.1	0.01	112	0[c]	119	132	0[c]	48.9	10.6	34.8
	0.1		140	0[c]	169	197	0[c]	89.2	16.6	32.6
B. Acid extraction[d]										
0.1		1.0	0[c]	83.6	0[c]	5.0	87.2	0[c]	15.3	27.2
	0.1		143	0[c]	140	99.0	0[c]	82.6	9.04	5.23
	0.1	1.0	172	0[c]	200	121	0[c]	57.0	10.4	8.54

[a] Numbers in parentheses are fraction numbers in which these compounds eluted from HPLC as described in Materials and Methods.

[b] Extraction was carried out using water containing 10 mM NaF and 4.5 mM *p*-nitrophenyl phosphate. Samples were heated to release FdUMP as described in Materials and Methods and finally extracted with 6% perchloric acid at 4° for 1.0 hr. Samples were then processed for HPLC analysis as described in Materials and Methods.

[c] <5.0 fmol detected.

[d] Conditions were identical to that of extraction using inhibitors (A) except that, following the 30-min drug exposure, cells were washed once with ice-cold PBS and immediately extracted with 6% perchloric acid at 4°. Cell extracts from 10 100-mm plates were quickly scraped and combined. After 1 hr at 4°, the combined extract was neutralized with KOH. Samples were then divided for free and bound FdUMP analysis as described in Materials and Methods; the precipitate was heated at 65° for 15 min in an attempt to release bound FdUMP. Samples were then processed as described in Materials and Methods.

itors of its deamination. When H_4U, an inhibitor of cytidine deaminase, was coadministered with FdC: (a) FdC was found to be incorporated to a small extent into the DNA of HEp-2 cells; however, no detectable incorporation of FdU into DNA was found as with equivalent concentrations of FdU; (b) no detectable FdC-related antimetabolites were found incorporated into the RNA (either cytoplasmic or nuclear) of HEp-2 cells; furthermore, no detectable RNA-level antimetabolite pools (FUra, FUrd, or FUMP) or FdU were found; and (c) a significant amount of FdUMP, the inhibitor of thymidylate synthetase, was detected; greater levels of FdUMP were formed than with equivalent concentrations of FdU. The results of these studies are consistent with our hypothesis that treatment of neoplastic cells with FdC and H_4U will result in the preferential conversion of a nontoxic, phosphorylase-resistant fluoropyrimidine (FdC) by the dCK-dCMPD pathway to substantial levels of FdUMP without the formation, or incorporation, of antimetabolites at the RNA level.

The coadministration of FdC and dH_4U, a potent inhibitor of both cytidine and deoxycytidylate deaminases (18), to log phase HEp-2 cells resulted in a channeling of FdC towards its incorporation into DNA (see Fig. 1). The incorporation of FdC, in place of deoxycytidine, into DNA was not surprising since FdCTP has been shown to be a substrate for α and β DNA polymerases from calf thymus (13), and since FdC has been shown to affect differentiation (17, 22). However, the extent of incorporation found previously (17) is not consistent with that found in HEp-2 cells. This discrepancy may be explained based upon the lack of CD and dCMPD enzymes in Chinese hamster ovary cells. Without deaminating enzymes, FdC incorporated at extremely high levels (17) since these cells do contain elevated dCK levels. Since many tumors contain elevated CD and dCMPD levels, the HEp-2 tumor model used in our studies probably represents more accurate conditions for the incorporation of FdC. Without labeled FdC, the amount of FdC detected in the DNA of mammalian cells would be subjected to considerable error using the methods previously described (17).

It is interesting that the treatment of HEp-2 cells with FdC did not result in detectable incorporation of FdU into DNA under any of the conditions tested. In contrast, HEp-2 cells treated with FdU did incorporate FdU into their DNA at levels comparable to those previously reported (3, 23). It is possible that the incorporation of FdU, derived from FdC, into DNA was simply below our detection limit ($<1.0 \times 10^{-9}$ mol/mol of DNA phosphate). Whether the lack of incorporation of FdU derived from FdC into the DNA of HEp-2 cells reflects increased repair (by uracil-DNA glycosylase), increased FdUTP degradation (by dUTPase) or some other allosteric regulatory events which prevent the incorporation of FdU derived from FdC into DNA remains to be determined. We are currently investigating the effect of various FdC treatments, with and without H_4U or dH_4U, on normal deoxynucleotide and deoxynucleoside pools to assess regulatory changes which may be occurring following treatment with FdC and H_4U or dH_4U.

The significance of the incorporation of FdC into DNA remains unclear. It is possible that the incorporation of FdC into DNA and its subsequent repair (if it is repaired) may lead to the production of small DNA fragments, as previously found with the incorporation of uracil or 5-fluorouracil into DNA (23–25). Even though the repair of misincorporated FdC into DNA may involve an entirely different set of enzymes than those involved in the repair of uracil or fluorouracil, the overall deleterious effects in a cell may be similar. The relationship between FdC incorporation into DNA and cytotoxicity will be explored in subsequent studies. The incorporation of FdC into tumor as compared to normal tissue DNA may provide a basis for selectivity against malignant cells (due to elevated dCK activities in tumor tissue) in a manner similar to that proposed for FdU (3). The effect of FdC incorporation into DNA on normal maintenance methylation patterns, both *in vitro* and *in vivo* with normal and tumor tissues, also warrants further studies. It is possible that a small portion of the antitumor activity of FdC (10) may be related to its effects on differentiation as proposed for 5-aza-2′-deoxycytidine (22, 26, 27).

