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INTERACTION OF DEOXYURIDINE WITH FLUOROURACIL AND DIPYRIDAMOLE IN A HUMAN COLON CANCER CELL LINE*

JEAN L. GREM,†§ R. TIMOTHY MULCAHY,† ELIZABETH M. MILLER,† CARMEN J. ALLEGRA‖ and PAUL H. FISCHER†¶

†Department of Human Oncology, University of Wisconsin Clinical Cancer Center, Madison, WI 53792; and the ‡Investigational Drug and ‖Clinical Pharmacology Branches of the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20892, U.S.A.

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Abstract—We have reported previously that dipyridamole increases the toxicity of 5-fluorouracil and alters fluorouracil metabolism in HCT 116 cells, producing a selective increase in fluorodeoxyuridine monophosphate (FdUMP) levels by blocking the efflux of fluorodeoxyuridine. Dipyridamole also blocks deoxyuridine efflux and prolongs the intracellular half-life of deoxyuridine monophosphate (dUMP). The significance of the effect of dipyridamole on FdUMP and dUMP levels was explored further. In cell growth experiments, 1–50 μM deoxyuridine enhanced the cytotoxicity of 5 μM fluorouracil in a dose-dependent manner, and ≥10 μM deoxyuridine increased the augmentation of fluorouracil toxicity produced by 0.5 μM dipyridamole. The effect of deoxyuridine on [6-^{3}H]fluorouracil metabolism was studied. After 4 hr, 25 μM deoxyuridine increased the amount of [^{3}H]FdUMP formed 2- to 4-fold relative to that of fluorouracil ± dipyridamole alone. The mechanism by which deoxyuridine increased FdUMP was examined by measuring the distribution of [2′-^{3}H]deoxyuridine metabolites following exposure of 25 μM deoxyuridine ±5 μM fluorouracil. Tritium appeared in the FdUMP peak at 4 and 24 hr in cells exposed to fluorouracil and deoxyuridine, indicating that [^{3}H]deoxyribose was transferred to fluorouracil. A large buildup of [^{3}H]dUMP was seen in cells exposed to fluorouracil plus deoxyuridine for 4 and 24 hr compared to exposure to [^{3}H]deoxyuridine alone, suggesting that dUMP may also inhibit catabolism of FdUMP. Since the increased FdUMP levels produced by dipyridamole did not appear to correlate with further depletion of thymidine triphosphate pools, the incorporation of [^{3}H]fluorouracil metabolites into nucleic acids was monitored by cesium sulfate density centrifugation. Fluorouracil-RNA increased as a function of time (1, 2 and 13 pmol/10^6 cells after 4, 8 and 24 hr), but fluorouracil-DNA was detected only after 24 hr (0.5 pmol/10^6 cells). Dipyridamole however, did not appear to alter the pattern of incorporation of fluorouracil into either RNA or DNA. Perturbations of endogenous dUMP levels by fluorouracil and dipyridamole were then studied. In cells exposed to fluorouracil alone, dUMP pools were unchanged from control at 2 hr, but they had increased 9-fold by 4 hr (3362 pmol/10^6 cells). Simultaneous exposure to fluorouracil and dipyridamole resulted in a 1.5-fold (566 pmol/10^6 cells) and 13.6-fold (5049 pmol/10^6 cells) increase over control dUMP levels after 2 and 4 hr respectively. The dUMP pools continued to enlarge through 24 hr. The effect of fluorouracil on DNA fragility was examined. In cells prelabeled with [^{14}C]thymidine, there was no evidence of single-strand breaks in high molecular weight DNA after 4 or 24 hr of exposure to fluorouracil alone or with dipyridamole as measured by alkaline elution. In contrast, fluorouracil produced alkaline labile sites in newly synthesized DNA. Alkaline labile sites were also produced by exposure to dipyridamole. Concomitant exposure to FUra with dipyridamole and/or deoxyuridine resulted in a striking increase in the alkaline labile sites in DNA. These results suggest that effects on deoxyuridine metabolism may be important components of the interaction between fluorouracil and dipyridamole.

We have reported previously that DP**, an inhibitor of nucleoside transport [1–3], enhances the cytotoxicity of FUra both in cell growth experiments and in viability assays measuring colony formation [4]. Several possible mechanisms by which DP augments FUra toxicity in HCT 116 cells, a human colon cancer cell line, have been investigated. Since FUra is converted to FdUMP, a potent inhibitor of dTMP synthase, we considered the possibility that inhibition of dThd salvage by DP might be of key importance

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§ Address reprint requests to: Dr. Jean Grem, Investigational Drug Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Executive Plaza North, Rm. 731, Bethesda, MD 20892.

¶ Current address: Pfizer Central Research, Eastern Point Road, Groton, CT 06340.

** Abbreviations: DP, dipyridamole; FUra, 5-fluorouracil; FdUrd, fluorodeoxyuridine; FdUMP, fluorodeoxyuridine monophosphate; FdUTP, fluorodeoxyuridine triphosphate; FUTP, fluorouridine triphosphate; dR-1-P, deoxyribose-1-phosphate; PBS, phosphate-buffered saline; PCA, percholoric acid; SDS, sodium dodecyl sulfate; cGy, centigray; TCA, trichloroacetic acid; FH_4, tetrahydrofolate; CH_2FH_4, 5-10-methylenetetrahydrofolate; KOH, potassium hydroxide; and KH_2PO_4, potassium phosphate.

in these cells. Although DP effectively inhibits the uptake* of physiological levels of nucleosides such as Urd and dThd, several lines of evidence suggested that inhibition of nucleoside salvage did not account for the increase in FUra toxicity produced by DP [4].

