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Dissociation of 5-Fluorouracil-induced DNA Fragmentation from Either Its Incorporation into DNA or Its Cytotoxicity in Murine T-Lymphoma (S-49) Cells[1]

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ABSTRACT

We have shown previously (W. B. Parker and P. Klubes, Cancer Res., *45:* 4249–4256, 1985) that uridine (10 μM) enhanced the cytotoxicity of 5-fluorouracil (FUra) in cultured mouse T-lymphoma (S-49) cells. Here we show, by the use of colony formation assays, that approximately 50% of the cytotoxicity of FUra plus uridine could be prevented by the simultaneous administration of thymidine (2.5 to 10 μM). In order to explain our observation of a thymidine-preventable component of the cytotoxicity of the FUra plus uridine combination, we examined the incorporation of FUra into DNA. The DNA from FUra-treated S-49 cells was purified by cesium chloride gradient centrifugation and degraded to nucleosides by DNase I and *Crotalus atrox* snake venom. 5-[^{3}H]-Fluoro-2′-deoxyuridine was not detected by high-pressure liquid chromatography in the hydrolysate of DNA from S-49 cells treated with 1.0 μM [^{3}H]FUra, 1.0 μM [^{3}H]FUra plus 10 μM uridine, or 2.4 μM [^{3}H]FUra. In contrast, 5-[^{3}H]fluoro-2′-deoxyuridine was detected in the DNA of L1210 cells treated with cytotoxic concentrations of either [^{3}H]FUra or 5-[^{3}H]fluoro-2′-deoxyuridine. Thus incorporation of FUra into the DNA of S-49 cells treated with cytotoxic concentrations of FUra was shown to be minimal or insignificant. Using alkaline elution techniques, however, fragmentation of the DNA was detected in S-49 cells treated with 1.0 μM FUra, 1.0 μM FUra plus 10 μM uridine, or 2.4 μM FUra (115-, 107-, and 159-rad equivalent single strand breaks, respectively). Most of the DNA fragmentation caused by FUra could be prevented by the inclusion of 2.5 μM thymidine with FUra during the incubation. Similar amounts of DNA fragmentation occurred with 1.0 μM FUra in either the presence or absence of 10 μM uridine. Because 1.0 μM FUra plus 10 μM uridine was more cytotoxic than 1.0 μM FUra alone, these results indicated that the enhancement of FUra cytotoxicity by uridine was not related to increased fragmentation of DNA.

INTRODUCTION

FUra[4] is used in the treatment of various tumors. The mechanisms responsible for the antitumor action of FUra have been studied since it was first synthesized in 1957 (1), and both the incorporation of FUra into RNA and the inhibition of TS by FdUMP play an important role in the toxicity of FUra (2). Recently, FUra has been shown to be incorporated into DNA (3–9). However, the importance of this latter effect to the cytotoxicity of FUra is not clear.

Uridine (10 to 50 μM) increases the cytotoxicity of FUra towards a mouse T-cell lymphoma (S-49) cell line (10, 11). We have shown that 50% of the cytotoxicity of the FUra-uridine combination was due to the enhancement by uridine of the anabolism of FUra. Furthermore, the increase in cytotoxicity of the combination was associated with increased FUra incorporation into RNA as well as the production of FUra analogues of uridine diphosphohexoses. However, the cytotoxicity of FUra plus uridine was not related to the inhibition of DNA synthesis due to FdUMP binding to TS (11). In this paper we show that a portion of the cytotoxicity of FUra or FUra plus uridine can be prevented by simultaneous treatment with dThd. In an attempt to explain the DNA-directed cytotoxicity of FUra or FUra plus uridine we examined both the incorporation of FUra into DNA and the fragmentation of DNA by FUra. We found that FUra was not incorporated into the DNA of S-49 cells at concentrations which resulted in 90% cell kill. Furthermore, DNA fragmentation was observed after FUra treatment, but there was no correlation between DNA fragmentation and cell kill. A preliminary report of our investigation has already appeared (12).

