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MODULATION OF 5-IODO-2′-DEOXYURIDINE METABOLISM AND CYTOTOXICITY IN HUMAN BLADDER CANCER CELLS BY FLUOROPYRIMIDINES

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Abstract—Iododeoxyuridine (IdUrd) potentiated the lethal but not the growth inhibitory properties of fluorouracil (FUra) and fluorodeoxyuridine (FdUrd) in human bladder cancer cells (T24). The rate of incorporation of IdUrd into DNA was enhanced by both fluoropyrimidines, but to a significantly greater extent by FdUrd. Both inhibition of iododeoxyridylate dehalogenation and the depletion of thymidine triphosphate pools contributed to the increased incorporation rate. Inhibition of dehalogenation accounted for 67% of the observed stimulation in the case of FUra, but only 37% of the increase produced by FdUrd. The depletion of dTTP pools, both in the presence and absence of IdUrd, was greater after FdUrd than FUra exposure. The observed increase in the rate of incorporation of IdUrd appears to account for the enhanced toxicity seen with FdUrd, but other factors may be involved in the case of FUra. Since FUra and IdUrd appear to be mutually potentiating and do not share a dependence on thymidine kinase activity, this drug combination warrants further investigation.

Successful treatment with many antineoplastic agents is limited by the emergence of drug resistance. One approach to this problem is the development of drug combinations designed to circumvent resistance or produce synergistic cytotoxicity. Since the biochemical pharmacology of IdUrd‖ and the fluoropyrimidines, FdUrd and FUra, suggests that these agents could be mutually potentiating, we have initiated studies of their interactions in human cancer cells.

The cytotoxicity of IdUrd, a thymidine analog, is dependent upon its metabolic activation to the 5′-triphosphate (IdUTP) and subsequent incorporation into DNA [1]. Since dTTP competes with IdUTP for incorporation into DNA, mechanisms which increase intracellular IdUTP pools, decrease dTTP levels, or both, could augment the cytotoxicity of IdUrd. Thymidylate synthetase activity is particularly important in this regard. Inhibition of this enzyme, which plays a key role in the *de novo* synthesis of dTMP, can reduce intracellular dTTP levels [2, 3]. In addition, it catalyzes an important inactivation reaction, the dehalogenation of IdUMP [4]. Deiodination is extensive, and substantial amounts of dUMP are formed and then converted to dTMP, also by thymidylate synthetase [5]. Thus, inhibition of this enzyme should enhance the intracellular ratio of IdUTP to dTTP.

FdUrd is phosphorylated by dThd kinase to FdUMP, a potent inhibitor of thymidylate synthetase [6, 7]. Consequently, it has been used to augment the incorporation of IdUrd into DNA [8–11]. The relative contributions of reduced dTTP pools and inhibition of IdUMP dehalogenation to the increased rate of incorporation, however, have not been clarified. Since thymidylate synthetase can be inhibited by FUra, its effects on the metabolism and cytotoxicity of IdUrd were also evaluated. This was of further interest since the activation of both FdUrd and IdUrd are dependent on dThd kinase, whereas FUra is activated by other enzymes [12, 13]. Thus, dThd kinase deficient cells, which are resistant to FdUrd [14–17] and IdUrd [1], would retain sensitivity to FUra [18]. In addition, the incorporation of FUra into RNA [12, 13, 19, 20] might produce increased cytotoxicity in cells in which IdUrd has been incorporated into the DNA.

Previous experiments combining FdUrd and IdUrd have shown enhanced cytotoxic and radiosensitizing activities [1, 8, 21]. Although trials in animal tumors have yielded additive or synergistic results [22–24], clinical evaluation has been limited [25–28].