A second possibility, DP-induced changes in FUra metabolism, was also considered [5]. DP alters the pattern of FUra metabolism and provides a selective increase in intracellular FdUMP levels without affecting the total amount of FUra incorporated into the acid-soluble fraction. Inhibition of the efflux of FdUrd appears to be primarily responsible for increased intracellular levels of FdUMP produced by DP. DP also inhibits the efflux of dUrd and prolongs the intracellular half-life of dUMP [5].

Although a high concentration of dThd, 25 μM, does not reduce the cytotoxicity of FUra, it does prevent the augmentation of FUra toxicity produced by DP [4]. Furthermore, 25 μM dThd prevents the increased FdUMP levels in cells exposed to FUra and DP [5]. A clear association between increased FdUMP levels and enhanced cytotoxicity, however, did not emerge. Taken together, the data suggested that depletion of dTTP pools did not fully account for the cytotoxic effects of FUra ± DP [4, 5]. dUrd shares the anabolic and catabolic pathways common to dThd and FdUrd. Since 25 μM dThd reverses the augmentation of FUra toxicity produced by DP and also diminishes the FdUMP levels, and because DP blocks dUrd efflux, the present experiments, designed to explore a potential role for modulation of dUrd metabolism in the augmentation of FUra toxicity by DP, were undertaken. Our findings indicated that dUrd does, in fact, increase the toxicity of FUra in HCT 116 cells and that DP can augment that effect. The FdUMP levels formed in the presence of dUrd were 2- to 4-fold higher than those seen with FUra alone or in combination with DP. In addition, FUra and DP were associated with marked expansion of the endogenous dUMP pools at 4 and 24 hr.

Studies were undertaken to determine if FUra was incorporated into the DNA of HCT 116 cells and, if so, whether DP could enhance the incorporation of FUra into DNA. In addition, the effects of FUra, DP and dUrd on elution of DNA in an alkaline elution assay were evaluated. The results indicate that the combination of FUra, DP and dUrd can produce significant changes in the alkaline-dependent stability of newly synthesized DNA.

MATERIALS AND METHODS

FUra, DP, most nucleosides and nucleotides, KH_2PO_4, tetrabutylammonium hydrogen sulfate, cesium sulfate, trizma acid and base, EDTA, phenol, chloroform, isoamyl alcohol and *d,l*-FH_4 were purchased from either the Sigma Chemical Co. (St. Louis, MO) or P-L Biochemicals (Milwaukee, WI). FUMP and FdUMP were purchased from Cal-Biochem-Behring (San Diego, CA). Sierra Biochemicals (Tucson, AZ) provided FUTP and FdUTP. Tri-*n*-octylamine and 1,1,2-trichlorofluoroethane were purchased from either the Aldrich Chemical Co. (Milwaukee, WI) or the Sigma Chemical Co. Perchloric acid was obtained from the Fisher Scientific Co. (Fairlawn, NJ). Moravek Biochemicals (Brea, CA) supplied the [6-^{3}H]FUra (26 Ci/mmol), [2′-^{3}H]dUrd (25 Ci/mmol), [^{3}H]dThd (25 Ci/mmol), and [^{14}C]dThd (56 mCi/mmol). The purities of the radioactive FUra and dUrd were checked by HPLC; over 97% of the tritium counts appeared in the fraction coeluting with either FUra or dUrd respectively. *Lactobacillus casei* dTMP synthase (10.2 nmol/ml) was from the New England Enzyme Center (Boston, MA). CH_2FH_4 was prepared by adding 5 μl of 37% (v/v) formaldehyde, 10 ml of 100 mM KH_2PO_4 buffer, pH 7.2, and 100 μl of 2-mercaptoethanol type I to 14 mg of FH_4; the solution was allowed to stand at room temp for 1 hr.

Cell culture. The characteristics of HCT 116 cells [6] and the methodology for cell culture, measurement of cell growth, colony formation and nucleoside uptake [4, 5] have been reported previously.

HPLC methodology. Separation of FUra metabolites via a reversed phase column was achieved using methodology that we previously reported [5], and will be referred to as Method 1. Separation of dUrd metabolites was achieved using a slight modification of the procedure for analyzing FUra metabolites, which will be referred to as Method 2. A gradient between buffers A (5 mM tetrabutylammonium hydrogen sulfate, 5 mM potassium phosphate, pH 7) and B (5 mM tetrabutylammonium hydrogen sulfate, 5 mM potassium phosphate, 50% methanol, pH 7) was used. Buffer A (100%) was run for 20 min at a flow rate of 2.0 ml/min, room temperature; the concentration of buffer B was increased from 0 to 80% between 20 and 40 min and maintained at 80% between 40 and 50 min. Authentic dUrd, FdUrd, dThd, dUMP, FdUMP, dTMP, dUTP, and dTTP were injected simultaneously as standard markers. Typical retention times were: dUrd, 4.5 min; FdUrd, 5.5 min; dThd, 9 min; dUMP, 17 min; FdUMP, 25 min; dTMP, 27 min; dUTP, 32 min; and dTTP, 38.5 min.

