A Novel Polypyrimidine Antitumor Agent FdUMP[10] Induces Thymineless Death with Topoisomerase I-DNA Complexes

Zhi-Yong Liao, Olivier Sordet, Hong-Liang Zhang, Glenda Kohlhagen, Smitha Antony, William H. Gmeiner, and Yves Pommier

Abstract

FdUMP[10], a 10mer of 5-fluoro-2'-deoxyuridine 5’-monophosphate (FdUMP), the thymidylate synthase inhibitory metabolite of 5-fluorouracil (FU), is most closely correlated with the DNA topoisomerase I (Top1) inhibitor camptothecin in the National Cancer Institute COMPARE analysis, but not with FU. FdUMP[10] exhibits more potent antiproliferative activity than FdUMP or 5-fluoro-2'-deoxyuridine (FdU) and is markedly more active than FU. Camptothecin-resistant P388/CPT45 cells lacking Top1 are cross-resistant to FdUMP[10] as well as to FdUMP, FdU, and the thymidylate synthase inhibitor raltitrexed (Tomudex). FdUMP[10] induces DNA single-strand breaks and Top1-DNA complexes. Such complexes are also observed in response to FdUMP, FdU, raltitrexed, and FU. The FdUMP[10]-induced Top1-DNA complexes are not inhibited by the caspase inhibitor z-VAD-fmk and form independently of apoptotic DNA fragmentation, indicating that they do not correspond to apoptotic Top1-DNA complexes. In biochemical assay, Top1 is directly trapped at uracil and FdU misincorporation sites. We propose that FdUMP[10] damages DNA by trapping Top1 at uracil and FdU misincorporation sites resulting from thymidylate synthase inhibition and thymine depletion. (Cancer Res 2005; 65(11): 4844-51)

Introduction

5-Fluorouracil (FU) was developed in the late 1950s (1) as an antimetabolite inhibiting RNA and DNA biosynthesis. FU and other fluoropyrimidines have been widely used for the treatment of human cancers, including colorectal, breast, and head and neck cancers for more than 40 years (2). The mechanism of action of FU has been ascribed to the inhibition of thymidylate synthase by its active metabolite 5-fluoro-2'-deoxyuridine 5’-monophosphate (FdUMP; ref. 3), induction of “thymineless death” (4, 5), and RNA synthesis inhibition (6).

In the past decades, significant research effort has continued to focus on developing strategies to enhance the antineoplastic activity of FU through biochemical modulation and different methods of administration (2, 7). More recently, FdUMP[10], a 10mer of FdUMP, was developed (8–11; Fig. 1). FdUMP[10] is 338-fold more potent than FU at inhibiting cell proliferation in the National Cancer Institute (NCI) 60 cell line screen (11). Unexpectedly, COMPARE analysis showed that the FdUMP[10] activity profile clearly differed from FU but was mostly closely correlated with camptothecins. Because camptothecin and its derivatives selectively trap topoisomerase I (Top1; refs. 12, 13), in this report, we studied the possible relationships between Top1 inhibition and the cellular mechanisms of action of FdUMP[10].

Mammalian Top1 is an essential enzyme, relaxing DNA supercoiling ahead of replication and transcription (14, 15). To relax DNA, Top1 reversibly creates transient single-strand breaks by forming covalent tyrosyl-phosphodiester bonds with the 3’ ends of the broken DNA. Once the DNA is relaxed, Top1 readily relegates the breaks and regenerates intact duplex DNA. Under normal physiologic conditions, these intermediates, referred to as the “Top1 cleavage complexes” are very transient: the relaxation step of the DNA cleavage/religation equilibrium is favored and only a small fraction of the DNA is cleaved at any given time (12, 13, 16). Top1 inhibitors, such as camptothecins, trap the cleavage complexes by inhibiting the relaxation step (13, 16, 17). Top1 cleavage complexes can also be trapped by a wide range of DNA alterations, including uracil misincorporations, base mismatches, abasic sites, nicks, oxidized bases, UV photo-lesions, and carcinogenic adducts (18, 19). Top1-DNA complexes also occur during apoptosis in response to oxygen radicals and mitochondrial dysfunction (20–22).

