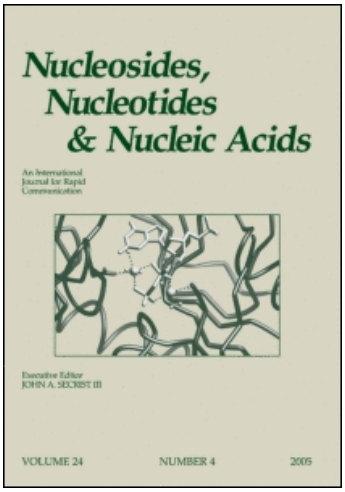

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Increased Cytotoxicity and Decreased In Vivo Toxicity of FdUMP[10] Relative to 5-FU

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Abstract: The efficacy of treatment with 5-Fluorouracil (5-FU) is limited, in part, by its inefficient conversion to 5-Fluoro-2'-deoxyuridine-5'-O-monophosphate (FdUMP). We present data indicating that FdUMP[10], designed as a pro-drug for intracellular release of FdUMP, is cytotoxic as a consequence of uptake of the multimeric form. FdUMP[10] is stable in cell culture medium, with more than one-half of the material persisting as multimers of at least six nucleotides after a 48 h incubation at 37 °C. FdUMP[10] is more than 400 times more cytotoxic than 5-FU towards human colorectal tumor cells (H630). FdUMP[10] also has decreased toxicity *in vivo*, with doses as high as 200 mg/kg/day (qdx3) administered to Balb/c mice without morbidity, compared to a maximum tolerated dose of 45 mg/kg/day for 5-FU using the same protocol. FdUMP[10] shows reduced sensitivity to OPRTase- and TK-mediated drug resistance, relative to 5-FU and FdU, respectively, and is much more cytotoxic than 5-FU towards cells that overexpress thymidylate synthase. Thus, FdUMP[10] is less susceptible to resistance mechanisms that limit the clinical utility of 5-FU. The increased cytotoxicity, decreased toxicity *in vivo*, and reduced sensitivity to drug resistance of FdUMP[10], relative to 5-FU, indicates multimeric FdUMP is potentially valuable as an anti-neoplastic agent, either as a single agent, or in combination with 5-FU.

Combination chemotherapy regimens including 5-fluorouracil (5-FU) as an adjuvant to surgery have proven effective at prolonging disease-free survival in patients treated for solid tumors.[1,2] For example, a large multicenter trial of 5-FU/levamisole reported prolonged disease-free and overall survival in patients with stage III colon cancer, compared with patients who received no treatment after surgery.[3] This benefit has persisted with continued follow-up.[4] More recently, the National Surgical Adjuvant Breast and Bowel Project (NSABP) reported a trial of stage II and III patients comparing the original 5-FU, semustine, vincristine (MOF) regimen to a weekly regimen of 5-FU plus high-dose leucovorin. This study demonstrated a statistically significant benefit for 5-FU/leucovorin in both overall and disease-free survival.[5] Taken together, randomized trials comparing adjuvant chemotherapy to surgery alone have shown a reduction in mortality of between 22% and 33% using protocols containing 5-FU. Because of the frequent incidence of solid tumors and high mortality rate, however, improved chemotherapeutic strategies are needed.[6]

5-FU, and other fluorinated pyrimidines, require metabolic activation for antitumor activity. The principal metabolite of 5-FU responsible for the antitumor effects is 5-fluoro-2'-deoxyuridine-5'-O-monophosphate (FdUMP), a potent inhibitor of thymidylate synthase (TS).[7] TS inhibition results in thymidylate levels insufficient for DNA replication and may contribute to intracellular signaling processes that eventually cause tumor cells to undergo apoptosis.[8] Although the clinical effectiveness of 5-FU may result, in part, from multiple mechanisms of activity such that transformed cells resistant to one mechanism are still susceptible to alternative mechanisms, e.g. inhibition of RNA processing, FdUMP production and TS inhibition are central to 5-FU therapeutic activity. The pathways for metabolic activation of 5-FU to FdUMP are the same as for the native nucleobase, Uracil.[9]

