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SEQUENCE-DEPENDENT INTERACTION OF 5-FLUOROURACIL AND ARABINOSYL-5-AZACYTOSINE OR 1-β-D-ARABINOFURANOSYLCYTOSINE

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Abstract—We studied the cytotoxicity of arabinosyl-5-azacytosine (Ara-AC), a dCyd antagonist which inhibits DNA synthesis, in combination with 5-fluorouracil (FUra) in two human colon cancer cell lines, HCT 116 and SNU-C4. Clonogenic assays done following sequential or concurrent 24-hr exposures to Ara-AC and FUra showed that the sequence Ara-AC followed by FUra resulted in more than additive lethality in the HCT 116 cell lines and additive lethality in the SNU-C4 cells. In contrast, the reverse sequence, FUra followed by Ara-AC, was antagonistic in both cell lines. A similar interaction between FUra and 1-β-D-arabinofuranosylcytosine (Ara-C) was evident in HCT 116 cells; at concentrations which individually diminished viability by 34 and 62%, respectively, the sequence Ara-C followed by FUra decreased viability by 97%. Pulse-labeling with [^{3}H]dUrd showed profound inhibition of DNA synthesis by the sequence Ara-AC followed by FUra, with over 90% inhibition lasting for up to 48 hr following Ara-AC exposure. When FUra preceded Ara-AC, however, earlier recovery from inhibition of DNA synthesis occurred. FUra pretreatment did not appreciably alter the quantity or distribution of [^{3}H]Ara-AC or [^{3}H]Ara-C nucleotides after a 4- to 6-hr exposure. Pre-exposure to FUra decreased Ara-AC incorporation into DNA by 37 and 73% at 6 hr in HCT 116 and SNU-C4, respectively. FUra pretreatment also inhibited Ara-C incorporation into DNA by over 50% at 6 and 24 hr. The antagonism of Ara-AC and Ara-C cytotoxicity by FUra pretreatment can thus be explained by diminished incorporation of the dCyd analogs into DNA resulting from inhibition of DNA synthesis by FUra-induced dTTP and dCTP depletion. In contrast, when Ara-AC or Ara-C preceded FUra, their incorporation into DNA was not disturbed, and prolonged inhibition of DNA synthesis was observed.

5-Fluorouracil (FUra) is commonly used alone or in combination with leucovorin in the treatment of metastatic colorectal carcinoma. Although the overall response rate observed with FUra and leucovorin appears to be increased when compared to FUra alone in some randomized clinical trials, less than half of the patients experienced an objective response, and few complete responses have been documented [1, 2]. Therefore, new strategies are needed to improve the clinical results achieved with FUra-based therapy. The identification of novel agents with therapeutic potential against colorectal carcinoma is of paramount importance. Fazarabine (Ara-AC, NSC 281272), an investigational antimetabolite with preclinical activity against murine and human colorectal cancer, is currently in Phase II clinical trials [3–10]. Ara-AC is metabolized intracellularly by deoxycytidine kinase (dCyd kinase; EC 2.7.1.74) to the triphosphate. Cytotoxicity is thought to result from DNA incorporation and inhibition of DNA polymerase (EC 2.7.7.7); incorporation into RNA does not occur [3–10]. Glazer and Knode [8] reported that a 24-hr exposure to 10 μM Ara-AC reduces the viability of the HT29 colon carcinoma line by 97%. Ara-AC on an intermittent schedule (days 1, 5, 9 and 13) inhibits the growth of the poorly differentiated CX-1 colon carcinoma xenograft by 93%, whereas 1-β-D-arabinofuranosylcytosine (cytosine arabinoside, Ara-C), azacytidine, dihydroazacytidine and 5-aza-deoxycytidine are inactive [6].

Based on the promising *in vitro* and *in vivo* activity of Ara-AC against human colon carcinoma, we wished to determine the cytotoxicity of this novel cytidine antagonist against two human colon carcinoma cell lines and the combined effects of FUra and Ara-AC given simultaneously or sequentially. Supra-additive or additive lethal effects were noted when Ara-AC was given for 24 hr prior to FUra, but antagonism was evident if the reverse sequence was employed. Similar results in clonogenic studies were obtained when Ara-C was substituted for Ara-AC. We studied the effects of sequential and simultaneous administration of FUra and Ara-AC on inhibition of DNA synthesis and the intracellular metabolism and DNA incorporation of Ara-AC and Ara-C in an effort to explain these sequence-dependent interactions.

MATERIALS AND METHODS

Materials. Ara-AC and [6-^{3}H]Ara-AC (80.0 Ci/mol) were supplied by the Developmental Therapeutics Program, Division of Cancer Treatment, NCI. Moravek Biochemicals (Brea, CA) supplied [6-^{3}H]Ara-AC (2.5 Ci/mmol), [^{3}H]Ara-C (20 Ci/mmol), [5-^{3}H]Urd (20 Ci/mmol), [*methyl*-^{3}H]dThd (25 Ci/mmol), and [6-^{3}H]dUrd (20 Ci/mmol). The

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radiopurity of Ara-AC was documented by injecting an aliquot onto a reversed-phase HPLC column (C8 μ-Bondapak Radial pack cartridge, Waters Millipore, Milford, MA) with water as the mobile phase. Approximately 99% of the counts coeluted in a single peak with non-radiolabeled standard Ara-AC. The [6-^{3}H]Ara-AC was stored at −70°, and maintained its integrity (as determined by HPLC) over the time the experiments reported herein were conducted. Proteinase K was purchased from Boehringer Mannheim (Indianapolis, IN). All other chemicals were obtained from either NIH Stores (Bethesda, MD) or the Sigma Chemical Co. (St. Louis, MO). Stock solutions of Ara-AC were prepared by dissolving the drug in 70% dimethyl sulfoxide (DMSO) and were stored at −70°. On the day of the experiments, dilutions of the Ara-AC stock solution were made in phosphate-buffered saline (PBS), and the concentration was determined by ultraviolet absorption spectrophotometry. The ARA-AC was sterile filtered and then kept on ice until the drug was added to the cells. The percentage of stock solution of Ara-AC remaining as parent drug was determined periodically by reversed-phase HPLC as described above.