MATERIALS AND METHODS

Chemicals and Supplies. FUra was obtained from the Drug Synthesis Branch, National Cancer Institute (Bethesda, MD). FdUrd was obtained from Hoffmann La Roche (Nutley, NJ). [6-^{3}H]FUra (20 Ci/mmol) and [6-^{3}H]FdUrd were obtained from Moravek Biochemicals (City of Industry, CA). [*methyl*-^{3}H]dThd (80 Ci/mmol) was obtained from ICN Pharmaceuticals (Irvine, CA). Proteinase K was obtained from Sigma Chemical Company (St. Louis, MO) or American Scientific Products (Bethesda, MD). *Crotalus atrox* snake venom was obtained from Sigma Chemical Company. DNase I was purchased from Worthington Biochemical Corp. (Freehold, NJ). All other chemicals were standard analytical grade unless otherwise noted.

Cell Culture Conditions. S-49 cells were obtained from Dr. Jonathan Maybaum, University of California School of Medicine, San Francisco, CA. The cells were grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 2.2 g of glucose/liter and 10% heat-inactivated horse serum (GIBCO), pH 7.4. The horse serum was inactivated by heating in a water bath at 56°C for 30 min. The cells were grown in a water-saturated 5% CO_2-95% air incubator at 37°C. Stock S-49 cells were stored in liquid nitrogen in a solution of 10% dimethyl sulfoxide in otherwise normal growth medium. Fresh cells were thawed every 9 mo during the course of the study to maintain genetic stability in the culture. The cells were assayed by Biofluids, Inc. (Rockville, MD) and found to be free from *Mycoplasma* contamination.

Cell Viability. Exponentially growing S-49 cells (1×10^5 cells/ml) were added to medium containing the desired concentration of drug(s). After 24 h of incubation, S-49 cells were cloned in a 0.5% agar solution which contained 20% heat-inactivated serum and 40% conditioned medium (13). Conditioned medium was collected from exponentially growing S-49 cell suspensions by centrifugation at $250 \times g$ for 5 min followed by filtration through a 0.22-μm filter. After 12 to 14 days, colonies (greater than 50 cells) were visible to the naked eye and could be counted. The cloning efficiency of S-49 cells was about 50%.

Isolation of DNA by Cesium Chloride Gradient Centrifugation. S-49 and L1210 cells, in exponential growth, were incubated with [^{3}H]FUra

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[4] The abbreviations used are: FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine monophosphate; dThd, thymidine; TS, thymidylate synthetase; HPLC, high-pressure liquid chromatography; UDGase, uracil-DNA glycosylase.

(20 Ci/mmol) or [^{3}H]FdUrd (20 Ci/mmol) for 12 h. The DNA was then isolated from the cells by treatment with NaOH followed by cesium chloride gradient centrifugation as described by Kufe and Egan (14). The DNA was degraded to deoxynucleosides by DNase I and *C. atrox* snake venom, and the deoxynucleosides were separated using a Waters C-18, 10-mm radial compression HPLC column (Milford, MA). The mobile phase was 10 mM sodium acetate, pH 4.5, with a flow rate of 2.0 ml/min.

Alkaline Elution Assays. The alkaline elution method as described by Kohn (15) was used to detect the presence of single strand breaks in DNA. S-49 cells to be used in these experiments (experimental cells) were labeled for 12 h with [^{3}H]dThd at 0.1 μCi/ml (80 Ci/mmol dThd) during treatment with FUra or the FUra plus uridine combination. Preliminary experiments demonstrated that DNA breaks were observed only when dThd labeling took place during drug exposure. During the labeling procedure, the final concentration of dThd in the medium, unless otherwise indicated, was 1.25 nM. This concentration is well below that which protects cells from either FUra or FUra plus uridine toxicity. Internal standard cells were labeled with [^{14}C]dThd (0.01 μCi/ml, 1 μM dThd) and received 300 rads of X-irradiation on ice prior to beginning the alkaline elution procedure, as described by Kohn (15).