Enzyme-deficient cell lines that inefficiently convert 5-FU to FdUMP are relatively resistant to 5-FU. In particular, resistance to 5-FU in L1210 cells has been reported to arise from decreased expression of orotic acid phosphoribosyl transferase (OPRTase), the enzyme that converts 5-FU to 5-fluorouridine-5'-O-monophosphate (FUMP). Resistance to 5-fluoro-2'-deoxyuridine (FdU) has been reported to result from decreased expression of deoxythymidine kinase (TK), the enzyme that converts FdU to FdUMP. Resistance to 5-FU due to overexpression of TS, and/or decreased conversion of 5-FU to FdUMP, frequently limits the effectiveness of 5-FU in the treatment of cancer.[10] Two chemical strategies have been employed to devise TS inhibitors that are not dependent upon OPRTase or TK for their TS inhibitory activity. One strategy is to design TS inhibitors that do not resemble dUMP, the native substrate for TS. Examples of drugs in clinical use employing this approach include the folate analogues ZD1694 (Tomudex) and BW1843U89.[11,12] Alternatively, pro-drug forms of FdUMP that do not require the same steps of anabolic activation as 5-FU may be utilized to circumvent resistance resulting from decreased metabolism of 5-FU to FdUMP. Examples of drugs designed using this strategy include the SATE-derivatives of FdUMP prepared by Imbach and co-workers,[13,14] the 5-fluoro-2'-deoxyuridine phosphoramidate analogs prepared by Borch and co-workers,[15] and the phosphonate analog of FdUMP prepared by Montgomery and co-workers.[16]

In contrast to these approaches, we are investigating FdUMP[N], oligodeoxynucleotides composed of some number, *N*, of FdUMP nucleotides[17,18] (FIGURE 1). We hypothesize that FdUMP is released from FdUMP[N] by 3'->5' exonucleolytic activity, an enzymatic activity associated with DNA polymerases. Previous studies from our laboratory have shown that FdUMP[N] with nuclease resistant