Cell culture and inhibition of colony formation. The characteristics of HCT 116 and SNU-C4 cells, two human colon adenocarcinoma cell lines, have been reported previously [11–13]. These cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, and 7% nondialyzed fetal bovine serum (all three obtained from Biofluids Inc., Rockville, MD).

For the clonogenic assay, serial dilutions of cells were prepared and approximately 300–500 cells were replicately plated in Costar 6-well (35 mm) plates. The cells were allowed to attach, and the desired drug concentration was added. Following a 24-hr exposure, the medium was gently aspirated and then replaced with fresh medium containing either PBS or the appropriate concentration of FUra, Ara-AC or Ara-C. The medium was gently aspirated 24 hr thereafter, and the cells were allowed to grow in drug-free medium for 5–7 additional days, at which time they were stained with 0.25% methylene blue in 50% methanol. Colonies, which were defined as greater than 50 cells for HCT 116 cells, and greater than 30 cells for SNU-C4, were enumerated. Cloning efficiency (mean ± SEM) was as follows: HCT 116, 34 ± 5%; SNU-C4, 37 ± 4%. The drug interactions were classified by the fractional inhibition method [14] as follows: when expressed as the fractional inhibition of colony formation, additive inhibition produced by both inhibitors occurs when $i_{1,2} = i_1 + i_2 - i_1 i_2$; synergism: $i_{1,2} > i_1 + i_2 - i_1 i_2$; antagonism: $i_{1,2} < i_1 + i_2 - i_1 i_2$.

The interaction of FUra with Ara-AC and Ara-C was also analyzed according to the median-effect principle of Chou and Talalay [15] assuming a mutually non-exclusive model. Multiple drug concentrations at a fixed ratio (FUra : Ara-C, 100 : 1; FUra : Ara-AC, 25 : 1) were evaluated. The data for fractional effects were determined from the fractional differences between the control and the treated groups and processed with an IBM computer program "Dose–effect analysis with microcomputers" by Chou and Chou (Biosoft, New Jersey; 1985, 1987). The combination index was used to signify antagonism (>1), additivity (=1), or synergism (<1) for the combination of drug 1 (Ara-AC or Ara-C) and drug 2 (FUra), and was derived from the equation: combination index = $(D)_1/(D_x)_1 + (D)_2/(D_x)_2 + (D)_{1,2}/(D_x)_{1,2}$, where $(D)_1$, $(D)_2$, and $(D)_{1,2}$ represent the concentration of drugs 1 and 2 and a mixture of drugs 1 and 2 in a specified ratio, and $(D_x)_1$, $(D_x)_2$, and $(D_x)_{1,2}$ are the concentrations of drug 1, drug 2 and their mixture that are required to affect a system x%.

Analysis of Ara-AC and Ara-C nucleotide formation. Approximately 2×10^5 cells were plated in 35-mm culture dishes and incubated for 24 hr. [^{3}H]Ara-AC (0.5 μM) or [^{3}H]Ara-C (0.1 μM) was then added to the dishes in the presence and absence of 10 μM FUra. The cells were incubated for 2–24 hr, depending on the particular experiment. After the desired interval of drug exposure, the cells were washed three times with iced PBS and then extracted with 1.2 mL of 60% methanol. A portion of the supernatant was counted in a liquid scintillation counter and the remainder was either stored at −70° (for HPLC analysis) or concentrated in a Speed-Vac concentrator (for DE81 filter disc assay).

For measurement of Ara-C nucleotide formation, the methanol soluble residue was reconstituted with ice-cold distilled water immediately prior to spotting equal aliquots onto quadruplicate Whatman DE81 impregnated filter paper discs (Millipore, Bedford, MA) [16]. An additional portion was placed directly into a scintillation vial and counted. Half the filter discs were washed free of soluble radioactivity, and the remaining radioactivity was quantitated. The amount of tritium retained on the discs after three washes less background was then calculated as a fraction of the tritium counts on the filter disc after zero washes. This fraction was then multiplied by the total methanol soluble counts to determine the Ara-C nucleotides present.

Metabolites were also analyzed according to a modification of the methods of Plunkett *et al.* [17, 18] using anion exchange HPLC. A Waters 600E multisolvent delivery system (Waters Millipore) was used with a Waters 990 photodiode array detector set to monitor wavelengths from 260 to 280 nm. A liquid scintillation detector (Radiomatic, Tampa, FL) on-line with the HPLC permitted analysis of the [^{3}H] counts eluted over time. An aliquot of the methanol soluble fraction was injected directly with nonradioactive standards into the HPLC system for analysis. An SAX Radial-Pak column (Waters) was developed using two buffers as the mobile phase: Buffer A was 0.007 M ammonium phosphate, pH 3.9, at room temperature; Buffer B was 0.75 M ammonium phosphate, pH 4.2, at room temperature. The following gradient was employed for analysis of [^{3}H]Ara-AC metabolites after a 4-hr exposure (2.2 μM; sp. act. 80.8 mCi/mmol): 100% Buffer A was run for 8 min, then a linear gradient to 70% Buffer A/30% Buffer B was run over the next 8 min, followed by a linear gradient to 28% Buffer A/72% Buffer B for 24 min. Standards for Ara-AC nucleotides are not available; thus, non-labeled Ara-C and cytidine nucleotides were used as a reference.

Retention times of non-labeled standards were as follows: Ara-AC, 2.4 min; Ara-CMP, 6.3 min; Ara-CDP, 21.0 min; UTP, 28.0 min; CTP, 30.7 min; Ara-CTP, 32.0 min; ATP, 33.7 min; and GTP 38.7 min. Tritium counts eluted in four primary peaks with retention times as follows: 2.0 min, 5.3 min, 20.0 min and 31.7 min; these were assumed to represent parent drug, mono-, di- and triphosphate, respectively.