Measurement of dUrd metabolites. The method for determination of dUrd intracellular metabolites was similar to that previously described for FUra metabolites [5]. Various combinations of [2′-^{3}H]dUrd (sp. act. = 0.4 μCi/nmol), FUra, and DP were added to the dishes to yield final concentrations of 25, 5 and 0.5 μM respectively. The cells were incubated with the compounds for 4- or 24-hr intervals prior to extraction with 0.5 N PCA. HPLC Method 2 was employed to analyze the reconstituted neutralized acid-soluble residue.

Incorporation of [6-^{3}H]FUra into nucleic acids. Equal numbers of HCT 116 cells were seeded into 75 cm^2 tissue culture flasks and allowed to incubate for 72 hr. The cells were then exposed to [6-^{3}H]FUra (5 μM; sp. act. = 4 μCi/nmol) + 0.5 μM DP for 4-, 8- or 24-hr intervals. Duplicate flasks containing non-radiolabeled FUra + DP were used to determine the cell number. At the end of the desired drug exposure, the cells from the flasks containing [6-^{3}H]FUra were washed with iced PBS/EDTA and then harvested

* Uptake implies the sum of membrane transport and subsequent incorporation of the nucleoside into the intracellular nucleotide pools.

with a rubber policeman. An aliquot of the initial cell suspension was spotted onto Whatman 3M filter paper strips for determination of the total acid-precipitable counts present. The cell pellet was digested with 0.5% SDS, 10 mM Tris/1 mM EDTA (pH 8.0), and 1.25 mg/ml Proteinase K overnight at 37°. The cells from the non-labeled FUra ± DP flasks were harvested by washing once with PBS/EDTA followed by trypsinization. An aliquot of the diluted cell suspension was counted in a Coulter counter. The following day, two phenol extractions followed by two extractions with chloroform/isoamyl alcohol (24:1) were used to extract the nucleic acids. The purified nucleic acids were analyzed by cesium sulfate density centrifugation. Briefly, the nucleic acids were diluted in 10 mM Tris/1 mM EDTA (pH 8.0) and mixed with a saturated solution of cesium sulfate to yield a starting density of 1.60 g/ml. An aliquot of the nucleic acid/cesium sulfate mixture was spotted onto Whatman 3M filter paper to permit an estimate of the recovery of the acid precipitable counts following the digestion and extraction procedures. The recovery ranged from 75 to virtually 100%. The sample was transferred to a $\frac{5}{8} \times 3$ inch polyallomer centrifuge tube (Beckman Instruments, Inc., Palo Alto, CA), placed in a Beckman Ti 70.1 rotor, and centrifuged in a Beckman model L5-65 ultracentrifuge at 55,000 rpm (20°) for 16 hr. The speed was then decreased to 42,000 rpm for an additional 45 min. Forty 250-μl fractions were collected from the top with a Buchler autodensiflow collector.

A refractometer was used to determine the refractive index of the samples, which permitted calculation of the density [7]. The density from the top to the bottom of the polyallomer tube ranged from 1.36 to 1.75 g/ml. The DNA peak was between 1.40 and 1.45 g/ml and the RNA peak was between 1.60 and 1.65 g/ml. Ethidium bromide staining was used to confirm the presence of nucleic acids in these peaks. A 50-μl aliquot from each fraction was spotted on Whatman 3M filter paper strips. After drying, the strips were washed three times in 5% PCA and twice in 95% ethanol. The acid-precipitable counts on the dried strips were quantified by liquid scintillation counting.

The remainder of the fractions containing DNA or RNA for each condition were then pooled and dialyzed against 1000 ml of 10 mM Tris/1 mM EDTA using a prepared dialysis membrane (mol. wt cutoff 12,000–14,000 daltons) and a microdialysis system (Bethesda Research Laboratories, Gaithersburg, MD) to remove the salt. The amounts of DNA and RNA were quantified by measuring the absorbance at 260 nm. The amounts of RNA and DNA per 10^6 cells were similar for cells exposed to either FUra alone or FUra + DP. After a 24-hr exposure, the average amount of RNA was 5.7 μg/10^6 cells (range 5 to 6.4) in FUra-treated cells versus 6.0 μg/10^6 cells (range 3.2 to 8.7) in cells expose to FUra + DP. The DNA content in FUra cells was 5.6 μg/10^6 cells (range 5.1 to 6.1) versus 6.4 (range 4.5 to 8.2) in FUra + DP cells.

Measurement of endogenous dUMP pools. dUMP pools were measured using a modified tritium release assay [8, 9] in which the amount of dUMP was the limiting substrate. Aliquots of neutralized acid-soluble extract were added to a reaction mixture containing 20 nmol CH_2FH_4 and 5 pmol [5-^{3}H]dUrd in a total volume of 200 μl. Known amounts of unlabeled dUMP were added to separate tubes. The reaction was initiated by the addition of excess dTMP synthase. After 30 min at 37°, the reaction was terminated by adding 100 μl of ice-cold TCA (20%); 200 μl of an albumin-coated charcoal slurry (pH 7.2) was then added and allowed to stand at room temperature for 10 min. The charcoal was then sedimented by centrifugation at 12,000 *g* for 15 min. A 200-μl sample of the supernatant fraction was assayed for [^{3}H]H_2O radioactivity. Assays were performed in duplicate. The amount of [^{3}H] released was inversely proportional to the concentration of cold dUMP in the reaction mixture. The standard curve of [^{3}H] release versus log pmol dUMP was linear from 200 to 1600 pmol. Coincubation with FdUMP at a molar ratio of 1:1 or 1:10 inhibited [5-^{3}H] release. The standard curve derived in the presence of FdUMP:dUMP at a ratio of 1:100, however, was similar to that obtained in the presence of dUMP alone.