The results of the present study indicate that IdUrd enhanced the lethality, but not the growth inhibitory effects, of fluoropyrimidines. The incorporation of IdUrd was increased markedly by these compounds. Quantitatively, inhibition of IdUMP dehalogenation

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‖ Abbreviations: IdUrd, 5-iodo-2′-deoxyuridine; FdUrd, 5-fluoro-2′-deoxyuridine; 5-FUra, 5-fluorouracil; FdUMP, 5-fluoro-2′-deoxyuridylate; IdUMP, IdUDP, and IdUTP, the 5′-mono-, di-, and triphosphates of IdUrd; PBS, phosphate-buffered saline; and HPLC, high pressure liquid chromatography.

was of primary importance in the case of FUra, whereas dTTP pool depletion played a greater role in the ability of FdUrd to augment IdUrd incorporation. The increased rate of incorporation of IdUrd into DNA appears to account for the enhanced toxicity in the case of FdUrd, whereas additional factors may be involved in the interactions with FUra.

MATERIALS AND METHODS

Materials. IdUrd, IdUMP, IdUTP, and dAMP were obtained from Calbiochem. (San Diego, CA), and, dTMP, dTDP, and dTTP were purchased from P-L Biochemical, Inc. (Milwaukee, WI). The Sigma Chemical Co. (St. Louis, MO) provided the dThd, FdUrd, FUra, and poly d(AT). [6-^{3}H]IdUrd, with a specific activity of 10 Ci/mmole, was supplied by Moravek Biochemicals, Inc. (Brea, CA); Amersham International furnished the [8-^{3}H]dATP and the [^{125}I] dUrd; and DNA polymerase I was obtained from Boehringer-Manheim (Indianopolis, IN). Alamine (tri-*n*-octylamine) and freon (1,1,2-trichlorotrifluoroethane) were purchased from the Aldrich Chemical Co. (Milwaukee, WI).

Cells. T24 cells, a human urinary bladder cancer cell line [29], were obtained from Dr. Ernest C. Borden (Wisconsin Clinical Cancer Center, Madison, WI). The cells were grown as monolayers in Eagle's Minimum essential medium with Eagle's salts and L-glutamine (Grand Island Biological Co., Grand Island, NY) supplemented with 10% newborn calf serum and 0.1 mM non-essential amino acids (K.C. Biologicals, Lenexa, KS), 2.0 mM L-glutamine, 60 μg/ml penicillin and 100 μg/ml streptomycin at 37° in a humidified 5% CO_2 atmosphere. Cells were removed from the plastic tissue cultureware with 0.1% porcine trypsin (K.C. Biologicals) with PBS [137 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, and 1.1 mM KH_2PO_4 (pH 7.4)] containing 2.5 mM EDTA, and then subcultured every 5 days. Cell numbers were determined using an electronic particle counter (Coulter Electronics, Hialeah, FL) with periodical confirmation by hemocytometer. These cells had a mean doubling time of 20 hr. DNA fluorochrome staining as described by Chen [30] demonstrated no evidence of mycoplasma.

Cell volume measurements were made using the Coulter counter and the Canberra series 35 Multichannel Analyzer. Modal cell volumes varied from approximately 2490 μm^3 to 1820 μm^3 at cellular densities ranging from about 1×10^4 to 4×10^4 cells/cm^2 respectively.

Cytotoxicity. The rates of cellular replication and efficiency of colony formation were used as measures of cytotoxicity. In the cell growth experiments, 4×10^3 cells/cm^2 were plated in 35 mm plastic dishes, incubated for 24 hr, and then exposed to drug(s) for an additional 24 hr. The cells were washed once with PBS and fresh medium was added. Duplicate dishes were plated and cell numbers were determined at 24, 48, and 72 hr following the addition of drugs.

In the viability experiments, 1×10^4 cells/cm^2 were plated and incubated for 24–48 hr. The cells were then exposed to drug(s) for 4, 8 or 24 hr after which they were washed with PBS and removed from the dishes with 0.1% trypsin in PBS/EDTA. The cells were diluted with medium, plated in triplicate in 35 mm plastic culture dishes at approximately 100 cells/dish and 200 cells/dish, and then incubated for 7–9 days. The colonies were stained with 1% methylene blue in methanol. Cells giving rise to colonies containing greater than 50 cells were considered viable.