For [^{3}H]Ara-C metabolites, a less protracted gradient was used: a linear gradient was run from 100% Buffer A to 70% Buffer A/30% Buffer B over 6 min, followed by an isocratic gradient for the next 10 min. A linear gradient was then run over 14 min to 21% Buffer A/79% Buffer B; 100% Buffer B was run for an additional 4 min to wash the column. The column was allowed to equilibrate for 15 min with 100% Buffer A between runs. The retention times of cold standards were as follows: Ara-C, 1.9 min; Ara-CMP, 6.0 min; Ara-CDP, 11.6 min; and Ara-CTP, 28.2 min.

For Ara-C-treated cells, the methanol precipitable fraction was washed three times with 1 mL of 60% cold methanol, dissolved in 0.5 N NaOH, and neutralized with 1 N HCl; the radioactivity was then determined.

Incorporation of Ara-AC into nucleic acids. Cells in logarithmic growth phase maintained in 75 cm^2 tissue culture flasks were exposed to [^{3}H]Ara-AC (0.5 μM; sp. act. = 2.5 μCi/nmol) with or without a prior 24-hr exposure to 10 μM FUra. The cells were harvested at 5–6 hr by washing three times with ice-cold PBS, incubating in 20 mM EDTA in PBS for 30 sec, and then incubating in PBS at 37° until the cells detached (2–10 min). The dislodged cells were washed once with 10 vol. of iced PBS, and collected by centrifugation. The supernatant was decanted, and the cells were incubated at 4° for 5 min in ice-cold lysis buffer (140 mM NaCl/1.5 mM $MgCl_2$, 10 mM Tris–Cl, pH 8.5, with octylphenoxy-polyethoxyethanol [19]. The cells were transferred to a microcentrifuge tube, and, following a 3-min centrifugation at 5000 g, the supernatant was separated, and extracted with 0.5 N perchloric acid (PCA). The nuclear pellet was incubated at 4° for 60 min in 0.5 N PCA, and then centrifuged at 8000 g for 2 min. Following an additional two washes with PCA, the acid precipitable material was dissolved in 0.5 N NaOH, neutralized, and then counted in entirety in a liquid scintillation counter.

We next determined into which nucleic acid species the [^{3}H]material in the nuclear pellet localized. Cells were exposed to [^{3}H]Ara-AC for 5–6 hr; the nuclear pellet was isolated by centrifugation as described above and digested for 3 hr in a lysis buffer containing 0.4 mg/mL proteinase K, 10 mM Tris/10 mM EDTA (pH 7.4), 0.4 M NaCl, and 0.4% sodium dodecyl sulfate (SDS) at 65°. The nuclear digest was then extracted three times with an equal volume of phenol/chloroform and precipitated at −70° by adding 0.1 vol. of 3 M sodium acetate (pH 8.2) and 2.5 vol. of absolute ethanol. The precipitate was collected by centrifugation, washed once with 2 mL of 70% ethanol, dissolved in 2 mL Tris/EDTA (10 mM/1 mM, pH 8.0) and repurified by repetition of phenol/chloroform extractions and ethanol precipitation. The precipitate was reconstituted in Tris/EDTA (as above) and subjected to cesium sulfate density centrifugation. The samples were centrifuged at 40,000 rpm (140,000 g) in a Ti 70.1 fixed angle rotor at 20° for 36–40 hr in a Beckman L8-60M Ultracentrifuge [20, 21]. Twenty-four 0.25-mL fractions were collected from the bottom to the top of the gradients, precipitated with 10% trichloroacetic acid (TCA), and collected onto Whatman type HA filters. The filters were washed three times with 5% TCA, once with 95% ethanol, and then placed in a liquid scintillation vial containing RPI 3a70B scintillation fluid. RNA was precipitated from fractions 4 to 10, and the DNA from fractions 16 to 22.

Measurement of dTTP and dCTP pools. Exponentially growing cells were exposed to PBS or 10 μM FUra for 4 hr. For the dTTP assay, the cells were extracted with 0.5 N PCA followed by neutralization with trichloro-trifluoroethane/tri-*n*-octylamine (3.4:1.5), and then lyophilized. The enzymatic assay used for measurement of dTTP and dCTP was a modification of the method of Solter and Handschumacher as described by Hunting and Henderson [22–24]. Because of poor recovery of dCTP with PCA extraction, an alternate extraction method was necessary. The cells were extracted with 3 mL of distilled water with 230 mM 2-mercaptoethanol and boiled for 2 min, following which the cell extract was centrifiged to pellet the protein precipitate. The samples were then lyophilized. Recovery of external dCTP standard from the cell extract was satisfactory with this extraction procedure. The standard curve was linear from 5 to 100 pmol dCTP.

RESULTS

Effects of FUra and Ara-AC on cell growth and viability. In clonogenic assays, both HCT 116 and SNU-C4 cells were relatively sensitive to Ara-AC, with IC_{50} values (mean ± SEM) of 0.45 ± 0.07 and 1.8 ± 1.4 μM, respectively, for a 24-hr exposure. Ara-AC undergoes considerable spontaneous hydrolysis in aqueous medium, and its concentration decreases continually with a half-life in RPMI medium at 37° of 3.9 hr [25, 26]. Thus, the concentration of Ara-AC would be expected to decrease to only 2% of the initial concentration after 24 hr of incubation; the IC_{50} values reported above underestimate the sensitivity for a 24-hr exposure in which the concentration of parent drug is replenished continually.

HCT 116 and SNU-C4 cells exposed to FUra for 24 hr had IC_{50} values of 11.7 ± 1.7 and 2.8 ± 0.95 μM, respectively. For each cell line, simultaneous exposure to 10 μM dThd, a concentration which by itself did not affect cell viability, partially reversed the lethality of FUra (Fig. 1) (the colony number increased from 64 to 80% of control in HCT 116, and from 58 to 73% of control in SNU-C4).