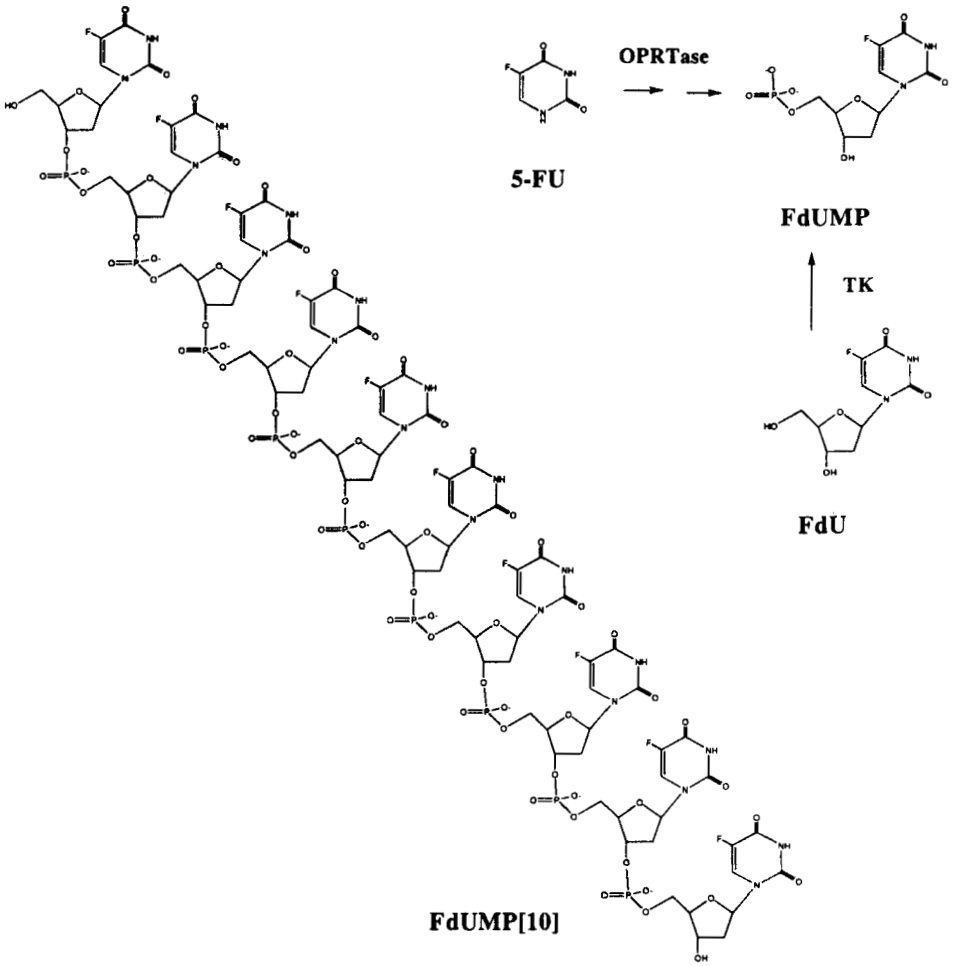

FIGURE 1. Structure of FdUMP[10] investigated in the present study. FdUMP (upper right) can be produced by metabolism of 5-FU with OPRTase and other enzymes, and from FdU by metabolism with TK. Cell culture and HPLC analyses indicate that FdUMP[10] is taken into cells as the 10mer, and as shorter oligomers.

phosphorothioate backbones are far less effective at inhibiting cell proliferation compared to FdUMP[N] with a nuclease susceptible phosphodiester backbone.[17] To investigate whether the observed cytotoxicity of FdUMP[N] results from cellular uptake of the multimer, we have investigated the effects of FdUMP[10] in cell lines either proficient or deficient in the expression of OPRTase or TK. The cytotoxicity of FdUMP[N] should be independent of OPRTase or TK expression, unless the multimers are substantially degraded to 5-FU or FdU in culture medium, or after internalization of the multimer into the cell. FdUMP[10] was selected as a representative multimer because it is in the range of lengths shown to have high activity previously.[17] We report here that FdUMP[10] inhibits the proliferation of both OPRTase and TK proficient/deficient pairs of cell lines equally well, indicating cytotoxicity does not result from degradation to 5-FU or FdU. Further, we show by high performance liquid chromotography (HPLC) analysis that FdUMP[10] remains largely in multimeric forms of length 6 or greater even after 48 h of incubation at 37 °C in cell-free culture medium. The relevance of these cell culture and stability studies to the *in vivo* situation is underscored by the reduced *in vivo* toxicity of FdUMP[10] relative to 5-FU. The maximum tolerated dose (MTD) of FdUMP[10] is much greater, in terms of molar content of fluorinated pyrimidine, than that of 5-FU, indicating rapid degradation to 5-FU does not occur in plasma. The increased cytotoxicity and decreased *in vivo* toxicity of FdUMP[10], compared to 5-FU, indicates multimeric fluorinated pyrimidines may be useful for the treatment of cancer.

EXPERIMENTAL METHODS

Synthesis of FdUMP[10]. 5-Fluoro-2'-deoxyuridine (Sigma; St. Louis, MO) was converted to 5'-*O*-(4,4'-Dimethoxytrityl)-5-Fluoro-2'-deoxyuridine 3'-(Cyanoethyl *N,N*-Diisopropylphosphoramidite) [FdU-amidite] according to standard procedures.[19] 5'-*O*-(4,4'-Dimethoxytrityl)-5-fluoro-2'-deoxyuridine was attached to controlled pore glass beads (CPG) using the procedure of Damha, et al.,[20] and the derivatized CPG was subsequently packed into 10 μmol columns. Each column was subjected to nine coupling cycles with the FdU-amidite using the standard coupling cycle for the ABI 380-B DNA synthesizer. The resulting decamers were cleaved from the solid support by treatment with ammonium hydroxide (28%

v/v for 90 min at room temperature), and desalted using Sephadex G-25 column chromatography. The FdUMP[10] product was purified using polyacrylamide gel electrophoresis (PAGE; 20% gel). The band containing FdUMP[10] was cut from the gel and the oligonucleotide product was recovered by crushing and soaking the acrylamide. The gel-purified FdUMP[10] was desalted using size-exclusion chromatography, and analyzed by mass spectrometry. The FdUMP[10] solution concentrations were determined from absorbance measurements at 260 nm using the conversion 33 μg/ODU. The concentrations of solutions of 5-fluorouracil (Sigma), 5-fluoro-2'-deoxyuridine (Sigma) and 5-fluoro-2'-deoxyuridine 5'-O-monophosphate were determined using values of 7.07, 8.91, and 8.80 x $10^3 M^{-1}cm^{-1}$ for the extinction coefficients. dUMP[8] was prepared in a manner analogous to that described for FdUMP[10].