It is apparent from the data that, at very high concentrations, FdC may compete with H_4U or dH_4U for cytidine deaminase allowing some conversion of FdC to the RNA level. The result of such conversion (either by competition by FdC for CD or when FdC is administered alone) is the ultimate incorporation of FUMP derived from FdC into the RNA of HEp-2 cells. In contrast to the administrations of FdC and H_4U or dH_4U, the administration of FdU to log phase HEp-2 cells for 24 hr resulted in extensive incorporation of FUMP into both cytoplasmic and nuclear RNAs (Table 2). These results are consistent with previous studies (5, 28, 29) which have demonstrated FUMP incorporation into the RNA of mammalian cells treated with FdU or FUra.

It is interesting that the extent of incorporation of FUMP into RNA derived from FdU was similar to that found with FdC when administered alone (Table 2). From the data in Table 2, it is clear that the enzyme pattern in HEp-2 cells favors a strong equilibrium for FdC to be rapidly converted to RNA-level antimetabolites and ultimately incorporate into RNA; FdC was metabolized by both the dCK-dCMPD and CD-dTK pathways. Since no apparent incorporation of FdU into the DNA of HEp-2 cells was found when FdC was administered alone and since very few FdUMP pools were formed (all detectable FdUMP formed was bound to thymidylate synthetase), catabolic processes must have actively converted FdU formed from FdC to FUra. FUra once formed was then converted to FUrd and FUMP, which was then incorporated into RNA (see Fig. 1). Such nonspecific incorporation of FUMP into the RNA of cells would contribute no antitumor selectivity since there is no significant difference between phosphorylase activities in normal human tissue compared to that present in human malignant tissue (11, 30, 31). Even though in some tumors thymidine phosphorylase activities are significantly elevated above those of normal human tissue (30), the inherently high levels of uridine

and thymidine phosphorylase present in systemic circulation (serum) and in normal tissue would allow extensive FUMP incorporation and thereby lead to general toxicity rather than to a neoplastic-specific mechanism of action. As a consequence, we propose that the same extent of incorporation of FUMP following FUra or FdU treatments would occur in actively dividing normal as in tumor tissue; *in vivo* studies to support the proposal that the drug combination FdC + H_4U would avoid such general toxic events are presently under way.

Results of antimetabolite pool size analyses following exposure of HEp-2 cells to FdU or FdC with and without H_4U or dH_4U are consistent with RNA and DNA incorporation data. The coadministration of H_4U or dH_4U to inhibit cytidine deaminase effectively blocked the formation of any RNA-level antimetabolites derived from FdC; this restriction has been shown not to be related to the inhibition of thymidine phosphorylase, but solely to the inhibition of cytidine deaminase (Table 5). Without the coadministration of a cytidine deaminase inhibitor, exposure of log phase HEp-2 cells to FdC resulted in the formation of essentially equal amounts of all antimetabolites at the ribose and deoxyribose levels, a result similar to that of cells exposed to FdU. FdCMP pool levels were elevated by the administration of H_4U or dH_4U in a manner identical with the incorporation of FdC into DNA following H_4U or dH_4U coadministration. Thus, the inhibitions of CD (by H_4U) and both CD and dCMPD (by dH_4U) diverted the anabolism of nontoxic levels of FdC towards the formation of FdCMP and ultimately to 2- and 25-fold increases in the incorporation of FdC into DNA.

The total levels of FdUMP derived from the coadministration of FdC and H_4U were found to be consistently greater than those formed following the administration of FdU. The net result is a greater pool size level of free FdUMP which would be used later to inhibit newly synthesized TS; newly synthesized TS could lead to a deinhibition of previously inhibited tumor cells. In contrast, it is believed that the cytotoxicity caused by FUra, specifically to HEp-2 cells, is not directly related to the formation of FdUMP, the potent inhibitor of TS; the growth-limiting event for HEp-2 cells exposed to FUra was not the inhibition of TS due to an inherently low level of 5,10-methylene tetrahydrofolate (32, 33), a necessary component in the formation of the inhibitory, ternary TS complex. Indeed, in the presence of excess folates, the growth-limiting event following exposure to FUra became the inhibition of TS (34). Therefore, we are currently investigating the effect of exogenously supplied folate intermediates upon the cytotoxicities of FUra or FdU compared to those of FdC with and without H_4U or dH_4U upon HEp-2 cells. Such comparative studies with FUra, FdU, and FdC + H_4U both *in vitro* and *in vivo* would allow the uncoupling of RNA incorporation and TS inhibition and settle a currently controversial matter: whether TS inhibition, or RNA incorporation and the subsequent inhibition of RNA maturation, is the major mechanism of antineoplastic action of 5-fluoropyrimidines, including FdC.

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