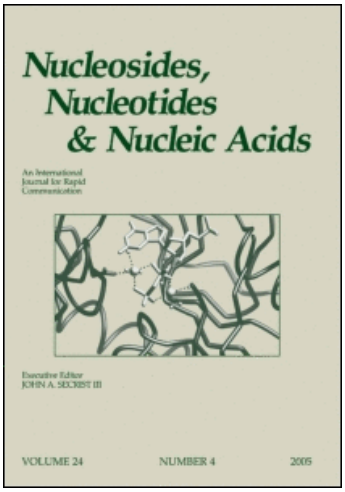

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Enhanced DNA-Directed Effects of FdUMP[10] Compared to 5FU†

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ABSTRACT

FdUMP[N] molecules and conjugates are much more effective at inhibiting the proliferation of human tumor cells than is the widely used anticancer drug 5-fluorouracil (5FU). We have evaluated the inhibition of thymidylate synthase (TS), the extent of DNA damage, cell cycle arrest, and the induction of apoptosis by FdUMP[10] and 5FU in the human colorectal cancer cell line HT29. The magnitude and duration of TS inhibition following exposure of HT29 cells to FdUMP[10] at 1×10^{-8} M was greater than that which occurred following exposure of these cells to 5FU at 1×10^{-6} M. FdUMP[10] exposure also resulted in much more extensive DNA damage to HT29 cells than occurred following exposure to 100-fold higher concentrations of 5FU. Although exposure of HT29 cells to both drugs resulted in S-phase arrest, more complete accumulation of cells in S-phase was achieved following FdUMP[10] exposure at much lower drug concentrations. FdUMP[10] was also much more effective at inducing apoptosis in HT29 cells than was 5FU. The results are

†In honor and celebration of the 70th birthday of Professor Leroy B. Townsend.

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consistent with FdUMP[10] being much more efficient that 5FU at inducing DNA damage that results in apoptotic cell death in colon cancer cells.

Key Words: Fluoropyrimidine; 5FU; Cancer chemotherapy; Thymidylate synthase.

INTRODUCTION

Fluoropyrimidine Chemotherapy

Fluoropyrimidines (FPs) are among the most widely used anticancer drugs for the treatment of solid tumors. The most widely used FP for cancer chemotherapy is 5-fluorouracil (5FU).[1] 5FU is not active as the nucleobase, but rather is metabolized to deoxyribonucleotide and ribonucleotide forms that interfere in critical cellular processes.[2] The principal 5FU metabolite that is responsible for anticancer activity is FdUMP (Figure 1). FdUMP forms a stable ternary complex together with a reduced folate co-factor that inhibits the cellular enzyme thymidylate synthase (TS).[3] TS is the sole enzyme responsible for the de novo synthesis of thymidylate (dTMP). Inhibition of TS results in thymidylate depletion and an imbalance in cellular deoxyribonucleotide pools. Ultimately, cells exposed to 5FU undergo ''thymineless cell death'' as a consequence of TS-inhibition.[4,5] Resistance to 5FU arises mainly as a consequence of TS overexpression, consistent with TS being the clinically relevant target of FP chemotherapy.[6]