Cell Culture. The antiproliferative activities of FdUMP[10], 5-FU and FdU were evaluated for pairs of cell lines resistant to either 5-FU or FdU due to deficiencies in the metabolic conversion of these drugs to FdUMP. L1210 cells (murine leukemia) (ATCC; Manassas, VA) were used to assess the antiproliferative activity of these drugs in cell lines resistant to 5-FU due to decreased expression of OPRTase.[21] L1210 cells were cultured using Dulbecco's modified Eagle's medium containing high glucose, L-glutamine, sodium pyruvate (110 mg/L), and supplemented with 5 μM 2-mercaptoethanol and 10% horse serum. LM cells (murine fibroblast; ATCC) were used to assess the relative effectiveness of these drugs for a cell line resistant to FdU due to decreased expression of TK.[22] LM cells were cultured using Minimum Essential Medium containing Earle's salts and supplemented with 10% Nu-serum IV, and for TK-deficient cells, BdUrd. Enzyme-proficient L1210 and LM cell lines served as controls. The effectiveness of these drugs was also assessed for the human colorectal tumor cell line H630 and the 5-FU resistant cell line H630-1 that expresses TS at 6-10 times higher levels than parental, H630 cells. These cell lines were cultured using RPMI medium 1640 containing L-glutamine and supplemented with 10% Nu-serum IV. All culture media were supplemented with penicillin/streptomycin.

MTT Assays. The inhibition of cellular proliferation by FdUMP[10], 5-FU, FdU and FdUMP was assessed using an assay for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) formazon.[23] For attachment dependent cells, 25,000 cells were plated in 200 μL of medium at 37 °C

overnight in an air atmosphere containing 5% CO_2. The medium was replaced the next day, and cells were exposed to the drugs for 72 h. The medium was then removed from the plates, and replaced with 100 μL of fresh medium and 25 μL of MTT solution (5 mg/mL in phosphate buffered saline), per well. Following 2 h incubation, 100 μL of MTT solvent (20% w/v sodium dodecyl sulfate dissolved in dimethylformamide:water [1:1], pH 4.7) were added to each well and the plates incubated overnight. The plates were analyzed the following morning on a Titerek Multiscan Plus 96-well plate analyzer (v. 1.4). The number of viable cells in each well containing cells treated with drug was calculated from the absorbance at 570 nm, compared to untreated controls. The means and standard deviation at each concentration for each drug were calculated using the statistical methods described below. MTT assays were run in duplicate or triplicate to ensure the statistical significance of the results. MTT assays using non-attached cell lines were performed using similar methods. Cells (25,000 per well) were grown overnight in 100 μL of medium at 37 °C. The next day, drug was added to each well. All doses of each drug were delivered in 50 μL of medium. Following 72 h exposure, 38 μL of MTT reagent were dispensed to the wells. The plates were incubated for 4 h and 100 μL of MTT solvent were added per well. The plates were analyzed as described for the non-attached cell lines.

Statistical Analyses. Two statistical methods were used to fit the data obtained in the MTT assays. The first model fit the data using the equation:

$$\text{Cell Activity} = A + B/\{1 + \exp(c^*[\log_{10}(\text{conc}) + d])\} \text{ where:}$$

A = The lowest level of cell activity of the five concentrations (excludes concentration = 0).

B = The difference between the highest and lowest mean levels of cell activity.

c = Multiplier for the smoothness of the curve.

d = The $\log_{10}$(concentration) producing cell activity of A + B/2 or $\log_{10}(IC_{50})$.