Alkaline elution assays. The alkaline elution filter assay described by Kohn [10] was used to measure single-strand breaks in mature and nascent DNA. For the former studies, cells were incubated with [^{14}C]dThd (0.05 μCi/ml) or [^{3}H]dThd (0.05 μCi/ml), the latter to serve as an internal reference, for 48 hr; then the radioactive medium was aspirated and replaced with fresh medium. Following an additional 24-hr chase period to allow the label to become associated with high molecular weight DNA, FUra and DP were added to the dishes in various combinations to yield final concentrations of 5–50 μM and 0.5 μM respectively. After a 4- or 24-hr exposure period, the cells were harvested for alkaline elution assay. One of the control dishes and the cells that served as the internal standard were irradiated on ice with 300 cGy X-rays prior to alkaline elution.

For alkaline elution studies examining the effect of drug exposure on nascent DNA, the cells were incubated with [^{3}H]dThd (0.018 μCi/ml) for 4 hr concurrent with drug exposure. The final concentrations of FUra, DP, and dUrd were 5, 0.5 and 25 μM respectively. After the drug treatment, the medium was aspirated and replaced with fresh medium. The cells were then prepared for elution according to the technique recently reviewed by Kohn *et al.* [11]. HCT 116 cells labelled with [^{14}C]dThd (0.05 μCi/ml) were used as internal standards and were irradiated on ice with 300 cGy of X-rays immediately before the alkaline elution procedure. Preliminary experiments were done to determine the amount of [^{3}H]dThd that would permit adequate labeling of DNA without producing significant single-strand breaks from the tritium itself. The [^{3}H]dThd thereby selected for use in these experiments (0.18 μCi/ml, 0.16 μCi/dish) did not alter significantly the control elution rate.

RESULTS

Modulation of FUra cytotoxicity by dUrd. DP could augment FUra cytotoxicity by altering the intracellular concentration of key nucleotides other than FdUMP by perturbing the efflux of nucleosides

such as dUrd. First, it was necessary to determine a concentration of dUrd which could overcome the transport block imposed by a fixed concentration of DP. The uptake of 0.5 to 500 μM dUrd into the acid-soluble cell fraction after 1 hr of exposure was measured in the presence and absence of 0.5 μM DP. At 10 and 25 μM dUrd, the uptake of [^{3}H]Urd (108 and 284 pmol/10^6 cells respectively) in the presence of DP was approximately 50% compared to a control with dUrd alone (260 and 537 pmol/10^6 cells respectively; data not shown). A series of experiments were performed to determine the influence of dUrd on growth inhibition produced by FUra. The use of sufficient dUrd to cause substantial incorporation of dUrd into acid-soluble metabolites despite the presence of DP allowed an examination of the ability of dUrd to modulate the growth inhibition produced by the combination of FUra + DP.

Figure 1A demonstrates that dUrd enhanced the cytotoxicity of 5 μM FUra in a dose-dependent manner, whereas dThd in concentrations of 1 to 25 μM did not (Fig. 1B). Furthermore, dUrd in concentrations ≥10 μM increased the augmentation of FUra toxicity produced by DP (Fig. 1A). In contrast, 25 μM dThd reversed the augmentation of FUra toxicity produced by DP (Fig. 1B).

To determine if dUrd altered the viability of HCT 116 cells in the same manner as cell replication, clonogenic assays were performed. Exponentially growing cells were exposed to drug for 8 or 24 hr and then cloned. The 24-hr data, presented in Table 1, indicated that 25 μM dUrd modestly increased the cytotoxicity of FUra. DP produced an augmentation of FUra lethality. The augmentation of FUra lethality produced by DP was somewhat increased by the addition of dUrd. These results are in contrast to those previously obtained with dThd [4]. A 25 μM concentration of dThd did not protect HCT 116 cells from FUra lethality and reversed rather than enhanced the lethality produced by the combination of FUra + DP [4]. An 8-hr exposure to 5 μM FUra alone or in combination with either 25 μM dUrd or 0.5 μM DP did not reduce the viability of HCT 116 cells (data not shown). These interesting differences prompted an examination of the effects of dUrd and FUra metabolism.

Effect of dUrd on FUra metabolism. We have reported previously that 25 μM dThd reduces FdUMP levels to less than half those measured in the presence of either FUra alone or FUra + DP [5]. dThd prevents the enhanced intracellular retention of FdUMP produced by DP in cells preloaded with FdUrd, and it also inhibits the incorporation of extracellular [^{3}H]FdUrd into intracellular FdUMP [5]. These two observations suggested that competition for dThd kinase or feedback inhibition of the enzyme by expanded dTTP pools might be operative. For comparison, the effect of 25 μM dUrd on the levels of free FUra nucleotides in the cell was determined 4 hr following the exposure to 5 μM FUra + 0.5 μM DP. The data, presented in Fig. 2, indicate that 25 μM dUrd indeed altered the pattern of FUra metabolism. dUrd increased the amount of FdUMP formed 4-fold relative to that of FUra alone, and approximately 2-fold relative to that of FUra + DP. In contrast, the FUTP levels were highest in cells exposed to FUra alone (12.9 pmol/10^6 cells). The addition of dUrd to FUra lowered the FUTP levels by 25% to 9.9 pmol/10^6 cells, and the FUTP levels in cells exposed to DP + dUrd were decreased by 40–45% (7.1 to 7.8 pmol/10^6 cells). The total acid-soluble metabolites was similar for all four