Uptake of IdUrd. The uptake of IdUrd into the acid-soluble and acid-insoluble pools of T24 cells was determined in the following manner. Exponentially growing cells, plated at variable densities between 4×10^3 and 2.5×10^4 cells/cm^2 per 35 mm plastic dish (between 6.3×10^3 and 1×10^4 cells/cm^2/100 mm dish), were utilized for all experiments. At the time of drug exposure there were between 4×10^4 and 8.5×10^4 cells/cm^2/35 mm dish (between 1.4×10^4 and 4×10^4 cells/cm^2/100 mm dish). Cells were usually incubated for 4 or 8 hr after the addition of 3 or 30 μM IdUrd ([6-^{3}H]IdUrd at a specific activity of 0.67 or 1.67 μCi/nmole) and/or 1 μM FdUrd; or, 1, 10 or 100 μM FUra. In the experiments using [^{125}I]dUrd the specific activity was 0.33 μCi/nmole. The medium was aspirated after incubation; cells were washed three times with ice-cold PBS and then extracted with 1 ml or 3 ml of cold 0.5 N $HClO_4$. After centrifugation, the acid-soluble material was collected and neutralized with 2 vol. of alamine-freon [31], and a portion was counted in ACS (Amersham Corp.) using a Searle Mark III 6880 liquid scintillation spectrometer. The remainder of the supernatant fraction was frozen. The pellet was dissolved in 5 N NaOH and counted in ACS after neutralization with 5 N HCl for determination of the amount of radioactivity incorporated into DNA. All experimental conditions were repeated several times.

Distribution of metabolites. The pattern of incorporation of [^{3}H]IdUrd into IdUMP, IdUDP, IdUTP, and dTTP was determined using a Spectra-Physics high pressure liquid chromatographic system. Exponentially growing cells were exposed to 3 μM [^{3}H]IdUrd (sp. act. = 0.67 or 1.67 μCi/nmole) and either 1 μM FdUrd or 1, 10 or 100 μM FUra. After incubation for 1, 4 or 8 hr, the medium was removed; the cells were washed three times with ice-cold PBS, extracted with 0.5 N $HClO_4$ for 15 min, and then scraped from the dish with a rubber policeman. The contents from each dish were centrifuged at 2000 *g* for 5 min, and the acid-soluble fractions were removed and then neutralized with alamine-freon. Neutralized extracts were lyophilized, the residue was dissolved in 100 μl of distilled water, and 45 μl portions were injected onto a Whatman Partisil SAX column (250 mm × 4.6 mm; 10 μm particle size) to separate the nucleotides. Authentic dThd, dTMP, dTDP, dTTP, IdUMP and IdUTP were injected simultaneously as markers. Separation on the anion-exchange column was achieved utilizing a linear increase in 0.3 M NaH_2PO_4 (pH 5.0) from 0 to 40% buffer during the first 4 min. This solvent composition was then run isocratically from 4 to 7.5 min followed by a 0.2-min linear gradient to 100% buffer which was maintained for an additional 12.3 min.

The flow rate was 2 ml/min, and absorbance was recorded at 254 nm. Forty-five 0.4-ml fractions were collected over the 20 min, and the amount of radioactivity was quantified.

DNA polymerase assay for dTTP. The assay for deoxyribonucleoside triphosphates is a modification of the previously reported enzymatic method of Solter and Handschumacher [32] as described by Hunting and Henderson [33]. Control cell populations were plated in duplicate, and cellular extracts were prepared as described above. The supernatant fractions were frozen, evaporated to dryness under vacuum, and then diluted in 100 μl of water. Poly d(A-T) (2 absorbance units/ml, Sigma Chemical Co.) was used as the template for assaying dTTP in a total assay volume of 170 μl. The reaction was initiated by the addition of 0.75 units of DNA polymerase I (Kornberg polymerase; Boehringer-Mannheim, Indianapolis) and 20 μl samples were collected at 45 and 60 min and spotted on Whatman 3MM paper strips. The reaction was linear from 5 to 100 pmoles dTTP and was proportional to the volume of cellular extract. dTTP pools are expressed as pmoles/10^6 and were found to decrease as cell density increased over the range of 1.4×10^4 to 4×10^4 cells/cm^2.