Two different methods of estimation of model parameters were used. The first method involved non-linear regression using the Gauss-Newton method of SAS PROC NLIN to simultaneously estimate the values of all four model parameters (A,B,c,d). This method failed in a few cases where c and d were highly correlated. A second approach fit all the experimental data except for 5-FU in the L1210-5-FU cell line. In this alternative approach, A and B were estimated from the plateaus that occurred at high

and low levels of drug concentration, and SAS PROC NLIN was then used to fit the above model. The methods were in agreement where both converged, while the second approach converged in all cases. Point estimates, and 95% confidence intervals for the IC_{50} values, were obtained by these methods.

HPLC Analysis. FdUMP[10] was incubated at 37 °C in RPMI medium 1640 containing L-glutamine and supplemented with 10% Nu-serum IV, the same medium used to culture the H630 cells. Aliquots of material were removed at 1, 4, 8, 24, 48, and 72 h and analyzed using a Waters 600E HPLC system controller and a Waters μBondpak C_{18} column (Milford, MA). Material was eluted using mixtures of 0.1 M triethylammonium acetic acid (solvent A), and 80% aqueous acetonitrile (solvent B). Solvent B was linearly increased from 6% to 13% over 15 min, and from 13% to 17% over 30 minutes. Standards of 5-FU, FdU, and FdUMP were utilized to characterize their retention times using this gradient.

MTD for 5-FU and FdUMP[10]. The MTD for 5-FU and FdUMP[10] was evaluated in Balb/c mice. The MTD was based on animal weight and mortality. Each of five female, Balb/c mice (8-12 weeks of age) (Charles River Laboratory Animals; Wilmington, MA) were injected with either 5-FU at 30, 40, 50, or 60 mg/kg/day, or with FdUMP[10] at 180 or 200 mg/kg/day. Doses of either drug were administered in 0.2 mL of saline solution by intravenous push administration using a qdx3 dosing schedule. The concentration of FdUMP[10] was determined from the optical density at 260 nm of the stock FdUMP[10] solution [1 OD unit = 33 μg/mL]. Animal weight, clinical toxicity, and mortality were determined daily.

RESULTS

FdUMP[10] Inhibits H630 Cell Growth. Human colorectal tumor cells are among the most frequent targets of 5-FU chemotherapy. The inhibitory activity of FdUMP[10] against human colon tumor cells (H630) was compared

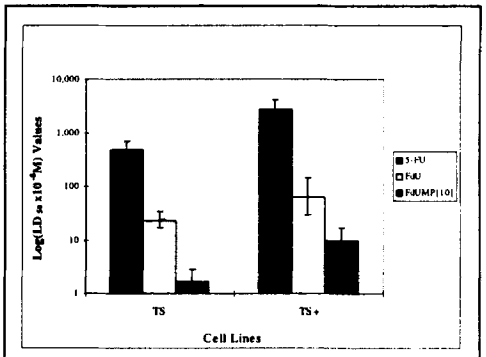

FIGURE 2. Relative IC_{50} values for FdUMP[10], 5-FU and FdU in human colorectal tumor cell lines H630 (TS) and H630-1 (TS+). H630-1 cells overexpress TS 6-10 fold relative to H630 cells. FdUMP[10] is much more cytotoxic than 5-FU towards both cell lines.

to 5-FU and FdU using an MTT assay. A comparison of IC_{50} values reveals that FdUMP[10] is 400 times more effective (per mole) or 40 times more effective (per mole of fluorinated pyrimidine) than 5-FU at inhibiting H630 cell proliferation (FIGURE 2). This significant difference in potency between FdUMP[10] and 5-FU must arise from mechanistic differences between these two drugs, under the conditions utilized in this assay. The relative activity of FdUMP[10] for H630 cells was also greater than expected (based on molar content of fluorinated pyrimidine) when compared to FdU, although in this instance the relative potency more closely correlates with fluorinated pyrimidine content (18-fold increase).