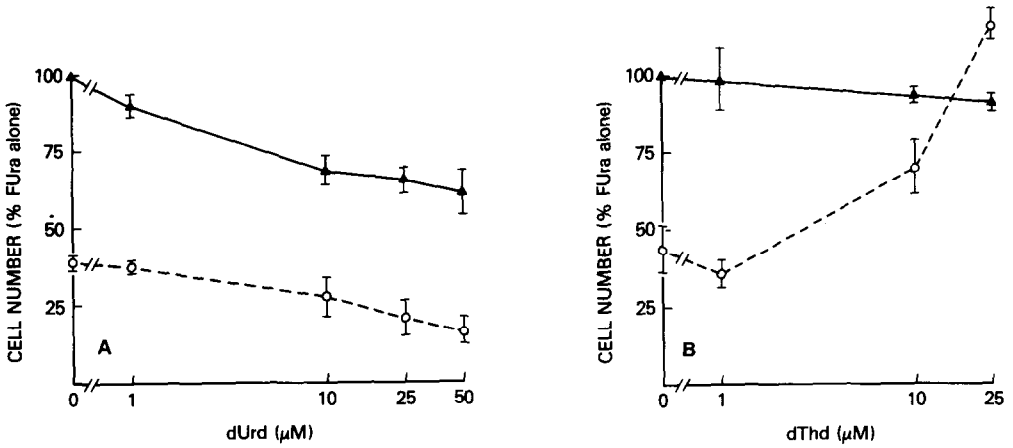

Fig. 1. Modulation of the cytotoxicity of FUra alone and in combination with DP by dUrd and dThd. Exponentially growing HCT 116 cells were exposed to 5 μM FUra in the presence (○) or absence (▲) of 0.5 μM DP and the indicated concentrations of either dUrd (A) or dThd (B). After 72 hr of drug exposure, the cells were trypsinized and enumerated by Coulter Counter. The data represent a compilation of multiple experiments, each done in triplicate. The data are presented as the percentage of cell number (mean ±SE, N ≥ 3) compared to that observed with FUra alone. The cell number of the group treated with FUra alone averaged 33% of the control cell number (mean control cell number was 6.6×10^5 cells; range, 3.3×10^5 to 1.1×10^6). Exposure to 1, 10 or 25 μM dUrd alone did not produce any inhibition of cell growth compared to control. The cell number was 90% of the control after a 72-hr exposure to 50 μM dUrd. A 1–25 μM concentration of dThd did not inhibit cell growth compared to the control.

Table 1. Effect of dUrd on the lethality of 5-fluorouracil ± dipyridamole in HCT 116 cells

Experimental condition	Mean % control cell survival (range)	Mean % FUra cell survival (range)
dUrd	96 (89–104)	—
FUra	72 (59–84)	—
dUrd + FUra	55 (48–62)	78 (74–81)
FUra + DP	44 (39–49)	62 (59–65)
FUra + DP + dUrd	40 (36–44)	57 (52–62)
DP	79 (76–82)	—
DP + dUrd	86 (85–86)	—

Exponentially growing cells were exposed to drugs for 24 hr. The concentrations employed were 5 μM FUra, 0.5 μM DP, and 25 μM dUrd. Serial dilutions were performed on the trypsinized cells, and 100–200 cells were cloned in triplicate dishes in drug-free medium. Following an 8-day incubation, the cells were stained, and colonies containing 50 or more cells were enumerated. The data are presented as the mean fractional survival compared to either the control group or to cells exposed to FUra alone (mean and range; N = 2). The plating efficiency for the controls averaged 50%.

conditions, ranging from 43 to 49 pmol/10^6 cells, with free FUra base accounting for 50–65% of the total acid-soluble pool (data not shown).

The increase in FdUMP levels in the presence of dUrd could be a consequence of two factors: enhanced formation of FdUrd by dThd phosphorylase with the deoxyribose moiety contributed from dUrd, or decreased catabolism of FdUMP and FdUrd. The first possiblity was tested by labeling the cells with [2′-^{3}H]dUrd in the presence or absence of 5 μM FUra. Following a 4- or 24-hr interval, the acid-soluble fraction was separated, neutralized, and lyophilized, and the constituents were analyzed by HPLC to determine the distribution of [2′-^{3}H]dUrd metabolites. The results, presented in Table 2, indicate that radioactivity could be detected in the fraction coeluting with FdUMP at both time points in cells exposed to dUrd + FUra. No radioactivity appeared in the peak coeluting with the FdUMP standard in cells exposed to dUrd alone. This suggested that [^{3}H]deoxyribose was indeed being transferred from dUrd to FUra via dThd phosphorylase. A tremendous buildup of dUMP (100- to 300-fold) was seen in cells exposed concurrently to FUra for 4 and 24 hr compared to that obtained with exposure to dUrd alone. Similar observations have been made by other investigators, and the accumulation of dUMP is presumed to be related to the inhibition of dTMP synthase by FdUMP [12–15]. The huge excess of dUMP relative to FdUMP suggests that the catabolism of FdUMP might be inhibited. After 4 hr, the dTTP level in the FUra + dUrd cells was reduced to about 40% of that observed in cells treated with dUrd alone. Surprisingly, after 24 hr, the dTTP pools were approximately two times larger in the FUra/dUrd cells. This is in agreement with our previous determinations of dTTP pools by an enzymatic assay: dTTP pools were decreased by a factor of 2 in cells exposed to FUra compared to control for the initial 8 hr of exposure, but by 24 hr the dTTP pools were actually 2-fold larger in FUra-treated cells [4]. The incorporation of the [2′-^{3}H] label into the acid-precipitable fraction can serve as an index of DNA synthesis, and FUra inhibited the incorporation by 46 and 62% after 4 and 24 hr respectively.