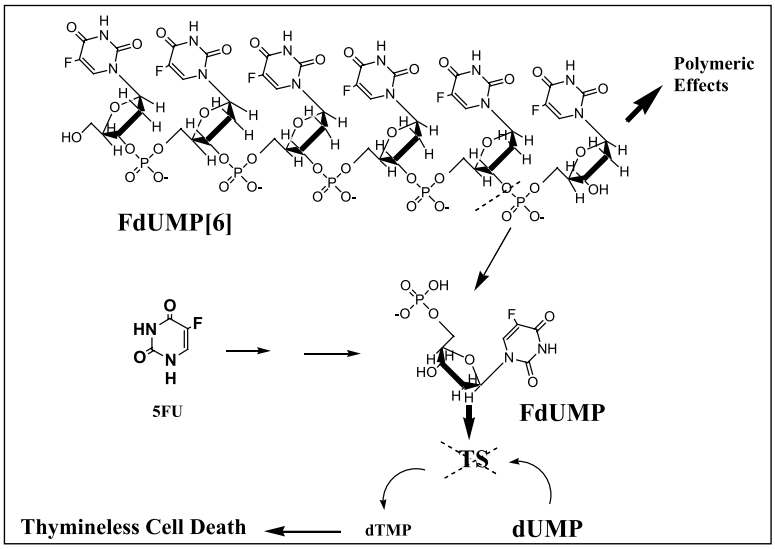

Figure 1. Structure of FdUMP[6], the FdUMP[N] of length 6. FdUMP[N] are much more efficiently metabolized to the TS-inhibitory metabolite FdUMP than is the widely-used fluoropyrimidine 5FU. Exposure of cancer cells to FdUMP[N] results in thymineless cell death. FdUMP[N] are also cytotoxic through polymeric effects, the origin of which is still being elucidated.

The FPs currently available for cancer chemotherapy are rarely curative and have serious dose-limiting toxicities associated with their administration. 5FU is very inefficiently converted to FdUMP.[2] The majority of the administered dose of 5FU is rapidly converted to such toxic metabolites as 2-fluoro-β-alanine (FBAL) and FUTP. FBAL contributes to the cardio- and neurotoxicities[7] associated with 5FU chemotherapy while FUTP has been implicated in the GI-tract toxicity that accompanies 5FU chemotherapy.[8] 5-fluoro-2′-deoxyuridine (FdU) has been evaluated as an alternative to 5FU for FP chemotherapy. In principle, the deoxyribonucleoside should much more efficiently target the TS/DNA-locus of FP chemotherapy relative to the nucleobase.

Although in cell culture studies a substantial improvement in cytotoxic activity for FdU relative to 5FU has been observed, little benefit is derived clinically from the use of FdU relative to 5FU.[9] The lack of a clinical benefit for FdU relative to 5FU stems from the rapid degradation of FdU in vivo to release the nucleobase, 5FU.[10] Thus, FdU serves mainly as a pro-drug for 5FU in vivo. Recently, the 5FU pro-drug capecitabine (xeloda) has become widely used clinically. Capecitabine (xeloda) is orally bioavailable and has shown reduced toxicity in vivo relative to 5FU.[11] Overall response rates to capecitabine are not, however, substantially better than 5FU.[12]

FdUMP[N] compounds can, potentially, overcome many of the drawbacks associated with the use of alternative FPs for cancer chemotherapy. FdUMP[N] can serve as a pro-drug for the direct release of FdUMP.[13,14] Thus, FdUMP[N] can selectively intervene in the TS/DNA-locus of FP activity. FdUMP[10], the prototype FdUMP[N], has been evaluated in both cell culture[15–17] and in animal models of human cancer.[18] FdUMP[10] was 338-fold more potent than 5FU, on average, in the NCI 60 cell-line screen. Importantly, FdUMP[10] was efficacious towards human colorectal cancer xenografts in nude mice when administered intravenously indicating it has pharmacological properties consistent with its use as a drug.[18] FdUMP[10] is also exceptionally well-tolerated in vivo.[15]

The mechanism by which exposure of cells to FdUMP[N] compounds results in cell death requires further clarification. FdUMP[N] can be degraded extracellularly to release FdUMP which, in turn, can be dephosphorylated to FdU. FdUMP[N] may also be taken into cells in polymeric form and serve as a pro-drug of FdUMP, as well as potentially eliciting polymeric effects. In the present study, we investigate the intracellular effects that occur to HT29 human colorectal cancer (CRC) cells following exposure to 5FU and FdUMP. HT29 is a well-recognized model of human CRC.[19] HT29 cells were somewhat less sensitive than average to FdUMP[10], but more sensitive than average to 5FU in the NCI 60 cell line screen. Thus, HT29 cells provide a rigorous system for investigating the effects of FdUMP[10]. The results confirm that FdUMP[10] intervenes specifically in the TS/DNA-locus of FP activity. Specifically, TS is inhibited in HT29 cells to a greater extent and for a longer duration following exposure to FdUMP[10] compared to a 100-fold greater concentration of 5FU. Substantially greater DNA damage is also observed in HT29 cells exposed to FdUMP[10] compared to 100-fold higher concentrations of 5FU.