RESULTS AND DISCUSSION

Modulation of cytotoxicity. The effects of IdUrd and the fluoropyrimidines, alone and in combination, on T24 cell proliferation (Fig. 1) and ability to form colonies (Figs. 2 and 3) were evaluated. The concentration of IdUrd used in these experiments, 3 μM, produced a minimal effect. A 24-hr exposure to 30 μM IdUrd reduced the viability of T24 cells by 50% (data not shown). In the growth inhibition experiments, the cells were exposed to the drugs for 24 hr and then incubated for an additional 48 hr in drug-free medium prior to counting. Under these conditions, 1 μM FdUrd and 100 μM FUra, whether as single agents or in combination with 3 μM IdUrd, inhibited growth 75–80% (Fig. 1). Similarly, IdUrd did not increase significantly the 31% inhibition of growth produced by 10 μM FUra. In contrast, in the clonogenic assays, IdUrd increased the lethality of both FdUrd (Fig. 2) and FUra (Fig. 3). FdUrd did not reduce significantly the viability of T24 cells after either 4 or 8 hr of exposure. Colony formation, however, was reduced substantially by the addition of 3 μM IdUrd (Fig. 2). After a 24-hr exposure, FdUrd alone and in combination with IdUrd reduced colony formation more than 85%. Similarly, IdUrd diminished the viability of cells exposed to FUra (Fig. 3). The percentage of cells surviving 4- and 8-hr exposures to 100 μM FUra was reduced from 40% to 11% and 18% to 3%, respectively, following the

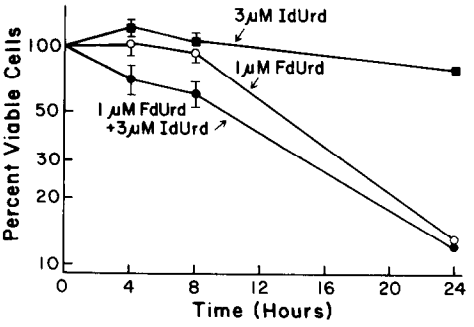

Fig. 2. Effects of IdUrd and FdUrd on the viability fo T24 cells. Exponentially growing cells were exposed to 3 μM IdUrd (■), 1 μM FdUrd (○), or both agents (●) for the times indicated. The cells were then washed free of drug and cloned. After 9 days of incubation the cells were stained, and colonies containing 50 or more cells were enumerated. The data are presented as the mean ± S.E. (N = 4) for the 4- and 8-hr time points. The 24-hr points are derived from a single experiment done in triplicate. The mean plating efficiency for the control cells was 0.46 ± 0.04 at 4 hr and 0.46 ± 0.02 at 8 hr.

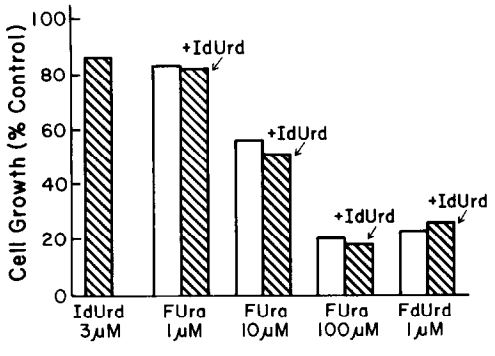

Fig. 1. Effects of IdUrd, FUra and FdUrd on T24 cell growth. Cells were plated at a density of 4×10^3 cells/cm^2 and then incubated for 24 hr. The cells were then exposed to IdUrd (3 μM), FUra (1, 10, 100 μM), or FdUrd (1 μM) for 24 hr. The hatched bars depict cells exposed to 3 μM IdUrd alone, or in combination with the indicated concentration of FUra or FdUrd. The cells were then washed with phosphate-buffered saline, renourished with fresh medium, and incubated for an additional 48 hr prior to counting. The data are presented as the percent of control cell growth. A variety of growth experiments were performed and similar results were obtained.