In this report, we show high COMPARE correlation between FdUMP[10] and several camptothecin derivatives, cross-resistance of Top1-deficient cells to FdUMP[10], FdUMP, 5-fluoro-2'-deoxyuridine (FdU), and raltitrexed, and induction of Top1-DNA cleavage complexes in mouse and human cancer cells treated with FdUMP[10], FdUMP, FdU, and raltitrexed. We also show induction of Top1 cleavage complexes by FdU incorporation in DNA oligonucleotides. A model is presented to explain Top1 trapping by FdUMP[10] treatment and thymidine depletion.

Materials and Methods

Chemicals and enzymes. Camptothecin was provided by M.E. Wall (Research Triangle Institute, Research Triangle Park, NC). FU, FdU, and FdUMP were from Sigma (St. Louis, MO) and raltitrexed was a gift from Dr. Godefridus J. Peters (VU University, Amsterdam, the Netherlands). FdUMP[10] was prepared in Dr. William H. Gmeiner’s laboratory. [3H]Thymidine and [14C]thymidine were purchased from Perkin-Elmer Life Sciences (Boston, MA). [32P]Cordycepin 5’-triphosphate and terminal deoxynucleotidyl transferase were purchased from DuPont-New England Nuclear (NEW, Boston, MA) and Life Technologies (Gaithersburg, MD). Human recombinant Top1 was purified as described (23).

Cell lines and cultures. Mouse leukemia P88 and P88/CPT45 cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated FCS (Invitrogen) and 10 μM/L β-mercaptoethanol (21, 22, 24, 25). Human leukemia CEM cells and colorectal carcinoma HCT116 cells were cultured in DMEM containing 10% FCS. The human colon cancer HCT-C18 cell line was a kind gift from Dr. Edward Chu at Yale University (New Haven, CT) and were maintained in RPMI 1640 containing 10% FCS supplemented with 10 μM/L thymidine (26). The HCT-CHis-TS(+) cell line was provided by Dr. Yves Pommier (Building 37, Room 5066, Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, NIH, Department of Health and Human Services, Bethesda, Maryland and Department of Cancer Biology, Wake Forest University School of Medicine, Winston-Salem, North Carolina)

Requests for reprints: Yves Pommier, Building 37, Room 5066, Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, NIH, Department of Health and Human Services, Bethesda, Maryland and Department of Cancer Biology, Wake Forest University School of Medicine, Winston-Salem, North Carolina

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line was established by stable transfection of HCT-C18 cells with human His-tagged thymidylate synthase cDNA (27). Murine FMA3/0 and FMA/TK– mammary carcinoma cells were a gift from Dr. Godefridus J. Peters (28). FMA3/0 and FMA3/TK– cells were maintained in RPMI 1640 containing 10% fetal bovine serum.

3-[(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays for drug cytotoxicity. Drug cytotoxicity was measured using standard 3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 72 hours drug exposure. The absorbance was measured at 560 nm using a Bio-Tek Microplate Reader (Molecular Devices, Palo Alto, CA).

DNA single-strand break induction by FdUMP[10]. DNA single-strand breaks were assayed by alkaline elution according to published protocols (29, 30).

Detection of covalent topoisomerase I-DNA complexes in cells. Top1-DNA complexes were isolated using the immunocomplex of enzyme (ICE) bioassay (25, 31, 32). Briefly, 10⁶ cells were lysed with 1 mL of 1% sarkosyl. After Dounce homogenization, cell lysate was layered on cesium chloride step gradients and centrifuged at 165,000 g for 20 hours at 20°C. Fractions (500 µL) were collected from the bottom, diluted with an equal volume of 25 mmol/L sodium phosphate buffer (pH 6.6), and applied to Immobilon-P membranes (Millipore, Bedford, MA) by using a slot-blot vacuum manifold. Top1-DNA adducts were detected by immunoblotting using the C21 mouse monoclonal Top1 antibody obtained from Dr. Yung-Chi Cheng (Yale University).