EXPERIMENTAL METHODS

Synthesis of FdUMP[10]. The compound 5′-O-(4,4′-dimethoxytrityl)-5-fluoro-2′-deoxyuridine-3′-O-(cyanoethyl-N,N-diisopropyl phosphoramidite) was prepared in two

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steps by a method similar to the one described previously.[20] Both reactions were carried out in flame-dried glassware and under an argon atmosphere. Briefly, 5′-O-(4,4′-dimethoxytrityl)-5-fluoro-2′-deoxyuridine was prepared by stirring a solution of 5-fluoro-2′-deoxyuridine (ChemImpex) in dry pyridine with 4,4′-dimethoxytrityl chloride (Aldrich) overnight at room temperature. The mixture was washed with diluted with methylene chloride, extracted with saturated sodium bicarbonate and sodium chloride solutions, and the solvent was evaporated and chromatographed with 98% methylene chloride-2% methanol to elute the impurities and then with 90% methylene chloride-10% methanol to elute the product, which was dried under vacuum. The product was then reacted with cyanoethyl-N,N-diisopropyl phosphonamidic chloride and diisopropyl ethyl amine in dry tetrahydrofuran at room temperature for three hours. The solution was diluted with ethyl acetate, washed with saturated sodium chloride solution, the solvent was evaporated, and the product was purified by column chromatography using 80% ethyl acetate-20% hexane containing a trace of triethylamine. Controlled Pore Glass (CPG) was derivatized with 5′-O-(4,4′-dimethoxytrityl)-5-fluoro-2′-deoxyuridine using the procedure reported by Damha, et al.[21] The oligodeoxynucleotide, FdUMP[10], was prepared using an Applied Biosystems 394 automated DNA synthesizer on a 10-micromolar scale using a 0.1 M solution of the phosphoramidite in dry acetonitrile. The derivatized CPG (0.31 g) was placed in an empty 10-micromolar synthesis column.

TS catalytic activity. HT29 cells were plated at a density of 3×10^6 cells in 100 mm^2 plates. The cells were grown overnight in RPMI 1640 medium with 10% dFBS. Either 5FU (4×10^{-6} M) or FdUMP[10] (4×10^{-8} M) was then added to each Petri dish, and the cells were incubated for 24, 48, or 72 h. The cells were then collected from each plate, sonicated in 300 μL of 25 mM Tris HCl, pH 7.4, and then centrifuged at 10,000 × g for 30 min. The supernatants were then assayed for protein content and TS catalytic activity. TS catalytic assays were performed in a final volume of 200 μL containing 75 μM 5,10 methylene tetrahydrofolate, 10 μM dUMP, 100,000–150,000 dpm of ^{3}H-dUMP, 100 μM 2-mercaptoethanol, 25 mM KH_2PO_4, pH 7.4. Aliquots of the cytosolic extract (400 μg) were added to the reaction buffer. The reaction mixtures were then incubated for 30 min at 37°C. The reactions were stopped by the addition of 100 μL of 20% TCA. 200 μL of charcoal solution (10 g activated charcoal, 0.25 g BSA, 0.25 g Dextran, in 100 μL of water) were then added and the solution was maintained at room temperature for 10 min and then centrifuged for 30 min at 10,000 × g. 200 μL of the supernatant were then added. The results are presented by % of control using the formula:

$$(\text{sample}_{\text{dpm}} - \text{blank}_{\text{dpm}})/(\text{control}_{\text{dpm}} - \text{blank}_{\text{dpm}}) \times 100\%$$