RESULTS

dThd Reversal of the Cytotoxicity of Either FUra or FUra plus Uridine. Treatment of S-49 cells for 24 h with 1.0 μM FUra did not decrease cell viability, whereas treatment with either 1.0 μM FUra plus 10 μM uridine or 2.4 μM FUra killed approximately 90% of the cells (Fig. 1). The addition of dThd (2.5 to 10 μM) to cells treated with 1.0 μM FUra plus 10 μM uridine prevented approximately 50% of the cytotoxicity seen with this combination. dThd (10 μM) did not inhibit the incorporation of [^{3}H]uridine into RNA (data not shown), indicating that dThd did not compete with uridine for uptake. Furthermore, dThd could only prevent 25% of the toxicity of 2.4 μM FUra. These results indicated that only part of the cytotoxicity of both FUra and FUra plus uridine was DNA directed.

Incorporation of FUra into the DNA of S-49 and L1210 Cells. Cesium chloride gradient centrifugation was used to separate [^{3}H]FUra-containing DNA from [^{3}H]FUra-containing RNA. S-49 cells were incubated for 12 h with 1.0 μM [^{3}H]FUra, 2.4 μM [^{3}H]FUra, or 1.0 μM [^{3}H]FUra plus 10 μM uridine. The incubation time for this experiment was 12 h, because after 12 h, the same amount of DNA per cell was recovered from either untreated cells or cells treated with FUra. In contrast, after 24 h of incubation the recovery of DNA from cells treated with either 1.0 μM FUra plus 10 μM uridine or 2.4 μM FUra was less than the recovery from cells treated with either 1.0 μM FUra alone or no drug. The effect of 1.0 μM FUra, 1.0 μM FUra plus 10 μM uridine, or 2.4 μM FUra on S-49 cell viability after 6 or 12 h of incubation was qualitatively similar to that seen after 24 h of incubation (data not shown).

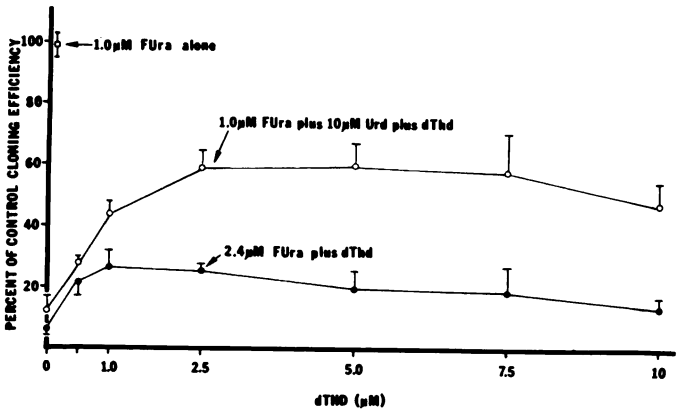

Fig. 1. Effect of dThd on the cloning efficiency of S-49 cells treated with either FUra or FUra plus uridine (*Urd*). S-49 cells were incubated with either 1.0 μM FUra plus 10 μM uridine or 2.4 μM FUra in the presence or absence of varying concentrations of dThd (0.5 to 10 μM). After 24 h, cells from each treatment were collected and cloned in agar containing the same concentration of dThd as during the 24-h incubation. After 2 wk, the colonies were counted, and the cloning efficiency was determined for each treatment. The cloning efficiency of control cultures was about 50%. *Points*, mean of 3 experiments; *bars*, SE.

DNA labeled with [^{3}H]dThd bands at a density of 1.70 g/ml in the gradient which is formed during centrifugation. Because the density of RNA in cesium chloride is greater than 1.78 g/ml (16), any RNA that was not degraded during incubation with NaOH should be in the pellet obtained after centrifugation. Furthermore, the hybridization of RNA with DNA was prevented by incubation of the DNA samples in a 50% formamide-50% Tris-EDTA solution at 80°C for 30 min prior to cesium chloride centrifugation (5).