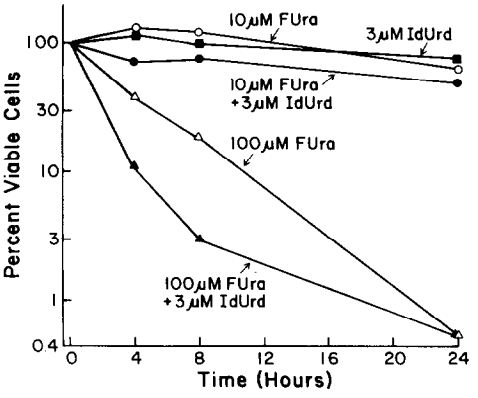

Fig. 3. Effects of IdUrd and FUra on the viability of T24 cells. Exponentially growing cells were exposed to 3 μM IdUrd (■), 10 μM FUra (○), 3 μM IdUrd and 10 μM FUra (●), 100 μM FUra (△) or 3 μM IdUrd and 100 μM FUra (▲) for the indicated periods of time. The cells were then clones as described in Fig. 2. The data are the average of two experiments, each done in triplicate.

inclusion of 3 μM IdUrd. No colonies were detected after a 24-hr exposure to 100 μM FUra. IdUrd also potentiated the lethality produced by 10 μM FUra. Thus, IdUrd potentiated the lethal, but not the growth, inhibitory properties of the fluoropyrimidines. Since the toxicity of IdUrd is thought to be dependent on its incorporation into DNA and its growth inhibitory effects are often delayed [1], these results are not surprising.

In this regard, it should be noted that numerous colonies of 10–20 cells were seen, but not counted, in the drug-treated groups. The morphology of the cells was irregular, and the colonies were not tightly packed as in the control population. These aborted colonies may account for the differences seen between the growth inhibition and viability assays.

Effects of FdUrd and FUra on the metabolism of IdUrd. Experimental approach: Accurate assessment of the effects of the fluoropyrimidines on IdUrd metabolism necessitates a variety of measurements since changes in the uptake of [^{3}H]IdUrd into the acid-soluble and -insoluble fractions provide an incomplete picture of IdUrd metabolism. A determination of the influence of FUra and FdUrd on the accumulation of the mono-, di-, and triphosphates of IdUrd and on the size and derivation of dTTP pools was needed. The extent to which IdUMP is dehalogenated and subsequently converted to dTTP determines the percentage of tritium incorporated into DNA as the halogenated nucleotide. These measurements were also necessary to compare fluoropyrimidine-induced changes in the ratio of intracellular IdUTP to dTTP.

In order to do this, an analysis of [^{3}H]IdUrd metabolism by HPLC and an enzymatic assay for total intracellular IdUTP and dTTP were done. The metabolism of [^{3}H]IdUrd to IdUMP, IdUDP and IdUTP and its conversion to [^{3}H]dTTP were quantified using HPLC. Coupled with the total dTTP and IdUTP pools, obtained with the enzymatic assay, both the amount and the derivation of the various pools could be determined.

A typical HPLC profile of the [^{3}H]IdUrd metabolites which accumulate intracellularly is shown in Fig. 4. In T24 cells exposed to 3 μM [^{3}H]IdUrd for 4 hr, approximately 60% (60 ± 2.4%; mean ± S.E.; N = 7) of the labeled nucleotides was IdUMP. Of the labeled 5′-triphosphates, 42% (42 ± 4%; mean ± S.E.; N = 7) was [^{3}H]IdUTP and 58% ± 4%; mean ± S.E.; N = 7) was [^{3}H]dTTP. Similar metabolic patterns were seen in cells exposed to IdUrd for either 1 or 6 hr (data not shown). The high levels of [^{3}H]dTTP formed emphasize the need to accurately determine the pattern of IdUrd metabolism. Interpretation of experiments measuring the incorporation of radioactivity into DNA is dependent on these data. For example, under the above conditions, for every molecule of [^{3}H]IdUTP utilized for DNA synthesis, about 1.4 molecules of [^{3}H]dTTP would be incorporated.