Flow cytometry. For the flow cytometry studies, 2×10^6 HT 29 cells were plated in 100 mm^2 petri dishes in media identical to that described above for the cell culture studies. These cells were maintained at 37°C and incubated overnight in a 95%air/5% CO_2 atmosphere. Stock solutions of 5FU and FdUMP[10] were then added to the media in each Petri dish to final concentrations of 1.0×10^{-6} and 1.0×10^{-7} M for 5FU and 1.0×10^{-8} and 1.0×10^{-9} M for FdUMP[10]. Cells were incubated in the presence of drug for 48 h. Following drug treatment, cells were suspended by trypsin-EDTA treatment, and then collected by centrifugation. Pellets of frozen cells

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were stored at − 20°C until analysis by flow cytometry. One hour prior to analyzing the drug-treated cells by flow cytometry, 1×10^6 cells were re-suspended in 0.5 mL of an ice-cold solution consisting of 50 mg/mL propidium iodide in 0.5 mM citrate buffer, pH 7.4, with 0.6% NP40 and 37 μg/mL Rnase A. Cells were maintained in this solution, on ice, for 15 minutes. The samples were then analyzed for DNA content using a FACScan (Becton Dickinson). The flow cytometry data were analyzed using CELL Quest and Mod Fit LT 1.0 software (Verity Software House, Inc.). Cell debris and fixation artifacts were gated out.

COMET assays. COMET assays were performed using the protocol supplied by Trevigen, Inc. (Gaithersburg, MD). HT29 cells (1×10^6) were grown overnight using 60 mm^2 plates. Drugs were added for 24 h after which the medium was removed, the cells were washed twice with ice-cold PBS, and the volume was adjusted to obtain cell densities of 2×10^5 cells/mL in cold PBS. The cells were combined with low-melting agarose (at 42°C) at a ratio of 1:10 (v/v), and 50 μL were immediately transferred onto a Cometslide™. The slides were placed flat at 4°C in the dark for 10 min, and then immersed in a pre-chilled lysis solution at 4°C for 30 min. Excess buffer was removed from the slides which were then immersed in freshly prepared alkali solution (pH > 13; 0.6 g NaOH, 250 μL of 200 mM EDTA, pH 10.0 in 49.75 mL of de-ionized water) for 60 min at room temperature in the dark. Slides were removed from the alkali solution and washed by immersion two times in TBE buffer for 5 min. Slides were transferred to a horizontal electrophoresis apparatus and electrophoresed (at 1 V/cm) for 10 min. Following electrophoresis, slides were placed in ethanol for 5 min and air-dried. Slides were then spotted with 50 μL of diluted SYBR Green (one drop anti-fade) onto each circle of dry agarose, and viewed by epifluorescence microscopy.

Time-lapse video microscopy. HT 29 cells were grown in RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum. 1×10^6 cells were plated in 25 cm^2 flasks overnight. Drugs (FdUMP[10] or 5FU) were then added to the medium and the flasks were then placed on a microscope stage fitted with a 5% CO_2, 37°C incubation chamber. Cell proliferation and the induction of apoptosis were monitored for 72 hours by video microscopy.

RESULTS AND DISCUSSSION

The GI_{50} values from the NCI 60 cell line screen indicate that FdUMP[10] differs mechanistically from 5FU, and also FdU. The HT29 human colon tumor cell line is a well-recognized model system for investigating the effects of novel anticancer drugs for the treatment of colon cancer. In particular, it has been used to study the effects of 5FU,[22,23] and folate-based TS inhibitors such as tomudex.[24] The HT29 cell line has a mutant p53 gene (mp53) and has been shown to undergo S-phase arrest without induction of acute apoptosis following exposure to 5FU/LV.[25] FdUMP[10] displayed equal activity towards HT29 cells in the presence and absence of leucovorin (LV). Lipofectin, a mediator of ODN uptake, also did not enhance the antiproliferative activity of FdUMP[10] towards HT29 cells. The effects of 5FU towards HT29 cells could be reversed by dT, implicating TS-inhibition and DNA damage as being responsible for the prolonged S-phase arrest in this mp53 cell line.