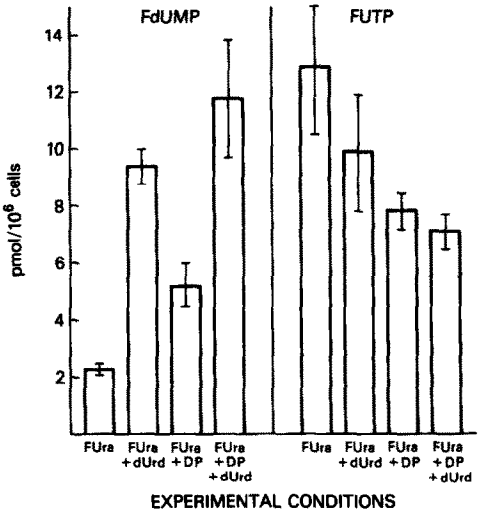

Fig. 2. Effect of dUrd on FUra metabolism. Exponentially growing HCT 116 cells were exposed to different combinations of [^{3}H]FUra (5 μM; sp. act. = 0.5 μCi/nmol), 25 μM dUrd and 0.5 μM DP for 4 hr. The cells were extracted with PCA, and the acid-soluble fraction was collected, neutralized and lyophilized. An aliquot of the reconstituted residue was analyzed by HPLC Method 1. The radioactivity eluting with the FdUMP and FUTP standards was quantified by liquid scintillation counting. The data are presented as pmol of metabolite per 10^6 cells (mean ± range, N = 2).

Effect of DP on the incorporation of [6-^{3}H]*FUra into nucleic acids.* Since the incorporation of FUra into RNA [16–25] and DNA [26–31] has been suggested as a key cytotoxic effect, the incoporation of [6-^{3}H]FUra into HCT 116 nucleic acids was moni-

Table 2. Effect of 5-fluorouracil on deoxyuridine metabolism

	Total acid-soluble pool	Total nucleoside pool	dUMP	FdUMP	dTMP	dTTP	Total acid-precipitable pool
	(pmol metabolite/10^6 cells)						
4 hr							
dUrd	563	388	15	ND	12	62	482
dUrd + FUra	1,993	407	1,443	20	20	24	259
24 hr							
dUrd	582	394	23	ND	12	92	10,671
dUrd + FUra	7,807	282	6,637	234	234	204	4,108

Exponentially growing HCT 116 cells were exposed to [2′-^{3}H]dUrd (25 μM; sp. act. = 0.4 μCi/nmol) in the presence or absence of 5 μm FUra for 4 or 24 hr. The distribution of dUrd metabolites in the acid-soluble fraction was determined by HPLC Method 2. The 4- and 24-hr exposures represent separate experiments, with a single determination of each time point. [^{3}H] counts were present in the peak coeluting with the FdUMP standard in the cells exposed to [2′-^{3}H]dUrd plus FUra, but [^{3}H] was not detectable (ND) in the peak coeluting with FdUMP in cells exposed to [2′-^{3}H]dUrd alone. The counts in the total acid-precipitable fraction were determined by washing the pellet three to five times with 0.5 N PCA. The pellet was solubilized by treatment with 0.8 ml of 3 N KOH for 60 min at 37°, and the radioactivity was quantified by liquid scinitillation counting.

tored by cesium sulfate gradient centrifugation which separates RNA (banding between density 1.60 and 1.65 g/ml) and DNA (banding between density 1.40 and 1.45 g/ml). After a 24-hr exposure to [6-^{3}H]FUra (5 μM; sp. act. = 4 μCi/nmol), FUra was detectable in both RNA and DNA (Fig. 3).

The amount of tritium activity was much greater (by 25-fold) in RNA than in DNA, in agreement with the findings described by other investigators [26–29]. The amount of FUra-RNA increased as a function of time (1, 2.1, and 13 pmol/10^6 cells after 4, 8 and 24 hr), consistent with our previous observation that FUTP increases as a function of time

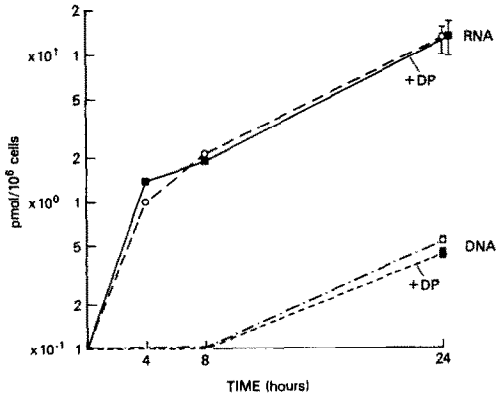

Fig. 3. Incorporation of [6-^{3}H]FUra in HCT 116 nucleic acids. Exponentially growing cells were incubated with [6-^{3}H]FUra (5 μM, sp. act. = 4 μCi/nmol) in the presence (■) or absence (○) of 0.5 μM DP for 4-, 8-, and 24-hr intervals. The total cellular nucleic acids were purified by phenol and chloroform extraction and analyzed by cesium sulfate density centrifugation. The acid-precipitable radioactive counts banding in the RNA and DNA regions were determined. The data are presented as pmol FUra incorporated into either RNA (straight line) or DNA (broken line) per 10^6 cells as a function of duration of exposure. The data from the 4- and 8-hr time points are from a single experiment. The data from the 24-hr time point is expressed as the mean ± range (N = 2).