Incorporation of thymidine into DNA. HCT116 cells in exponential growth phase were first labeled with 0.025 µCi/mL of [³H]thymidine for 48 hours at 37°C. After FdUMP[10] treatment for 48 hours, cells were pulse-labeled with 1 µCi/mL of [³H]thymidine for 10 minutes at 37°C. After washing the cells twice with cold HBSS, cells were scraped in 4 mL of cold HBSS. One hundred microliters of 10% trichloroacetic acid was added to the 1-mL aliquot. Precipitation was done on ice for 2 hours with interval mixing with vortex every 10 minutes. Precipitates were collected by centrifugation for 10 minutes at 13,000 rpm at 4°C. To the precipitate, 0.5 mL of 0.4 N NaOH was added, and mixtures were incubated overnight at 37°C. Radioactivity was counted by dual-label liquid scintillation with a liquid scintillation counter.

[³H]Thymidine incorporation (percent control) was calculated as the ratio of [³H]:[¹⁴C] in the treated samples over that in the untreated samples.

Effect of 5-fluoro-2'-deoxyuridine on topoisomerase I cleavage complexes. We designed model oligonucleotides containing one or two FdU incorporated immediately 3' from a preexisting Top1 cleavage site (33, 34) to study the effect of FdU incorporation on the Top1-DNA cleavage complexes (see Fig. 7A). Oligonucleotides were purchased from MWG Biotech Co. (High Point, NC). 3' Labeling of single-stranded oligonucleotides and annealing were done as described (33, 34). Duplex DNA substrates were generated by annealing a 3'-labeled upper strand [5'AAAAAGACTTGGAAAAATTTT*-3'] with three types of FdU (F) modified 22mer lower strand (FdU + 1, FdU + 2, and FdU + 1 + 2; see Fig. 7A). Top1 reactions were done as described (33, 34).

Results

Relationship between topoisomerase I and antiproliferative activity of FdUMP[10]. In the 60 cell lines from the NCI Developmental Therapeutics Program (DTP), FdUMP[10] [National Service Center (NSC) 697912] is 338-fold more effective than FU (NSC 19893; based on mean GI50 values; ref. 11). COMPARE analysis (http://dtp.nci.nih.gov; ref. 35) was used to reveal the potential mechanism of action of FdUMP[10]. Table 1 shows the Pearson correlation coefficients for the compounds with the highest correlation, as well as FU, which ranked 237th among all the compounds/extracts deposited in the DTP database. The best correlated compounds, besides FdUMP[10], chosen as the seed, and therefore has a Pearson correlation coefficient of 1, include four camptothecin derivatives (compounds ranked no. 1, 3, 8, and 9 with Pearson correlation coefficient ≥0.74), four FdU derivatives [ranked no. 2, 4 (FdU), 5, and 6; see Fig. 1 for structures], and 1-beta-D-arabinofuranosylcytosine (ara-C; ranked no. 7).

This COMPARE result prompted us to test whether the camptothecin-resistant P388/CPT45 cells, whose resistance to
camptothecin is due to Top1 deficiency (21, 24, 25) were also cross-resistant to FdUMP[10]. As shown in Fig. 2, P388 cells are highly sensitive to FdUMP[10] and P388/CPT45 cells are cross-resistant to FdUMP[10] with a resistance index of 12.5 (IC50s for FdUMP[10] in P388 and P388/CPT45 cells are 0.004 and 0.05 μmol/L, respectively). Comparatively, FU is markedly less potent than FdUMP[10] (~100-fold in P388 cells) and its resistance index in P388/CPT45 cells is only 1.8 (IC50s for FU are 0.55 and 1 μmol/L in P388 and P388/CPT45 cells, respectively). P388/CPT45 cells were also cross-resistant to both FdU and FdUMP (Fig. 2, middle), and to the thymidylate synthase inhibitor, raltitrexed (Fig. 2, bottom right). The differential sensitivity of P388/CPT45 to FdUMP[10] and FU suggested different mechanism(s) of action between FdUMP[10] and FU. The COMPARE analysis and response profile of P388/CPT45 cells suggested that Top1 contributes, at least in part, to the antiproliferative activity of FdUMP[10], FdU, FdUMP, and to the thymidylate synthase inhibitor raltitrexed.

DNA single-strand break induction by FdUMP[10]. One characteristic of Top1 inhibition by camptothecin is the trapping of Top1, which can be detected as the induction of DNA single-strand breaks by alkaline elution (30, 36). FdUMP[10] (0.1 μmol/L) induced detectable single-strand breaks as early as 4 hours after treatment (Fig. 3). Single-strand break induction increased further with time, and after 15 hours treatment, FdUMP[10] produced extensive single-strand breaks, reaching about 500 rad equivalents. To test whether these single-strand breaks may be induced by Top1 cleavage complexes, we measured FdUMP[10]-mediated Top1 trapping in cells (21, 31, 32, 37).