The fluoropyrimidines substantially altered the pattern of IdUrd metabolism. As an example, the changes in the HPLC profile produced by 100 μM FUra are shown in Fig. 5. Note the greatly diminished [^{3}H]dTTP peak and the increased levels of [^{3}H]IdUTP. The percentage of labeled triphosphates present as IdUTP increased to 92% after exposure to FUra. Under these conditions almost all of the radio-activity incorporated into the DNA was derived from [^{3}H]IdUTP. FdUrd was more effective than FUra in blocking the dehalogenation of IdUMP. After exposure to 1 μM FdUrd, [^{3}H]dTTP peaks were generally undetectable, and [^{3}H]IdUTP formation was typically increased over 200% (207 ± 24%; mean ± S.E.; N = 4).

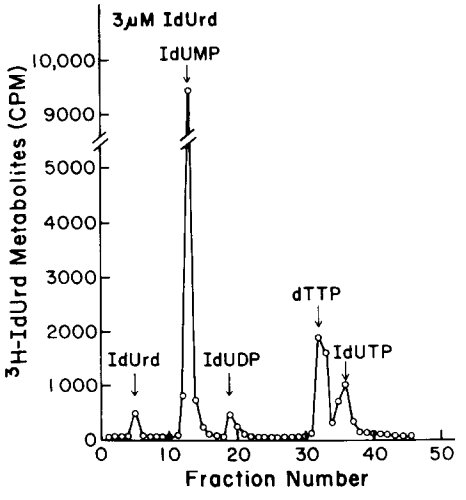

Fig. 4. HPLC profile of radiolabeled metabolites extracted from T24 cells following a 4-hr exposure to 3 μM [^{3}H]IdUrd. Analysis of the acid-soluble metabolites was conducted as described in Materials and Methods.

The overall effects of the fluoropyrimidines on the uptake of [^{3}H]IdUrd into the acid-soluble and acid-insoluble pools are summarized in Table 1. FUra produced a very modest dose-dependent increase in the uptake of IdUrd into the acid-soluble pool. IdUrd uptake was decreased modestly by 1 μM FdUrd,

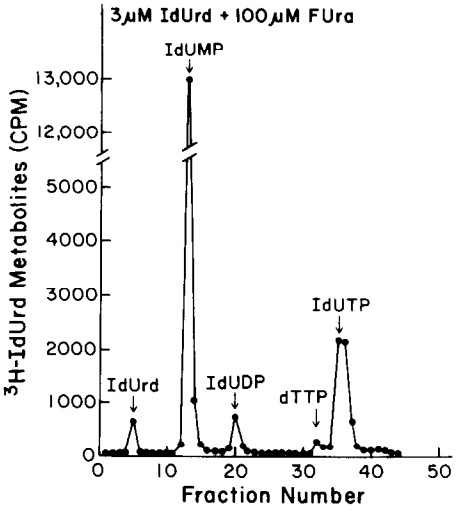

Fig. 5. HPLC profile of radiolabeled metabolites extracted from T24 cells following a 4-hr exposure to 3 μM [^{3}H]IdUrd and 100 μM FUra. Analysis of the acid-soluble metabolites was conducted as described in Materials and Methods.

Table 1. Effect of fluoropyrimidines on the uptake of [^{3}H]IdUrd*

Condition 3 μM [^{3}H]IdUrd	Acid-soluble metabolites (% control)	Acid-insoluble metabolites (% control)	Percent of labeled triphosphate present as [^{3}H]IdUTP	Incorporation into DNA derived from IdUTP† (% control)
1 μM FUra	103 + 1 (2)	103 + 9 (2)	41 + 5 (2)	103
10 μM FUra	105 ± 1 (3)	124 ± 9 (3)	69 ± 9 (4)	204
100 μM FUra	107 ± 8 (4)	159 ± 6 (4)	92 ± 215 (4)	348
1 μM FdUrd	88 ± 5 (4)	235 ± 19 (4)	99 ± 1 (4)	553

* Data are presented as the mean ± S.E. for N = 3 or 4 or as the mean ± range for N = 2. For the controls (3 μM IdUrd), the average amount of uptake into the acid-soluble fraction was 101 ± 17 pmoles/10^6 cells (N = 8). Incorporation into the acid-insoluble fraction averaged 220 pmoles/10^6 cells/4 hr in cultures of low density (1.5×10^4 cells/cm^2) and 80 pmoles/10^6 cells/4 hr in cultures of high density (4×10^4 cells/cm^2).