(7.2, 27.2 and 44.6 pmol/10^6 cells after 2, 4 and 24 hr) [5]. DP did not appear to alter the amount of [^{3}H]FUra metabolites into RNA. The amount of [^{3}H]FUra detected in the DNA fraction at 24 hr was small (approximately 0.5 pmol/10^6 cells) and was not increased by exposure to FUra + DP. The incorporation of [^{3}H]FUra into DNA was checked to earlier time points to make certain a transient peak had not been missed. Following a 4- or 8-hr exposure, no radioactivity appeared in the DNA fraction whether or not DP was present.

The augmentation in FUra lethality produced by DP could not be attributed to obvious differences in the amount of FUra incorporated into either DNA or RNa. Even though FUra-RNA was present after 4- and 8-hr exposures in the range of 1 to 2 pmol/10^6 cells, an 8-hr exposure to 5 or 10 μM FUra was not associated with a decline in clonogenic survival whether or not DP was simultaneously present (data not shown).

Accumulation of dUMP in cells treated with FUra and DP. We next evaluated whether perturbations of dUMP metabolism occurred following exposure to FUra/DP. In the presence of 5 μM FUra, marked expansion of the dUMP pools occurred by 4 hr (Table 3). A slight increase in dUMP pools was noted by 2 hr following simultaneous exposure to 0.5 μM DP and FUra; by 4 hr, the dUMP levels rose to over 5000 pmol/10^6 cells (13.6-fold over control). The ratio of dUMP levels in the presence of FUra/DP relative to that observed with FUra alone was 1.5 at both the 2- and 4-hr time points. The dUMP pools were even larger at 24 hr; however, the highest dUMP levels were seen in FUra-treated cells rather than in those treated with FUra/DP. The proportion of dUMP to free FdUMP levels (Table 3, Fig. 2) was more than 3 logs higher at 4 hr, and was over 2 logs higher at 2 and 24 hr [5]. These data are consistent with the hypothesis that accumulation of dUMP might contribute to FUra toxicity in this cell line. The modest increase in dUMP in the presence of FUra/DP relative to FUra alone, however, may not fully account for the enhanced FUra toxicity.

Table 3. Effect of 5-fluorouracil ± dipyridamole on dUMP pools

Duration of exposure (hr)	dUMP pool size (pmol/10^6 cells)		
	DP	FUra	FUra + DP
2	—	350 ± 95 (95%)*	566 ± 111 (153%)
4	364 ± 123 (98%)	3362 ± 738 (906%)	5049 ± 1938 (1359%)
24	650 ± 410 (175%)	9618 ± 613 (2592%)	7166 ± 1142 (1932%)

Exponentially growing cells were exposed to 5 μM FUra with or without 0.5 μM DP for 2, 4 and 24 hr. The acid-soluble fraction was separated, neutralized and lyophilized. An aliquot of the resuspended residue was assayed for dUMP levels by a modified [5-^{3}H]dUrd release assay in the presence of excess dTMP synthase and 100 μM CH_2FH_4 cofactor as described in Materials and Methods. The data, presented as mean dUMP pool size ± SE, are a compilation of 3–6 separate experiments. The endogenous (control) dUMP pools were 371 ± 111 pmol/10^6 cells.
* Percent control.

Effect of FUra exposure on DNA fragmentation. We previously noted that the level of free FdUMP increases as a function of duration of exposure [5]. Despite the accumulation of free FdUMP over time, FdUDP and FdUTP were not detected, presumably because FdUTP is an excellent substrate for deoxyuridine triphosphatase [15, 32, 33]. Nevertheless, a small amount of FdUTP was incorporated into DNA.

The effect of FUra exposure on the fragmentation of DNA was assessed by alkaline elution [10, 11]. In experiments in which the [^{14}C]dThd was allowed to incorporate into high molecular weight DNA prior to drug treatment, there was no evidence of single-strand breaks following exposure to 5 or 50 μM FUra ± 0.5 μM DP (data not shown).

To test the possibility that these drugs could affect the stability of newly synthesized DNA, the cells were treated with radiolabeled dThd simultaneously. The amount of [^{3}H]dThd used during drug exposure did not alter significantly the control elution rate. In experiments in which the DNA was labeled concomitantly with FUra exposure, the DNA elution pattern was biphasic, suggesting the presence of small DNA species which elute from the filters rapidly and larger fragments which elute more slowly (Fig. 4). This pattern contrasts with that of the control which was nearly linear. Exposure to DP alone resulted in elution profiles indistinguishable from that observed for FUra treatment, whereas treatment with FUra in combination with dUrd or DP resulted in an increase in the proportion of fast eluting species. The proportion of fast eluting species was greatest following simultaneous exposure to all three agents.