While FdUMP[10] was considerably more growth inhibitory overall in the NCI 60 cell line screen, the HT29 cell line was considerably less sensitive than average to FdUMP[10] ($GI_{50} = 5.01 \times 10^{-7}$ M; Avg. $GI_{50} = 7.1 \times 10^{-8}$ M). Conversely, the HT29 cell line was somewhat more sensitive than average to 5FU ($GI_{50} = 6.3 \times 10^{-6}$ M; Avg. $GI_{50} = 2.4 \times 10^{-5}$ M). Our studies evaluated TS-inhibition, cell-cycle arrest, DNA damage, and induction of apoptosis following exposure of HT29 cells to FdUMP[10], relative to that which occurs following exposure to 5FU. The results establish that the same cellular processes are perturbed by these two different types of FPs, although to different extents and over different time periods.

TS Inhibition in HT29 Cells

TS is the principal cellular target of FP chemotherapy,[26] and exposure of colon cancer cells to pharmacologically relevant concentrations of a more ideal FP (e.g., FdUMP[10]) would be expected to inhibit TS more extensively, and for longer time periods, than exposure to 5FU. Exposure of colon cancer cells (e.g. HT29) to 5FU is known to inhibit TS catalysis.[23] TS catalytic activity was measured in HT29 cells following exposure to either FdUMP[10] (at 4×10^{-8} M) or 5FU (at 4×10^{-6} M) for 24, 48, or 72 h. TS activity was measured by 3H-release from [5–3H]dUMP using the assay previously described.[27] The results are presented in Table 1.

TS catalytic activity was reduced to a greater extent and for a longer duration in HT29 cells following exposure to FdUMP[10] at 4×10^{-8} M than following exposure to 5FU at 4×10^{-6} M at all time points. Since TS expression is autoregulated via binding of TS protein to TS mRNA,[28,29] the time course of TS inhibition following exposure of HT29 cells to the two FPs is particularly important for assessing the relative activities of FdUMP[10] and 5FU. FdUMP[10] is expected to serve as a pro-drug for FdUMP (and FdU) over a sustained period of time while 5FU is expected to rapidly enter HT29 cells and undergo metabolism to yield numerous metabolites (Figure 1). TS inhibition is expected to reduce cellular dTTP, and enlarge dUMP/dUTP pools.[23] It is the imbalance in the ratio of dUTP/dTTP nucleotide pools that is believed to be responsible for initiating processes that result in DNA damage that ultimately is lethal to colon cancer cells. As described below, this more complete inhibition of TS following exposure of HT29 cells to FdUMP[10] results in substantially greater DNA damage.

Cell-Cycle Distribution of HT29 Cells

Exposure of various colon cancer cell lines to 5FU, or other FPs, results in extensive cell-cycle arrest. Cell-cycle arrest may occur in S-phase or at the G2/M

Table 1. TS catalytic activity in HT29 cells.[a,b]

	24 h	48 h	72 h
5FU (4×10^{-6} M)	5.3%	11.5%	23.4%
FdUMP[10](4×10^{-8} M)	0.01%	2.2%	14.2%

[a]Percentages of TS catalytic activity relative to untreated cells.
[b]400 micrograms of protein were used in all assays.

Table 2. Cell-cycle distribution of HT29 cells following exposure to 5FU and FdUMP[10].

Drug	Concentration	G0–G1	G2-M	S-phase
Control		72%	6%	22%
5FU	1×10^{-6} M	46%	6%	48%
5FU	1×10^{-7} M	69%	7%	24%
FdUMP[10]	1×10^{-9} M	11%	27%	63%
FdUMP[10]	1×10^{-10} M	58%	10%	32%

checkpoint depending on the cell line, the type of FP, and the conditions of exposure.[30] The type of cell-cycle arrest induced by FPs in cell lines is informative concerning the mechanism of cytotoxicity. HT29 cells have mp53, and are sensitive to FPs by a TS/DNA-directed mechanism.[23] Due to the expression of mp53, HT29 cells may incur considerable DNA damage without initiating an apoptotic cascade, although delayed apoptosis occurs in HT29 cells following exposure to 5FU, even in the absence of wtp53 expression.[22] We used flow cytometry to evaluate the effects of FdUMP[10] exposure to the cell-cycle distribution of HT29 cells. The cell cycle distribution of HT29 cells was analyzed following exposure to 1.0×10^{-6} and 1.0×10^{-7} M for 5FU and 1.0×10^{-8} and 1.0×10^{-9} M for FdUMP[10]. Cells were incubated in the presence of drug for 48 h. The results are shown in Table 2.