† These values were calculated from the average changes in the percentage of labeled triphosphate present as [^{3}H]-IdUTP. In the controls, 42 ± 4% (N = 7) was [^{3}H]IdUTP.

probably as a result of competition for phosphorylation by thymidine kinase [34]. The incorporation of IdUrd into DNA was strongly enhanced by both fluropyrimidines. FUra produced dose-dependent increases in the incorporation of radioactivity into the acid-soluble fraction and in the percentage of triphosphates present as IdUTP. Consequently, the rate of incorporation of IdUrd into DNA was calculated to increase by 348% after exposure to 100 μM FUra (Table 1). FdUrd was more effective and more potent in this regard. FdUrd (1 μM) increased the rate of incorporation of IdUrd into DNA by a striking 553%. The effect of the fluoropyrimidines on the incorporation of IdUrd into DNA was also measured directly using [^{125}I]dUrd. In this case, dehalogenation of iododeoxyuridylate resulted in the loss of the radioactive label, and the incorporation of only IdUrd is detected. As shown in Fig. 6, 1 μM FdUrd also increased the incorporation of [^{125}I]dUrd into DNA more effectively than 100 μM FUra. Between the second and fourth hours of incubation with 3 μM [^{125}I]dUrd, 10,000 cpm were incorporated into the DNA; the incorporation was increased to 32,000 cpm by 100 μM FUra and to 45,000 cpm by 1 μM FdUrd. The inhibition of IdUMP dehalogenation and the subsequent increase in intracelular [^{3}H]IdUTP produced by the fluoropyrimidines do not, however, fully explain the magnitude of the increased rates of incorporation of IdUrd into DNA. Average increases of 234 and 207% in IdUTP levels account for 67% of the stimulation produced by 100 μM FUra, but only 37% of the FdUrd effect respectively.

The overall rate of incorporation of IdUrd into DNA depends not only on IdUTP levels, but also on the size of the competing dTTP pool. Since the FUra and FdUrd are known to deplete intracellular dTTP, these pools were measured. Figure 7 shows the effects of the fluoropyrimidines alone, and in combination with IdUrd, on dTTP pools. Intracellular dTTP was decreased 22% by 3 μM IdUrd, 54% by 1 μM FdUrd, and 77% by the combination.

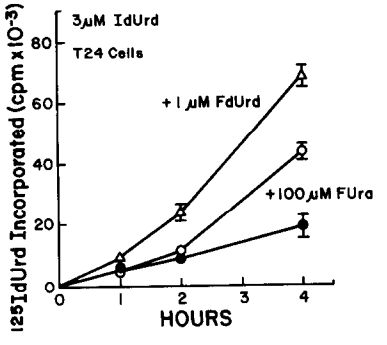

Fig. 6. Effect of the fluoropyrimidines on the incorporation of [^{125}I]dUrd into DNA. Exponentially growing T24 cells were exposed to 1 μM FdUrd (△) or 100 μM FUra (○) in the presence of 3 μM [^{125}I]dUrd (0.33 μCi/nmole). Analysis of the incorporation of IdUrd into the acid-insoluble fraction was carried out as described in Materials and Methods. The data are presented; the mean ± S.E. (N = 3) of the error was greater than the size of the symbols.