No [^{3}H]FdUrd was detected in the DNA of S-49 cells after treatment with 1.0 μM [^{3}H]FUra, 2.4 μM [^{3}H]FUra, or 1.0 μM [^{3}H]FUra plus 10 μM uridine when the DNA from these treatments was collected from the cesium chloride gradients, dialyzed, and degraded to deoxynucleosides by DNase I and *C. atrox* snake venom (Fig. 2). In contrast, when cells were incubated with [^{3}H]dThd, greater than 80% of the radiolabel found in the DNA of a cesium chloride gradient could be recovered from the dThd fraction after HPLC analysis (data not shown). This indicated that most of the radioactivity from [^{3}H]dThd in DNA was retained during its isolation, degradation, and analysis of HPLC. Furthermore, only 8 nmol of [^{3}H]FdUrd per mol of DNA nucleotide were found in the DNA of S-49 cells treated with 1.5 nM [^{3}H]FdUrd for 12 h (data not shown). Incubation of S-49 cells with 1.5 nM FdUrd for 24 h killed 70% of the cells (data not shown).

Using [^{3}H]FUra with a specific activity of 20 Ci/mmol, the technique shown in Fig. 2 should be sensitive enough to detect the incorporation of 17 nmol of FdUMP per mol of DNA nucleotide. Because both FUra or FdUrd have been shown to be incorporated into the DNA of various tumors (3–9), it was of concern that the inability to detect the incorporation of

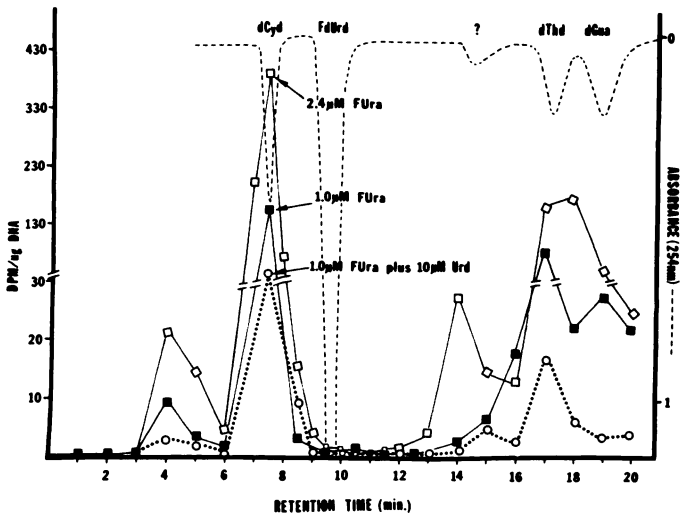

Fig. 2. HPLC of DNA hydrolysate obtained from S-49 cells treated with either [^{3}H]FUra or [^{3}H]FUra plus uridine. S-49 cells (8×10^5 cells/ml) were incubated with 1.0 μM [^{3}H]FUra (20 Ci/mmol), 1.0 μM [^{3}H]FUra (20 Ci/mmol) plus 10 μM uridine, or 2.4 μM [^{3}H]FUra (20 Ci/mmol) for 12 h. The DNA from each treatment was collected from cesium chloride gradients, dialyzed, and degraded to deoxynucleosides by DNase I and *C. atrox* snake venom. The deoxynucleosides were then separated using HPLC. The concentration of DNA was determined by its absorbance at 260 nm. This technique was sensitive enough to detect the incorporation of 17 nmol of FdUrd per mol of DNA nucleotide. *dCyd*, deoxycytidine; *Urd*, uridine; *dGua*, deoxyguanosine.