Clonogenic assays were performed with each cell line subjected to a 24-hr exposure to 5 μM FUra (SNU-C4) or 10 μM FUra (HCT 116) and 0.5 μM Ara-AC alone, concurrently or sequentially (Fig. 1). In HCT 116 cells, FUra or Ara-AC given alone each reduced colony formation to approximately 60% of

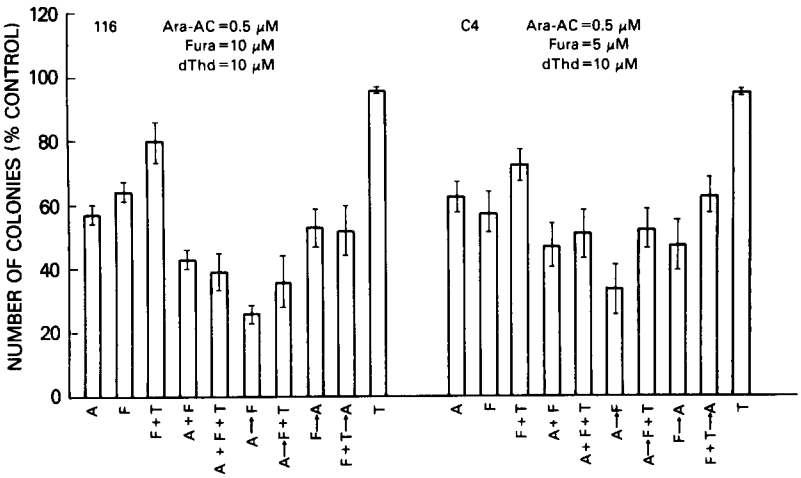

Fig. 1. Effect of sequence of administration of FUra and Ara-AC on the viability of two human colon carcinoma cell lines. HCT 116 and SNU-C4 cells were exposed to FUra (alone or with 10 μM dThd) or Ara-AC for 24 hr alone, concurrently or sequentially as described in Materials and Methods. The drug concentrations used for each cell line are indicated for HCT 116 cells (left) and SNU-C4 cells (right). The data, presented as mean number of colonies (as a percent of control) ± the SEM, are from four to nine separate experiments, each done in duplicate. The data for the dThd rescue experiments (mean ± SD) are from two experiments, each done in duplicate.

control. An additive effect of FUra and Ara-AC is predicted to reduce clonogenicity to 36% of control [14]. Figure 1 (left panel) shows that the sequence Ara-AC followed by FUra (each for 24 hr) produced a greater than additive effect (colony formation 26% of control), whereas the sequence FUra followed by Ara-AC produced less cytotoxicity than expected (colony formation 52% of control). The decrease in viability produced by the sequence Ara-AC → FUra was significantly different from that produced by exposure to Ara-AC alone ($P = 0.015$) or FUra alone ($P = 0.001$); in contrast, the colony formations produced by simultaneous administration or the sequence FUra → Ara-AC were not significantly different from that resulting from exposure to Ara-AC alone ($P = 0.27$ and 0.55, respectively). Concomitant exposure to 10 μM dThd with FUra partially reversed the lethality of the sequence Ara-AC → FUra (the colony number increased from 26 to 36% of control), suggesting that a DNA-directed effect of FUra may be important for the enhanced lethality when FUra was given after Ara-AC. In contrast, the addition of dThd did not affect the lethality observed with Ara-AC and FUra given either simultaneously or when FUra preceded Ara-AC.

In SNU-C4 cells (Fig. 1, right panel), the sequence Ara-AC followed by FUra produced additive lethality (observed colony number was 34% of control); addition of dThd during FUra treatment partially reversed the effect (colony number 52% of control).

We also determined if a similar effect occurred with Ara-C. As the sequence-dependent interaction of FUra and Ara-AC appeared most prominent in HCT 116 cells, this line was selected for additional studies. The IC_{50} of Ara-C for a 24-hr exposure was 0.08 ± 0.02 μM. Exposure to either 10 μM FUra or 0.1 μM Ara-C alone for 24 hr reduced viability (mean ± SEM) to 66 ± 1% and 38 ± 19% of control, respectively. A striking enhancement of lethality was observed with the sequence Ara-C → FUra: the cloning efficiency was decreased to only 3 ± 3% of control. If FUra preceded 0.1 μM Ara-C, the observed colony number (35 ± 21%) was similar to that seen with either drug alone, suggesting antagonism. With simultaneous exposure, a more than additive interaction occurred.

The fractional inhibition method does not take into account the shapes of concentration–response curves of the individual agents, and may, therefore, over- or underestimate a combination response when the concentration–response curve is non-linear. Therefore, the interaction of FUra with Ara-AC or Ara-C was also analyzed according to the median-effect principle of Chou and Talalay [15]. In one experiment, the combination index for the sequence Ara-AC followed by FUra (at a fixed concentration ratio of 1:20, respectively) was less than one (mean 0.32; range 0.29–0.35), at all points along the X-axis (fraction inhibited 0.05 to 0.95; data not shown), indicating synergism. A 1:25 concentration ratio was used in a separate experiment; the combination index for the sequence Ara-AC followed by FUra was approximately one (mean 0.98, range 0.95–1.12, data not shown) at all points along the X-axis, indicating additivity.