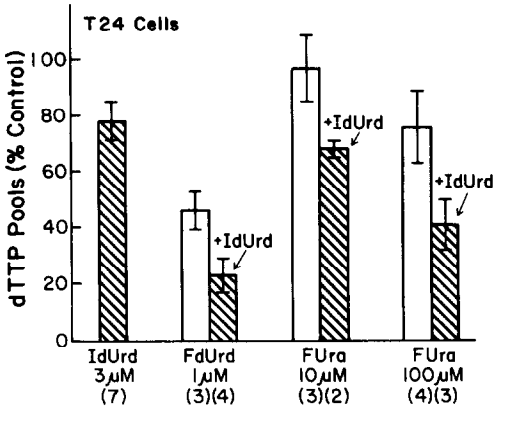

Fig. 7. Effects of IdUrd, FdUrd and FUra on dTTP pools in T24 cells. In these experiments, exponentially growing cells were exposed to 1 μM FdUrd, 10 μM FUra or 100 μM FUra for 4 hr in the presence (hatched bars) or absence of 3 μM IdUrd. Endogenous dTTP pools were determined using the DNA polymerase assay for IdUTP and dTTP and HPLC for IdUTP as described in Materials and Methods. The data are expressed as the mean ± S.E. for N ≥ 3 and as the mean + range for N = 2. The value of N is shown in the parentheses. The size of the endogenous dTTP pools varied with cell density. At higher densities (7×10^5 cells/cm^2) dTTP pools averaged about 34 pmoles/10^6 cells, whereas at lower densities (2×10^5 cells/cm^2) the pools were about 92 pmoles/10^6 cells.

The pools were reduced 3% by 10 μM FUra and 24% by 100 μM FUra. In combination with IdUrd, reductions in dTTP levels of 32 and 59% were achieved.

These effects, coupled with the increased levels of IdUTP, account for the enhanced rate of IdUrd incorporation into DNA produced by the fluoropyrimidines. On the average, the ratio of intracellular IdUTP to total dTTP was increased 6-fold, from 0.2 to 1.2, by 1 μM FdUrd. This value closely approximates the 553% increase in IdUrd incorporation noted in Table 1. Similarly, the 3.7-fold increase from 0.29 to 1.07 on the ratio of IdUTP to total dTTP produced by 100 μM FUra approximates the 348% increase presented in Table 1. In the case of FUra, inhibition of IdUMP dehalogenation is of primary importance and accounts for about two-thirds of the stimulation. Depletion of the dTTP pools appears to contribute more significantly to the FdUrd effect.

The magnitude of the increased incorporation of IdUrd into DNA produced by FdUrd would appear to account for the increased toxicity seen in this combination. The lesser stimulation elicited by FUra suggests that additional factors may be involved. Studies in other cell lines have demonstrated that a major proportion of FUra cytotoxicity may be secondary to incorporation into RNA [12, 13, 19, 20, 35], and IdUrd may increase such incorporation. Alternatively, the simultaneous incorporation of IdUrd into DNA and FUra into RNA may be of critical importance by inducing severely impaired RNA species. The potential of using this strategy to achieve synergistic cytotoxicity requires further investigation.

Of additional note, several quantitative differences, dependent on cellular density, were evident in these experiments. Cell volumes decreased from 2490 μm^3 at lower densities to 1820 μm^3 at higher cell densities. In addition, endogenous dTTP pools and IdUrd uptake were decreased in higher density cultures. Similar changes in cell volume, dTTP pools and fluoropyrimidine uptake have been reported by others [36] and are correlated with the status of cell culture growth.

The rationale for combining FUra and IdUrd appears to be validated by these experiments. Several additional factors suggest that further investigations of this combination are warranted. Although the stimulation of IdUrd incorporation into DNA by FUra was less than that seen with FdUrd, a similar increase in cell killing was evident. The molecular basis for this interaction is unknown, but may be related to the simultaneous incorporation of FUra into RNA and IdUrd into DNA. Since the cytotoxic effects of FUra are less S-phase dependent than those of FdUrd and IdUrd, a greater proportion of tumor cells may be susceptible to this combination. In addition, cells resistant to FdUrd and IdUrd by virtue of diminished thymidine kinase activity retain sensitivity to FUra [18].

Burchenal *et al.* [23] reported over 20 years ago that IdUrd potentiated the activity of FUra against murine leukemias *in vivo*, but we are unaware of further studies. Localized drug delivery of these agents, as would be possible with intravesical instillation in bladder cancer or with intrahepatic arterial infusions, may provide a good setting for evaluating the clinical utility of this combination.

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