[^{3}H]FUra into DNA in our system could be due to a technical problem rather than some characteristic of S-49 cells. Therefore, using our method, we measured the incorporation of both [^{3}H]FUra and [^{3}H]FdUrd into the DNA of L1210 cells. Incubation of L1210 cells with 1.0 μM [^{3}H]FdUrd (18 Ci/mmol) for 12 h resulted in the incorporation of 2700 nmol of [^{3}H]FdUMP per mol of DNA nucleotide (or 0.67 pmol per 10^7 cells; data not shown), which was comparable to that reported previously (3, 4). Treatment of L1210 cells with 1.0 μM FdUrd for 6 h kills 65% of the cells (4). L1210 cells treated with 20 μM [^{3}H]-FUra (0.5 Ci/mmol) for 12 h resulted in the incorporation of 340 nmol of FdUMP per mol of DNA nucleotide (or 0.44 pmol per 10^7 cells; data not shown). Incubation with 20 μM FUra for 24 h was 20 times the dose of FUra required to kill 90% of L1210 cells (data not shown). These results using [^{3}H]FUra or [^{3}H]FdUrd with L1210 cells indicated that the technique would have allowed us to detect the incorporation of [^{3}H]FUra or [^{3}H]FdUrd into the DNA of S-49 cells, if it were present.

Most of the radioactivity found in the DNA from treatment of S-49 cells with [^{3}H]FUra was associated with the nucleosides, deoxycytidine, dThd, deoxyguanosine, and deoxyadenosine (Fig. 2). This finding was unexpected, because the stock [^{3}H]-FUra was found to be 98% pure by HPLC. Two % of the radioactivity eluted with the solvent front and was probably either degradation products from the radiolysis of [^{3}H]FUra or other impurities. Since large amounts of label were used per treatment group (at least 0.4 mCi/determination), these small amounts of impurities, presumably, containing ^{3}H-labeled carbon fragments, might account for the labeling of the deoxynucleosides of DNA. Sawyer *et al.* (17) also observed the presence of [^{3}H]dThd in the DNA of mouse bone marrow cells treated with [^{3}H]FUra. For investigators who are studying the incorporation of FUra into DNA, these results indicated that it is imperative to verify that the ^{3}H label found in DNA is [^{3}H]-FdUrd.

Production of DNA Fragmentation by FUra or FUra plus Uridine. It was possible that the absence of [^{3}H]FUra in the DNA of S-49 cells was due to its efficient removal by UDGase (18, 19). The removal of [^{3}H]FUra from the DNA by UDGase could cause the fragmentation of DNA and result in cytotoxicity. To determine whether FUra or the FUra-uridine combination caused DNA fragmentation, S-49 cells treated with drug(s) were analyzed using alkaline elution methods (15). After 12 h of incubation with drugs and label, the cells were resuspended in fresh medium for 2 h to chase the ^{3}H label into the longer pieces of DNA. Treatment of S-49 cells with 1.0 μM FUra caused 115-rad equivalent single strand breaks in the DNA (Table 1). The addition of 10 μM uridine to cells treated with 1.0 μM FUra did not affect the amount of fragmentation found in the DNA (107-rad equivalent). Interestingly, treatment of S-49 cells with 1.0 μM FUra for 24 h did not kill any cells, whereas treatment with the FUra-uridine combination killed 90% of the cells (Fig. 1). Treatment with 2.4 μM FUra for 24 h caused more DNA damage (159-rad equivalent) than did 1.0 μM FUra or 1.0 μM FUra plus 10 μM uridine. Qualitatively similar results were obtained when there was no chase period before analysis by alkaline elution or when the chase period was lengthened to 6 h (data not shown). Very little DNA fragmentation was detected when 2.5 μM dThd was added to the above treatment period and the 2-h chase (Table 1). This concentration of dThd maximally protected against the DNA-directed component of the toxicity of either FUra plus uridine or 2.4 μM FUra (Fig. 1).

Table 1 *Effect of dThd on the fragmentation of DNA from cells treated with either FUra or FUra plus uridine*

Treatment[a]	Rad equivalent single strand breaks
10 μM uridine	5[b]
1 μM FUra	115
1 μM FUra plus 2.5 μM dThd	20
1 μM FUra plus 10 μM uridine	107
1 μM FUra plus 10 μM uridine plus 2.5 μM dThd	22
2.4 μM FUra	159
2.4 μM FUra plus 2.5 μM dThd	37

[a] Cells were treated with the indicated concentration of uridine, FUra, or dThd for 12 h as described in "Materials and Methods." After drug exposure, the cells were prepared for alkaline elution analysis, and the number of rad equivalent single strand breaks was determined as described previously (15).
[b] The results represent the means of 2 or 3 separate experiments. The variation about the mean was less than 20%.