The percent of HT29 cells in S-phase increases following exposure to either 5FU or FdUMP[10] in a dose-dependent manner. Exposure of cells to FdUMP[10] at 1×10^{-9} M resulted in S-phase arrest for 63% of cells while exposure of HT29 cells to 5FU at 1×10^{-6} M resulted in S-phase arrest for 48% of cells.

The results indicate that exposure of HT29 cells to FdUMP[10] results in substantially greater accumulation of these cells in S-phase relative to exposure of these cells to 5FU at concentrations that result in similar growth inhibition. Thus, S-phase cell-cycle arrest in HT29 cells occurs upon exposure to much lower concentrations of FdUMP[10] than is observed for 5FU. Other colon cancer cell lines are blocked at the G2/M checkpoint of the cell cycle following exposure to 5FU.[30]

DNA Damage

To assess the extent of DNA damage in HT29 human colon tumor cells following exposure to FdUMP[10] and 5FU, COMET assays were performed. HT29 cells (1×10^6) were grown overnight in 60 mm^2 petri dishes. Either 5FU or FdUMP[10]

Table 3. Tail moments from comet assays in HT29 cells.

Treatment	Concentration	Experiment 1	Experiment 2	Experiment 3
Control	NA	4	2	3
γ-irradiation	4 gray	17	15	20
5FU	1×10^{-6} M	6	14	14
FdUMP[10]	1×10^{-7} M	27	17	26
FdUMP[10]	1×10^{-8} M	16	14	10

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Table 4. Summary of apoptotic and proliferative activity in HT29 cells over 72 h.

Experiment	Apoptotic activity	Proliferative activity
Control (No treatment)	0 apoptotic events from 40 cells	111 division from 40 cells
5FU 4×10^{-6} M	3 apoptotic events from 40 cells	20 division from 40 cells
FdUMP[10] 4×10^{-8} M	20 apoptotic events from 40 cells	2 division from 40 cells

was then added to the culture medium and the cells were incubated in the presence of drug for 24 h. The medium was removed, and the cells were washed twice with ice-cold PBS. The cells were removed from the plate, and transferred to a centrifuge tube. The cells were counted, and the volume of the cell suspension was adjusted so that the final density was 2×10^5 cells per mL in cold $1 \times$ PBS. A summary of tail moment values is included in Table 3.

As is evident from Table 3, exposure of HT29 cells to FdUMP[10] for 24 h results in substantially greater tail moments than are observed for HT29 cells following exposure to 5FU at 10- to 100-fold greater concentration, also for 24 h.

Time-Lapse Video Microscopy

Time-lapse video microscopy was used to measure the number of apoptotic and proliferative events originating from a fixed number of drug-exposed cells over a defined time interval. HT29 cells were grown in RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum. 1×10^6 cells were plated in 25 cm^2 flask and incubated under standard conditions overnight. FdUMP[10] or 5FU was then added to the growth medium at the concentration indicated in Table 4. The plated cells were then placed on the stage of a microscope equipped with a video camera and an incubation chamber (with 5% CO_2, 37°C). Apoptotic and proliferative activity was monitored for 72 h.

SUMMARY

In summary, our studies evaluating the cellular consequences of FdUMP[10] exposure to HT29 cells compared to 5FU demonstrate that FdUMP[10] differs mechanistically from 5FU. Concentrations of FdUMP[10] that inhibit cell proliferation to a similar extent as 5FU inhibit TS enzymatic activity to a greater extent and for longer time periods. FdUMP[10] exposure also causes greater accumulation of HT29 cells in S-phase and induces greater DNA damage than 5FU. The results are consistent with FdUMP[10] intervening selectively in the TS/DNA locus of fluoropyrimidine activity.

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