When a 24-hr exposure to Ara-C preceded FUra exposure (at a fixed concentration ratio of 1:100, respectively), the combination index was less than one when the fraction inhibited was greater than 40% (Fig. 2A). This analysis indicates that synergism can be expected only when appreciable lethality occurs with the sequence Ara-C → FUra; when less than 40% lethality occurs, e.g. when concentrations

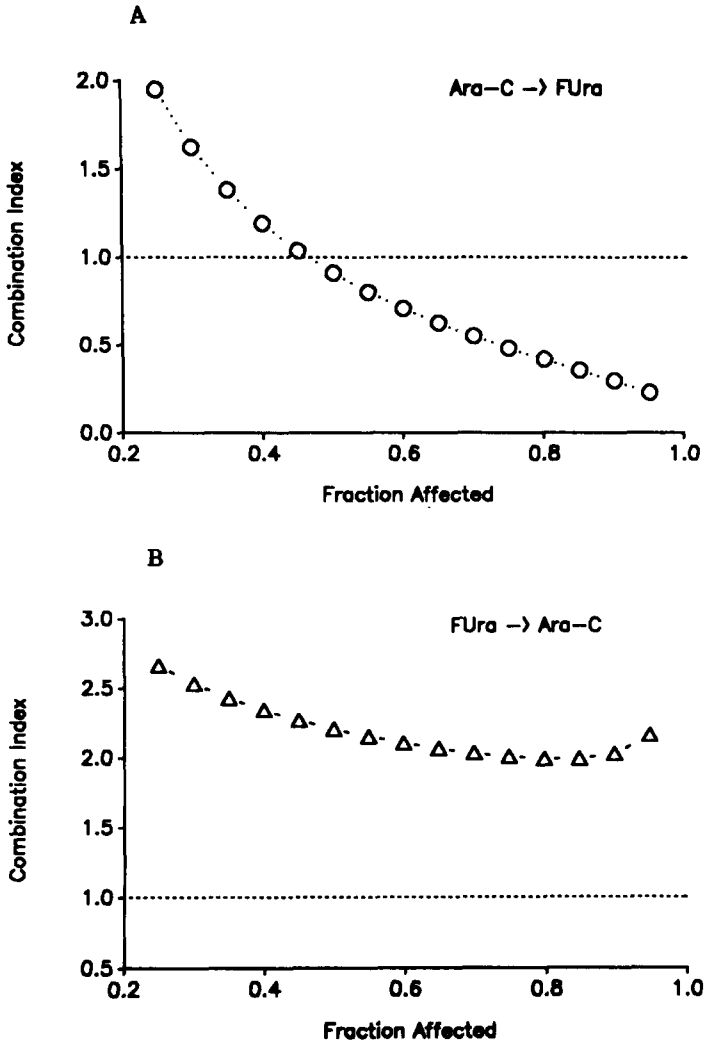

Fig. 2. Median effect analysis of FUra and Ara-C interaction. Clonogenic assays were performed in HCT 116 cells exposed to either FUra (at five concentrations ranging from 2.5 to 25 μM) or Ara-C (at five concentrations ranging from 0.025 to 0.25 μM) for 24 hr, followed by a 24-hr exposure to the second drug. A fixed concentration ratio of FUra to Ara-C of 100:1 was employed. The data were analyzed according to the median-effect principle of Chou and Talalay [15] assuming a mutually non-exclusive model. The data represent an average of two experiments, each done in duplicate. Figure 2A depicts the combination index versus the fraction affected for the sequence Ara-C followed by FUra. Figure 2B shows the results with the sequence FUra followed by Ara-C.

from the early, non-linear portion of the concentration-response curve for the two drugs were combined, there was a negative interaction of the two drugs. When the reverse sequence (FUra → Ara-C) was employed, in contrast, antagonism was evident over the entire concentration range (Fig. 2B).

Inhibition of DNA synthesis as a function of order of FUra administration. HCT 116 and SNU-C4 cells exposed to 10 or 5 μM FUra, respectively, showed maximum inhibition of DNA synthesis (75%) at the end of the 24-hr exposure (Table 1); complete recovery was evident by 96 hr (72 hr after FUra removal). Ara-AC, in contrast, produced a delayed effect; the greatest inhibition occurred 24–48 hr after drug removal. The sequence Ara-AC for 24 hr followed by FUra for 24 hr was associated with profound inhibition of DNA synthesis; 90% inhibition was seen at 48 and 72 hr. The sequence FUra followed by Ara-AC, however, produced much less inhibition of DNA synthesis and earlier recovery was noted. Thus, the enhanced lethality resulting from Ara-AC followed by FUra was associated with greater inhibition of DNA snythesis.

A 24-hr treatment with FUra produced transient inhibition of RNA synthesis in HCT 116 cells (25 and 53% inhibition at 24 and 48 hr, respectively) and SNU-C4 cells (27 and 70% inhibition at 24 and 48 hr, respectively), with complete recovery by 72 hr (data not shown). Ara-AC alone did not inhibit

Table 1. Sequence-dependent effect of Ara-AC and FUra on DNA synthesis

Cell line	DNA synthesis (% control)			
	24 hr	48 hr	72 hr	96 hr
HCT 116				
FUra, 10 μM	25 ± 12	59 ± 24	56 ± 14	104 ± 11
Ara-AC, 0.5 μM	70 ± 8	44 ± 15	24 ± 15	59 ± 18
Ara-AC + FUra	14 ± 8	24 ± 13	31 ± 14	59 ± 19
Ara-AC → FUra	70	9 ± 4	6 ± 3	52 ± 42
FUra → Ara-AC	25	38 ± 23	42 ± 20	76 ± 7
SNU-C4				
FUra, 5 μM	24 ± 2	40 ± 28	65 ± 10	105 ± 36
Ara-AC, 0.5 μM	53 ± 14	33 ± 2	33 ± 11	50 ± 7
Ara-AC + FUra	44 ± 27	10 ± 6	23 ± 12	40 ± 10
Ara-AC → FUra	53	12 ± 6	4 ± 3	22 ± 17
FUra → Ara-AC	24	21 ± 15	45 ± 13	55 ± 12

Exponentially growing cells were exposed to the indicated drug concentrations for 24 hr either alone, together or sequentially. Prior to the addition of [6-^{3}H]dUrd, the medium was gently aspirated and the cells were washed once with iced PBS. Fresh drug-free RPMI medium was replaced, and then a 30-min pulse with [6-^{3}H]dUrd (0.5 μCi/well; sp. act. = 20 μCi/nmol) was performed. The [^{3}H]dUrd pulse was performed at 24-hr intervals, and the amount of radioactivity incorporated into the acid precipitable fraction was determined. The data, from three separate experiments each done in duplicate, are presented as mean percent control DNA synthesis ± SEM. The absolute control values (mean ± SEM) for DNA synthesis are as follows (pmol [^{3}H]dUrd incorporated/10^6 cells): HCT 116: 24 hr, 12.2 ± 4.9; 48 hr, 6.6 ± 2.2; 72 hr, 3.9 ± 2.3; and 96 hr, 2.3 ± 1.0; SNU-C4: 24 hr, 14.8 ± 3.0; 48 hr, 9.1 ± 0.6; 72 hr, 5.9 ± 1.6; and 96 hr, 4.9 ± 0.7.