DISCUSSION

Our results indicate that the DNA-directed cytotoxicity of the FUra-uridine combination in S-49 cells was not related to either the incorporation of FUra into DNA or the fragmentation of DNA. This combination may be relevant to the treatment of patients with FUra, because uridine levels in human plasma are 2 to 9 μM (20). It is possible that the fragmentation of the DNA by FUra in our experiments could be due to the accumulation of [^{3}H]dThd-labeled Okazaki fragments (21), resulting from an inhibition of DNA synthesis after treatment with FUra. This explanation is unlikely for several reasons. If [^{3}H]dThd-labeled Okazaki fragments were present, then a pronounced biphasic elution pattern would be expected with the Okazaki fragments eluting with a shorter half-life than the larger DNA fragments (22). In this study, the elution of DNA from the filters was almost linear. Furthermore, incubation of S-49 cells with 1.0 μM FUra or 1.0 μM FUra plus 10 μM uridine did not result in significant inhibition of DNA synthesis (11). Okazaki fragments would not be expected to accumulate in cells with near control rates of DNA synthesis. Recently, Lonn and Lonn (23) have shown in human colon adenocarcinoma cells (WiDr) that the production of lesions in the DNA by FUra did not result in cytotoxicity. Furthermore, when methotrexate preceded FUra in these cells the FUra-induced DNA lesions were prevented, while cytotoxicity was increased. These results suggest that fragmentation of the DNA by FUra does not result in cytotoxicity. It is possible that the lesions observed here are transient and are eventually repaired.

S-49 cells appear to be unique as an *in vitro* model system in that insignificant incorporation of FdUMP into the DNA of cells treated with cytotoxic concentrations of FUra or FdUrd was found (Fig. 2). Danenberg *et al.* (24) found only 6.1 nmol of FdUMP per mol of DNA phosphate in L1210 cells treated with 1.0 μM FdUrd for 4 h. They considered this amount to be too small to make a major contribution to the cytotoxicity of FdUrd in L1210 cells. [^{3}H]FdUMP in the DNA hydrolysate of L1210 cells after treatment with [^{3}H]FdUrd was detected by its ability to form a tightly bound complex with TS (24). Using HPLC to identify [^{3}H]FdUMP in the DNA of L1210 cells treated with [^{3}H]FdUrd, the results in this paper and from other investigators (3, 4) have indicated that incorporation of FdUMP into the DNA of L1210 cells treated with FdUrd does occur. Furthermore, we have also shown that L1210 cells treated with FUra incorporated FdUMP into the DNA. In all other cell lines studied including MCF-7 (5), human lymphoblast 8866 (7), HL-60 (8), HeLa (9), and human colon adenocarcinoma (WiDr) (25), FUra and FdUrd were shown to be incorporated

into DNA. Schuetz *et al.* (6) have shown a correlation between the incorporation of FUra into the DNA of bone marrow cells and cytotoxicity.

There are a number of possible mechanisms which could account for the absence of FdUMP from the DNA of S-49 cells treated with FUra. Before the discovery that cells could incorporate both FUra and uracil into their DNA, it was believed that the action of deoxyuridine triphosphate nucleotidohydrolase and UDGase was responsible for the absence of FUra and uracil from the DNA (18, 19). It is now hypothesized that the removal of FUra from the DNA by UDGase could result in DNA strand breaks and cytotoxicity (9). If FUra treatment of cells increased the incorporation of dUMP into DNA, the fragmentation of DNA observed here in drug-treated cells could be due to the rapid removal of uracil residues by UDGase. The fragmentation of DNA observed in S-49 cells treated with either FUra or FUra plus uridine is consistent with these possibilities. However, further work is necessary to distinguish which of these possible mechanisms may account for DNA fragmentation in FUra-treated cells.

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