The development of resistance during treatment frequently limits the effectiveness of chemotherapy with 5-FU. Resistance to 5-FU occurs frequently due to overexpression of TS. The human colorectal tumor cell line H630-1 was used to assess the effect of TS overexpression on FdUMP[10], 5-FU, and FdU activities. H630-1 cells overexpress TS 6-10 fold, compared to the parental cell line.[10] The inhibitory activity of 5-FU, FdU, FdUMP, and FdUMP[10] against H630-1 cells was also monitored using an MTT assay. Similar inhibition was observed for H630-1 cells following co-incubation with FdUMP[10] as were observed for the H630 cell line (FIGURE 2). Specifically, the relative antiproliferative activity of FdUMP[10] against H630-1 cells, compared to 5-FU, was much greater than could be accounted for based on the molar equivalency of fluorinated pyrimidine (nearly 300-fold).

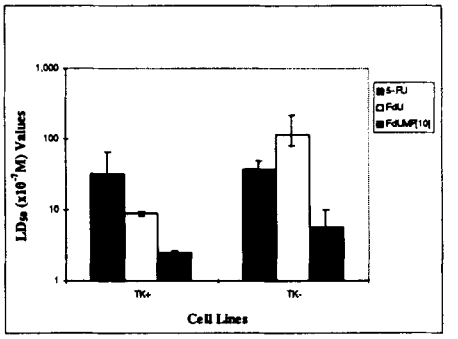

FIGURE 3. Relative IC_{50} values for FdUMP[10], 5-FU and FdU in a pair of cell lines that differ in TK expression. FdUMP[10] is less sensitive to TK expression than FdU, but unlike 5-FU, shows some dependency on TK expression.

FdUMP[10] Activity Does Not Require TK. A pair of murine fibroblast cell lines, [LM TK+] and [LM TK-], were used to assess the importance of TK expression for FdUMP[10] cytotoxicity. TK efficiently converts FdU to FdUMP, and a deficiency in TK activity causes resistance to FdU. The observation of resistance to FdUMP[10] in TK- cells would implicate degradation of FdUMP[10] to FdU as being responsible for the activity of FdUMP[10] in these cells. As expected, FdU is inhibitory to [LM

TK+] cells at sub-micromolar concentrations, but is considerably less active against the TK-deficient cells (FIGURE 3). 5-FU has similar activity against [LM TK+] and [LM TK-] cells. The cytotoxic activity of FdUMP[10] is greater than 5-FU for these cell lines, with IC_{50} values more than the one log lower than can be explained by molar equivalency of fluorinated pyrimidine. The IC_{50} value for FdUMP[10] is slightly increased in the TK deficient cell line relative to TK proficient LM cells, indicating degradation to FdU contributes somewhat to the cytotoxicity of FdUMP[10]. The IC_{50} values for 5-FU, FdU, and FdUMP[10] for the [LM TK+] and [LM TK-] cell lines are shown in FIGURE 3.

FdUMP[10]Activitity Does Not Require OPRTase. The murine leukemia cell lines [L1210-0] and [L1210-5-FU] were used to assess the importance of OPRTase for FdUMP[10] activity. OPRTase converts 5-FU to FUMP, and L1210 cells deficient in OPRTase activity are resistant to 5-FU.[20] Observation of resistance to FdUMP[10] in OPRTase deficient cells would implicate degradation to 5-FU as an intermediary step in the production of FdUMP from FdUMP[10]. In fact, the antiproliferative activity of FdUMP[10] was relatively insensitive to OPRTase expression (FIGURE 4). Similarly, the activity of FdU was not significantly different in OPRTase-deficient cells than in OPRTase-proficient cells.

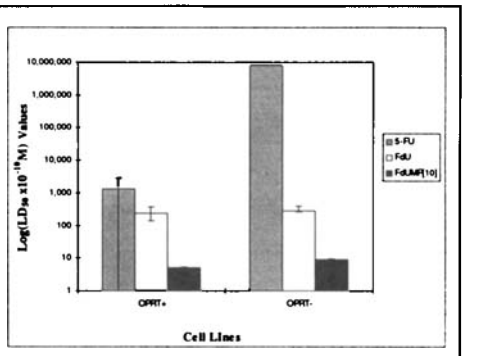

FIGURE 4. Relative IC_{50} values for FdUMP[10], 5-FU and FdU in a pair of cell lines that differ in OPRTase expression. FdUMP[10] is less sensitive to OPRTase expression than 5-FU, but unlike FdU, shows some dependency on OPRTase expression.