RNA synthesis (data not shown). For cells treated sequentially with Ara-AC followed by FUra, essentially no effect on RNA synthesis was observed in HCT 116 cells (16 and 8% inhibition at 48 and 72 hr, respectively) and SNU-C4 cells (33 and 26% inhibition at 48 and 72 hr, respectively), even though this sequence had greater effects on cell viability. Thus, it is unlikely that the combination of Ara-AC followed by FUra exerts its cytotoxicity through inhibition of total RNA synthesis.

Effect of FUra on Ara-AC metabolism and nuclear incorporation. The triazine ring of Ara-AC can undergo hydrolysis in aqueous conditions, with release of [^{3}H]formate from the 6-carbon position [3, 4, 8, 9]. The tritiated formate can then be used for *de novo* purine synthesis. Therefore, HPLC analysis of [^{3}H]Ara-AC metabolites was performed to elucidate the composition of the [^{3}H]nucleotide pool, and to determine the effect of FUra pretreatment on Ara-ACTP formation. After a 4-hr exposure of HCT 116 cells to Ara-AC (0.5 μM, sp. act. 80.8 mCi/mmol), four major tritium peaks were detected; the first co-eluted with Ara-AC standard. The three other peaks were assumed to represent Ara-AC mono-, di- and triphosphate, respectively, as the retention times were very similar to that of CMP, CDP and CTP. No tritium, however, co-eluted with ATP or GTP. Ara-ACTP accounted for the following percentage of tritium nucleotide counts (mean ± SEM) for Ara-AC alone and FUra preceding Ara-AC, respectively: HCT 116: 52 ± 6% and 48 ± 12%; SNU-C4: 43 ± 10% and 56 ± 13%. FUra pretreatment did not alter appreciably the distribution of Ara-AC nucleotides. Table 2 shows that the total amount of Ara-ACTP formed in each cell line following a 4-hr exposure to 0.5 μM Ara-AC was similar regardless of FUra pre-exposure.

Because of the lability of the triazine ring, tritium found in the acid precipitable fraction of the cell nucleus represents a composite pool with contributions from not only [6-^{3}H]Ara-AC incorporation into DNA, but potentially also from [^{3}H]purines in RNA and DNA. The HPLC analysis discussed above indicated that neither [^{3}H]ATP nor [^{3}H]GTP was present. These results are in agreement with those reported by Townsend and colleagues [9]. Townsend *et al.* have also shown that the majority of ^{3}H-label from DNA digests is in the form of Ara-AC metabolites provided that the duration of exposure is kept to less than 8 hr [9]. We therefore limited the duration of exposure to [^{3}H]Ara-AC to 4–6 hr.

Separation of the cell nucleus from cytosolic RNA was achieved by adding a nonionic detergent to the lysis buffer [19]. During a brief incubation, the detergent disrupts the cell membrane but does not affect the nuclear membrane [19]. Differential centrifugation was then used to isolate nuclear nucleic acids from RNA present in the cytosol [19]. FUra pre-exposure reduced Ara-AC incorporation into nuclear nucleic acids at 6 hr by 37 and 73%, respectively, in HCT 116 and SNU-C4 cells (Table 2).

Since the nuclear nucleic acids represent DNA as well as RNA, we wished to further document the nucleic acid species into which the radioactivity

Table 2. Effect of FUra on Ara-AC metabolism and nuclear incorporation

Treatment	Ara-ACTP (pmol/10^6 cells) HCT 116	SNU-C4
Ara-AC alone	14.1 ± 4.8	13.9 ± 4.3
FUra → Ara-AC	8.5 ± 3.8	13.6 ± 3.7
Treatment	**Nuclear incorporation (pmol/10^6 cells) HCT 116**	**SNU-C4**
Ara-AC alone	5.2 ± 3.4	7.4 ± 4.9
FUra → Ara-AC	3.3 ± 2.2	2.0 ± 1.4

Exponentially growing cells in duplicate flasks were exposed to either PBS or 10 μM FUra for 24 hr; then the cells were washed and fresh medium was added. Ara-AC was added (0.5 μM; sp. act. 80.8 μCi/μmol) for 4 hr; the cells were washed three times and extracted with 60% methanol. The radioactivity in an aliquot of the methanol soluble fraction was determined in a liquid scintillation counter and the remainder was analyzed by HPLC. The data for Ara-ACTP are from four to six separate experiments, each done in duplicate. For determination of nuclear incorporation, FUra-treated and control cells were exposed to [^{3}H]Ara-AC (0.5 μM; sp. act. 2.5 μCi/nmol) for 6 hr, and extracted in a lysis buffer containing a nonionic detergent as described in Materials and Methods. In separate experiments, the nuclear pellet was digested in detergent and proteinase K, and then the nucleic acids were purified and analyzed by cesium sulfate density centrifugation as described in Materials and Methods. Virtually 100% of the [^{3}H] localized in the DNA fractions. The acid precipitable material contained in the nuclear pellet was therefore considered to represent DNA. The data, from three separate experiments done in duplicate, are presented as means ± SEM.

localized. This was accomplished by digesting the nuclear pellet in detergent and proteinase K, purifying the nucleic acids in the nuclear pellet through phenol/chloroform extraction and ethanol precipitation, followed by cesium sulfate density centrifugation. After a 6-hr exposure to [^{3}H]Ara-AC with or without FUra pretreatment, virtually 100% of the radioactivity was precipitated from the DNA fractions (data not shown). Thus, the radioactivity from [^{3}H]Ara-AC present in the nuclear acid precipitable material represents DNA incorporation.