DISCUSSION

In summary, dUrd enhanced the cytotoxicity of FUra alone and of the combination of FUra + DP. The modulation of FUra toxicity by dUrd was in marked contrast to the dThd effect. Furthermore, dUrd and dTHd produced disparate effects on FUra metabolism: dUrd increased FdUMP levels, whereas dThd decreased FdUMP levels. These data suggest that dUrd may contribute to the mechanism by which DP augments FUra toxicity in HCT 116 cells. Both dThd and dUrd can serve as potential donors of dR-1-P, which is necessary in the formation of FdUrd from FUra catalyzed by dThd phosphorylase [34, 35]. The salvage enzyme dThd kinase can use dThd, dUrd or FdUrd as substrates for conversion to the nucleotide monophosphate level [36, 37]. Expansion of the intracellular dTTP pools by the administration of exogenous thymidine can decrease the activity of dThd kinase through feedback inhibition [38, 39], and thus would decrease activation of FdUrd to FdUMP. In contrast, dUMP and FdUMP are not readily converted to dUTP and FdUTP; therefore, product inhibition of dThd kinase would not be expected to occur. In the presence of FUra, dUMP levels accumulate behind the block of dTMP synthase. In cell lines in which FdUMP-mediated inhibition of dTMP synthase is growth

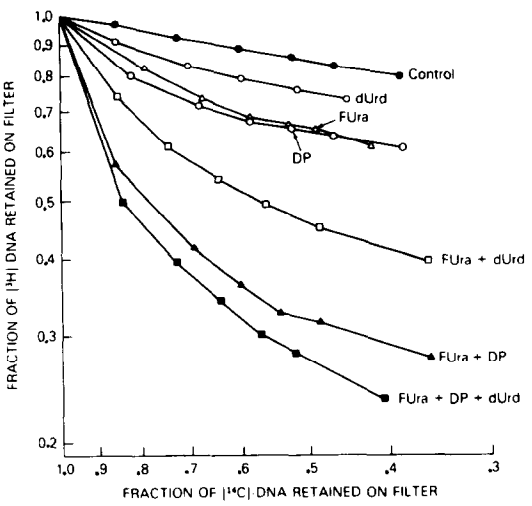

Fig. 4. Alkaline elution profiles for newly synthesized DNA. Exponentially growing HCT 116 cells were treated for 4 hr with 5 μM FUra, 0.5 μM DP and 25 μM dUrd alone and in various combinations. The cells were concurrently exposed to [^{3}H]dThd as described in Materials and Methods to permit labeling of nascent DNA strands. The cells were processed at 4° for alkaline elution. Cells prelabeled with [^{14}C]dThd served as the internal standard. The data are from a single experiment and are representative of four individual replicates.

limiting, an accumulation of dUMP has correlated with the ability of cells to resume cell growth and escape from the blockade of dTMP synthesis [40–48].

In HCT 116 cells, in contrast, the very large pool sizes of dUMP observed in the presence of FUra ± DP do not clearly correlate with escape from FUra toxicity. Rather the possibility that dUMP may be related to the enhanced cytotoxicity is suggested.

The buildup of dUMP and FdUMP may result in increased formation of dUTP and FdUTP. Incorporation of dUTP into DNA with subsequent alterations in DNA stability has been described with methotrexate and other antifols [49–52]. In the dUrd metabolism experiments described in this paper, radioactivity was not detectable in the peak coeluting with the dUTP standard. Similarly, we could not detect FdUTP in the [6-^{3}H]FUra metabolism experiments (the lower limit of sensitivity in the system utilized was approximately 0.2 pmol/10^6 cells). This is not surprising, since deoxyuridine triphosphatase can rapidly degrade both dUTP and FdUTP. Other investigators have been able to detect very small levels of FdUTP and dUTP in cells exposed to FdUrd [15, 29]. For example, Ingraham *et al.* [15] reported that FdUTP and dUTP pools of 0.12 and 0.055 pmol/10^6 cells were detected following a 6-hr exposure to 1 μM FdUrd. In addition, both FdUMP and dUMP residues were detected in the DNA of these cells.

A small amount of FUra was detectable in the DNA of cells exposed to [6-^{3}H]FUra for 24 hr, but none was detected in DNA following a 4- or 8-hr exposure. The total amount of FUra incorporated into DNA did not appear to be affected by DP. Other investigators have reported that the incorporation of FUra metabolites into DNA occurs during semiconservative DNA synthesis [30, 31]. The active removal of the fraudulent residues from DNA of FUra-treated cells by the normal repair mechanisms is thought to lead to fragmentation of DNA and contribute to the cytotoxic effects of FUra [15, 32, 33]. In fact, cells treated with FUra have been shown to contain DNA with alkaline labile bonds, and increased formation of DNA fragments has been observed [30, 31, 53, 54]. DNA fragmentation following FUra exposure has also been noted in the absence of detectable fluoropyrimidine incorporation into DNA [53]. Yoshioka *et al.* [54] reported a correlation between depletion of deoxyribonucleotide pools and DNA damage and detected an activity in cell lysate of mouse mammary FM3A cells treated with FdUrd that induced DNA double-strand breaks. Therefore, the effect of FUra ± DP on the stability of DNA was examined in HCT 116 cells. We did not detect any evidence of increased single-strand breaks in the high molecular weight DNA in the presence of FUra with or without simultaneous exposure to DP. A marked increase in the elution rate of DNA labeled at the time of FUra exposure was, however, clearly evident. Surprisingly, DP alone produced this effect. Incubation with FUra and either DP, dUrd or the combination enhanced the elution rate. Further studies will need to be done to clarify the significance of the incorporation of FUra into DNA as a mechanism of cytotoxicity. Similarly, the specific mechanism(s) by which DP augments the alkaline lability of DNA in this colon carcinoma cell line remains to be defined.

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