HPLC Analyses. The stability of FdUMP[10] was investigated by incubating the multimer in culture medium at 37 °C. Aliquots were removed periodically, and examined by reversed-phase HPLC to determine the relative amount of intact 10-mer, and to identify the degradatory products. The retention profile for the 48 h incubation time is compared to the starting material in FIGURE 5. Even after 48 h incubation, a substantial amount of 10mer remains, as well as appreciable concentrations of multimers of length 6, 7, 8 and 9. Monomeric FdUMP and FdU are detected as the first degradatory products of FdUMP[10]. The persistence of drug in the multimeric form under the incubation conditions used is consistent with the observed cytotoxicity resulting from cellular uptake of multimeric FdUMP.

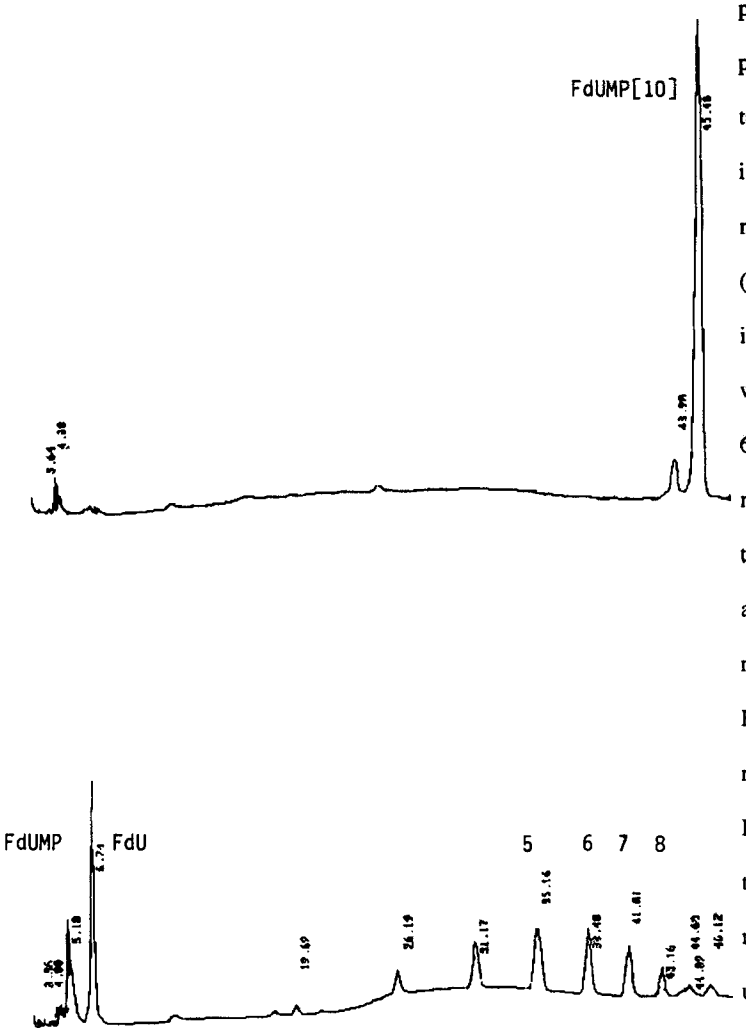

FIGURE 5. Reversed-phase HPLC retention profile for FdUMP[10] at t=0 (top) and after 48 h incubation in culture medium at 37 °C (bottom). FdUMP[10] is indicated in the top trace, while multimers of length 6-10 are indicated numerically in the bottom trace. FdUMP and FdU are the initial degradatory metabolites of FdUMP[10]. The retention of FdU and FdUMP is indicated in the bottom trace. 5-FU is not a major metabolite under these conditions.