Effect of FUra on Ara-C metabolism and DNA incorporation. Because [^{3}H]Ara-C is not subject to hydrolytic ring opening, analysis of Ara-C nucleotide formation was initially performed using the DE81 ion-exchange filter disc assay [16]. With FUra pre-exposure, no consistent change in Ara-C nucleotides was apparent after 6 hr; a 31–48% increase was evident by the end of the 24-hr exposure, but this increase was not statistically significant (Table 3). During simultaneous exposure to FUra and Ara-C, a 24–26% decrease in Ara-C nucleotides was noted in HCT 116 and SNU-C4 cells by the end of the 24-hr exposure (not statistically significant) (Table 3). The composition of the Ara-C nucleotide pool was also analyzed by ion-exchange HPLC analysis. Following a 6-hr exposure to 0.1 μM Ara-C, the Ara-CTP pool, 21.0 ± 0.8 pmol/10^6 cells (mean ± range; N = 2), accounted for 84% of the nucleotides (data not shown). Prior exposure to FUra did not alter the amount of distribution of Ara-C metabolites at 6 hr: the amount of Ara-CTP formed was 19.2 ± 0.4 pmol/10^6 cells, accounting for 81% of the nucleotides. These results are in agreement with the filter disc assay. The total amount of Ara-C nucleotides recovered at 6 hr was 4-fold higher with the HPLC assay. This disparity is probably related to the differences in processing the samples. An aliquot of the methanol soluble fraction was directly analyzed by HPLC without further processing, affording little opportunity for degradation of the nucleotides.

Measurement of [^{3}H]Ara-C methanol precipitable material was used as an index of DNA incorporation. Upon exposure to 0.1 μM Ara-C alone, the amount of Ara-C incorporated in DNA between 6 and 24 hr increased 7-fold (Fig. 3). When FUra was given simultaneously with Ara-C, no inhibition was noted within the first 6 hr, but after 24 hr, Ara-C incorporation was inhibited by 36%. When FUra was given alone for the initial 24 hr, subsequent administration of Ara-C resulted in much less DNA incorporation (52 and 67% inhibition at 6 and 24 hr, respectively).

Modulation of deoxyribonucleotide triphosphate pools by FUra. FUra (10 μM) produced a significant depletion of dTTP within 4 hr of exposure, reducing the pool size by 64% from 76 ± 9 (mean ± SEM; N = 7) to 26 ± pmol/10^6 cells (P = 0.0008). The dCTP pool was reduced following FUra exposure by 78% from 27 ± 7 (N = 5) to 6 ± 1 pmol/10^6 cells (N = 3; P = 0.07). Depletion of dTTP and dCTP, therefore, appears to contribute to the inhibition of DNA synthesis resulting from FUra treatment.

DISCUSSION

The apparent antagonism of lethality produced by exposure to FUra followed by Ara-AC or Ara-C appears to be explained by a negative effect of FUra on their incorporation into DNA. The decreased incorporation of Ara-AC and Ara-C resulting from FUra pre-exposure is not due to impaired metabolism of Ara-AC or Ara-C to the triphosphate forms. Exposure to FUra resulted in transient inhibition of DNA synthesis as a consequence of FdUMP-mediated inhibition of thymidylate synthase (TS; EC 2.1.1.45) and subsequent depletion of dTTP. Inhibition of DNA synthesis by FUra would seem to be a likely explanation for the reduced assimilation of Ara-AC and Ara-C into DNA.

The effect of dTTP depletion on other deoxyribonucleotide triphosphate pools is complex and depends on the cell type studied. Since ribonucleoside diphosphate reductase (EC 1.17.41) is under feedback control by dTTP and dATP, dTTP depletion resulting from inhibition of TS may result in increased dCTP production by this pathway [14, 27–32]. dTTP is also a feedback inhibitor of deoxycytidylate (dCMP) deaminase (EC 3.5.4.14);

Table 3. Effect of FUra on Ara-C nucleotide formation

Cell line	Treatment	Ara-C nucleotides (pmol/10^6 cells) 6 hr	24 hr
HCT 116	Ara-C alone	6.3 ± 2.2	5.4 ± 1.0
	FUra → Ara-C	5.4 ± 1.7	7.1 ± 2.1
	Ara-C + FUra	7.8 ± 4.8	4.0 ± 3.1
SNU-C4	Ara-C alone	6.8 ± 2.5	2.3 ± 1.2
	FUra → Ara-C	7.1 ± 1.5	3.4 ± 2.0
	Ara-C + FUra	14.6 ± 13.8	1.8 ± 1.7

HCT 116 and SNU-C4 cells were exposed to 0.1 μM [^{3}H]Ara-C (sp. act. 2.5 μCi/nmol) for 6 or 24 hr either during or following a 24-hr exposure to 10 μM FUra. Nucleotide formation was determined by a DE81 filter disc assay, as described in Materials and Methods. The data, from three separate experiments done in duplicate, are means ± SEM.

a decrease in dTTP could theoretically stimulate the conversion of dCMP to dUMP, thereby resulting in depletion of dCTP [14, 33]. In the HCT 116 cells, dTTP depletion was accompanied by a reduction in dCTP pools. No significant effect on Ara-ACTP or Ara-CTP formation was evident, however.

Other interactions between fluoropyrimidines and Ara-C which have been described include competition of dUMP (which accumulates behind the FdUMP-mediated blockade of TS) with Ara-CMP for phosphorylation by dCMP kinase (EC 2.7.4.14) [34]. In addition, Drake *et al.* [35] reported that FdUrd and FdUMP inhibit cytidine deaminase (EC 3.5.4.5) (K_i = 134 μM) and dCMP deaminase (K_i = 23 μM), respectively. We have previously analyzed the intracellular metabolism of FUra in HCT 116 cells [36]. The concentration of free FdUMP formed after a 4- or 8-hr exposure to 5 μM FUra is in the range of 2 to 30 μM [36]. Although the FdUMP concentration was in the range of the reported K_i for dCMP deaminase, deamination of Ara-C did not appear to be an important factor in our tissue culture model.