FdUMP[10] induces topoisomerase I-DNA complexes in cells. The ICE bioassay allows the detection of Top1-covalently linked to genomic DNA after cesium chloride gradient fractionation and subsequent immunoblotting with Top1 antibody (21, 25, 31, 32, 37). Genomic DNA sediments toward the high cesium chloride concentrations (fractions 6-11), whereas proteins stay on the top of the gradient (fractions 16-20) unless they are covalently bound to the DNA, in which case they sediment in the DNA-containing fractions. Figure 4 shows Top1 signals in the DNA-containing fractions for the three cell types tested when exposed to FdUMP[10]: the murine leukemia P388 cells (Fig. 4A and B), the human leukemia CEM cells (Fig. 4C and D), and the human colon carcinoma HCT116 cells (Fig. 4E and F).

FdUMP[10] can be degraded into FdUMP monomers by the 3’-5’ exonuclease enzymes, TREX1 and TREX2 (38). Consequently, FdUMP can act as a potent inducer of apoptosis by thymidylate depletion (3, 5, 39). Because we recently found that Top1-DNA complexes can be induced during apoptosis (20–22), we studied whether the general caspase inhibitor z-VAD-fmk could affect the formation of Top1-DNA complexes in FdUMP[10]-treated cells. Figure 4 (G-I) shows that z-VAD-fmk has almost no effect on FdUMP[10]-induced Top1-DNA complexes in P388 cells (compare B and G), and only a partial effect in CEM and HCT116 cells (compare D and H, and F and I, respectively). These results suggest that FdUMP[10] can induce cellular Top1 cleavage complexes independently of caspase activation and apoptosis.

**Cytotoxicity of FdUMP[10] is related to thymidylate depletion.** There are two routes leading to the synthesis of thymidylate in cells (2). One is via the *de novo* synthesis of thymidylate from dUMP catalyzed by thymidylate synthase. The other is via the salvage pathway from thymidine catalyzed by thymidine kinase. Here, the thymidylate pathway would be the major route, and the salvage pathway the minor route. The salvage pathway is less efficient than the *de novo* pathway, and its efficiency depends on the intracellular concentration of thymidine in the cell. The thymidylate synthase inhibitors, FdUMP[10] and FdU, would be expected to be more toxic than FU because they are more potent inhibitors of thymidylate synthase than FU. The COMPARE analysis and resistance profile of P388/CPT45 cells for FdUMP[10], FdU, FdUMP, FU, or raltitrexed. Experiments were done in triplicate, and average values. Points, average values; bars, SDs.

### Table 1. COMPARE analysis results for FdUMP[10] (NSC 697912) versus all compounds in the DTP open database

<table>
<thead>
<tr>
<th>Rank no.</th>
<th>Target ID</th>
<th>PCC</th>
<th>Chemical name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100880</td>
<td>0.781</td>
<td>Camptothecin sodium</td>
</tr>
<tr>
<td>2</td>
<td>674182</td>
<td>0.781</td>
<td>FdUMP derivative (see Fig. 1)</td>
</tr>
<tr>
<td>3</td>
<td>682298</td>
<td>0.773</td>
<td>Camptothecin derivative</td>
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<tr>
<td>4</td>
<td>27640</td>
<td>0.766</td>
<td>Deoxyfluorouridine (FdU; see Fig. 1)</td>
</tr>
<tr>
<td>5</td>
<td>704533</td>
<td>0.765</td>
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<tr>
<td>6</td>
<td>407335</td>
<td>0.756</td>
<td>FdU derivative (see Fig. 1)</td>
</tr>
<tr>
<td>7</td>
<td>668281</td>
<td>0.745</td>
<td>Cytosine arabinoside (ara-C)</td>
</tr>
<tr>
<td>8</td>
<td>618939</td>
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<tr>
<td>9</td>
<td>610456</td>
<td>0.741</td>
<td>Camptothecin hemisuccinate sodium salt</td>
</tr>
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**Note:** For details on COMPARE analysis, see [http://dtp.nci.nih.gov/dtpstandard/dwindex/index.jsp](http://dtp.nci.nih.gov/dtpstandard/dwindex/index.jsp).