In Vivo Toxicity. Administration of 5-FU is frequently limited by dose-limiting toxicities. To determine the *in vivo* toxicity of FdUMP[10], relative to 5-FU, a study was conducted in Balb/c mice. A plot of animal weight *versus* time post-injection, for 5-FU is shown in FIGURE 6. Maximal weight loss for the surviving animals occurred on day 8, and weight recovery did not occur until day 18. Injection of 50 mg/kg/dose of 5-FU, qdx3, resulted in 40% mortality, while the 60 mg/kg/dose injection resulted in 100% mortality. A similar study was done to establish the MTD of FdUMP[10]. Injection of 200 mg/kg/dose, qdx3, resulted in about 10% weight loss, but no mortality. In contrast to the weight loss observed for animals treated with the MTD of 5-FU, weight loss following the 200 mg/kg/dose of FdUMP[10] was maximal on day 5, with full recovery of weight on day 7, indicating FdUMP[10] has a different toxicity profile than 5-FU. A plot of the animal weight versus time post-injection for FdUMP[10] is also shown in FIGURE 6. The 200 mg/kg/dose of FdUMP[10] that induced weight loss, but no morbidity, contains more than a 50% greater molar content of fluorinated pyrimidine than the MTD of 5-FU (45 mg/kg/dose; qdx3). These data indicate that if equimolar doses are equipotent, FdUMP[10] will have an improved therapeutic index, relative to 5-FU.

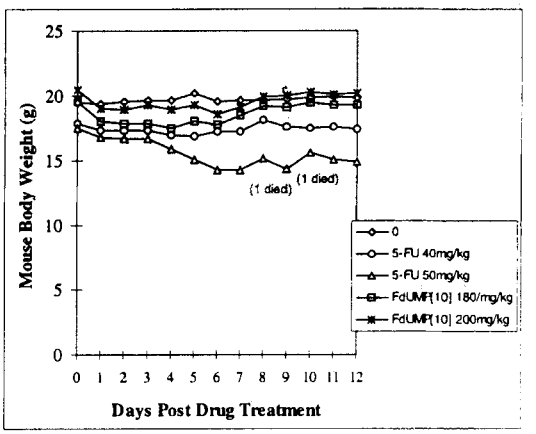

FIGURE 6. Effect of 5-FU and FdUMP[10] on the weight of Balb/c mice at the indicated doses. FdUMP[10] exhibited no morbidity at doses as great as 200 mg/kg/dose qdx3. All of the Balb/c mice treated with 60 mg/kg/dose of 5-FU (qdx3) died (data not shown).

DISCUSSION

Post-operative chemotherapy with TS inhibitory drugs remains central to effective strategies for the treatment of breast, colon and other cancers. We have previously reported that FdUMP[N] inhibits

cellular proliferation and that the antiproliferative activity of FdUMP[N] requires the enzymatically-labile phosphodiester backbone in the multimeric complex.[17,18] We now report that the antiproliferative activity of FdUMP[10] cannot be attributed solely to the release of 5-FU or FdU either prior to, or following, cellular uptake. This point is established by the activity of FdUMP[10] towards cell lines that are resistant to 5-FU and FdU and by the stability of FdUMP[10] in culture medium. The necessity of different, and fewer, steps of metabolic activation for FdUMP[10] compared to 5-FU suggests that delivery of fluorinated pyrimidine in multimeric form may have advantages in the treatment of tumors that are resistant to 5-FU due to deficiency in the anabolic activation of this drug.

A common cause of clinical resistance to 5-FU is the overexpression of TS.[24,25] 5-FU is metabolized to FdUMP inefficiently, and the toxicity of 5-FU prevents escalation of the dosages necessary to inhibit the proliferation of tumor cells that overexpress TS even moderately. The observed antiproliferative activity of FdUMP[10] in the cell lines utilized in the present study is due to uptake of the multimer into cells. Although the intracellular metabolism of FdUMP[10], and other multimers, requires further investigation, the cytotoxicity is consistent with efficient TS inhibition by degradatory products of the full-length multimer, especially FdUMP. Preliminary data from our laboratory shows FdUMP[10] has significantly reduced toxicity, relative to 5-FU, suggesting the potential for an improved therapeutic index during clinical administration. Additional studies are in progress to establish the potential clinical utility of these compounds for the treatment of cancer.

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