Since [^{3}H]formate released by hydrolytic ring opening Ara-AC can be used for purine synthesis, it is advisable to use brief drug exposures to minimize this confounding variable. HPLC analysis of [^{3}H]Ara-AC metabolites in the HCT 116 and SNU-C4 cell lines indicated that the [^{3}H]triphosphate accounted for the majority of the nucleotides, and no tritium was detected in the HPLC fractions coeluting with ATP or GTP standards after a 4-hr exposure. Our results are in agreement with those of Townsend *et al.* [9], who found that [^{3}H]Ara-AC nucleotide metabolites after a 3-hr exposure are phosphorylated products of the parent nucleosides, rather than a chemical breakdown product.

Separation of the nuclear nucleic acids by cesium sulfate density centrifugation indicated that essentially all of the tritium counts localized in the fractions coeluting with DNA. Townsend *et al.* [9] reported that enzymatic digests of DNA following an 8-hr exposure to [^{3}H]Ara-AC yield primarily the parent nucleoside. In contrast, Glazer and Knode noted that if the exposure time to [^{3}H]Ara-AC is 24 hr, approximately 50% of the tritium in DNA digests analyzed by HPLC is present as dGuo and dAdo. Furthermore, appreciable radioactivity is found in RNA after a 24-hr exposure, but virtually all the [^{3}H] is present in the fractions coeluting with Ado and Guo; none is detected as parent nucleoside [8].

Our results in human colon cancer cell lines using concurrent or sequential 24-hr drug exposures differ from previously published articles describing the interaction of fluoropyrimidines with Ara-C in murine leukemia/lymphoma cell lines. Grant and Cadman [33] reported that sequential exposure of L1210 cells to 1 μM FUra for 4 hr followed by 5 μM Ara-C for 1 hr results in synergistic cell killing in a clonogenic assay, whereas simultaneous administration of the reverse sequence results in additive or subadditive killing. The basis for this synergy was not fully explained. The pre-exposure to 1 μM FUra did not decrease dCTP or dTTP pools. A 4-hr pre-exposure to 100 μM FUra increased Ara-CTP pools by 2-fold, but comparable information on Ara-C metabolism or DNA incorporation following pre-exposure to 1 μM FUra, the concentration which produced synergy in the clonogenic studies, was not presented. Studies by Chu *et al.* [37] indicated that pretreatment of the murine lymphoma cell line L5178Y for 2 hr with low concentrations of FdUrd (0.01 μM) sensitizes them to the cytotoxicity of a 4-hr exposure to 3.3 μM Ara-C. This potentiation of cytotoxicity was observed even though FdUrd decreased the incorporation of [^{3}H]Ara-C into DNA [37]. In contrast to these earlier studies, we used human solid tumor cell lines rather than murine leukemic lines and employed longer exposure times: 24 hr for each drug. FUra pretreatment resulted in antagonism of the cytotoxicity of Ara-AC in the two colon carcinoma lines we studied, and antagonism of Ara-C lethality in HCT 116 cells. The optimal sequence was Ara-AC followed by FUra. No inhibition of Ara-AC incorporation into DNA occurred with this sequence, and the additional inhibition of DNA synthesis produced by FUra potentiated the cytotoxicity of the combination.

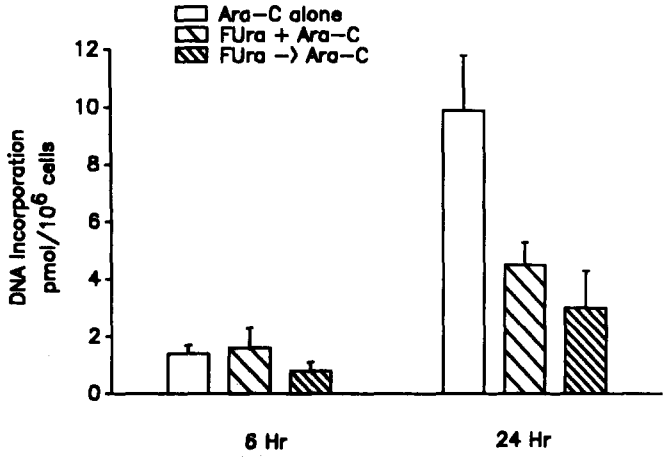

Fig. 3. FUra inhibition of Ara-C incorporation into DNA. HCT 116 cells in logarithmic growth phase were exposed to 0.1 μM [^{3}H]Ara-C (sp. act. 2.5 μCi/nmol) for 6 or 24 hr either concurrently with 10 μM FUra or after a 24-hr pre-exposure to 10 μM FUra. The radioactivity incorporated into methanol precipitable material was determined. The data, presented as pmol Ara-C incorporated/10^6 cells are from two (FUra + Ara-C; mean ± SD) or three (FUra → Ara-C; mean ± SEM) separate experiments, each done in duplicate.

Clinically achievable steady-state plasma concentrations of Ara-AC are in the range of 1.8 to 2.5 μM and 0.2 μM for a 24- and 72-hr infusion, respectively [25, 26]. If Ara-AC proves to have activity against colorectal carcinoma in the clinic, then a logical approach might involve combination with other active agents such as FUra. These observations concerning sequence-dependent additivity and antagonism have implications for the design of clinical trials with Ara-AC and FUra. An additional point is that Ara-C was quite potent against the HCT 116 cell, with an IC_{50} of about 0.1 μM, a clinically relevant concentration. The sequence of Ara-C followed by FUra produced a decrease in viability of over 90%. Ara-C is not thought to be an active agent against human colorectal cancer [38]. These observations suggest that *in vivo* studies of human colorectal cancer xenografts treated with Ara-C alone and followed by FUra may be of interest.

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