**Abbreviations:** Target ID, target identification, here using the NSC number of each compound; PCC, Pearson correlation coefficient.
we investigated the contribution of thymineless death to FdUMP[10]-mediated cellular effects by using the 10mer of thymidylate (T[10]) and measuring its effect on FdUMP[10]-induced cytotoxicity and [3H]thymidine incorporation inhibition. One micromole per liter of T[10] completely prevented the cytotoxicity of FdUMP[10] within the range of 0.001 to 0.1 μmol/L, and partially prevented the cytotoxicity of 1 μmol/L FdUMP[10] in HCT116 cells (Fig. 5). Evidence for thymineless-related cytotoxicity of FdUMP[10] was further provided by [3H]thymidine incorporation assays. [3H]thymidine incorporation rate was enhanced 1.8–8-fold in FdUMP[10]-treated HCT116 cells over the dose range of 0.01 to 1 μmol/L (Fig. 5B). These results indicate that thymineless death is the primary mechanism for the antiproliferative activity of FdUMP[10].

To further investigate the implication of thymidylate synthase, the antiproliferative activity of FdUMP[10] was compared in thymidylate synthase–deficient and complemented cells (26). Figure 5C shows ~40-fold resistance to FdUMP[10] in the thymidylate synthase–deficient cells. As expected, the thymidylate synthase–deficient cells are cross-resistant to FdU (~70-fold; Fig. 5C). To inhibit thymidylate synthase, FdU needs to be converted to FdUMP by thymidine kinase. As expected, thymidine kinase–deficient cells are highly resistant to FdU (~1000-fold; Fig. 5D). The thymidine kinase–deficient cells were also highly resistant to FdUMP[10]. These results suggest that FdUMP[10] acts as a potent inhibitor of thymidylate synthase after initial conversion to FdU.

**Topoisomerase I-DNA complexes generated by thymidylate synthase inhibitors.** We next tested whether the well-characterized thymidylate synthase inhibitors FdUMP, FdU, FU, and raltitrexed are also able to induce Top1-DNA complexes. FdUMP[10] was 20-fold more potent than FdU and 70-fold more potent than FU in HCT116 cells (Fig. 5A). Raltitrexed was 9-fold more potent than FdUMP[10]. Top1 cleavage complexes were readily observed with all drugs after 48 hours exposure with concentrations as low as 0.1 μmol/L for FdU, FdUMP, and raltitrexed, and starting at 1 μmol/L for FU (Fig. 6B–E). These results show that similarly to FdUMP[10], FU, FdU, FdUMP, and raltitrexed also induce Top1-DNA complexes.
is almost 30-fold slower than removal of Uracil by uracil-DNA glycosylase (40). We designed four sets of oligonucleotides containing a high-affinity Top1 cleavage site (refs. 25, 33, 34; see Fig. 7A). All wobbled mismatched substrates [containing a deoxy-guanosine (dG):FdU at the +1, +2, or at both the +1 and +2 sites from the Top1 cleavage site] increased Top1 cleavage in the presence or the absence of camptothecin (Fig. 7B, compare lanes 2 and 3). FdU incorporation in the absence of camptothecin was at least as efficient as camptothecin for enhancing Top1 cleavage. Enhancement of Top1-DNA complexes was also greater when FdU was positioned at the +1 site than at the +2 site. Reversibility of FdU-induced Top1-mediated DNA cleavage was studied in the FdU-incorporated substrates, indicating that, like camptothecin, FdU misincorporation enhances almost completely reversible in the FdU-incorporated substrates, of FdU-induced Top1-mediated DNA cleavage was studied in HCT116 cells after FdUMP[10] exposure. HCT116 cells (prelabeled with [14C]thymidine) were treated with 0.01, 0.1, or 1 μmol/L of FdUMP[10] for 48 hours and then pulse-labeled with 1 μCi/mL [3H]thymidine for 10 minutes. After precipitation with 100% trichloroacetic acid, radioactivity was counted by liquid scintillation. [3H]Thymidine incorporation (percent control) was calculated as the ratio of [3H]/[14C] in the treated samples over the untreated control samples. Dashed line, exogenous thymidine incorporation in untreated cells (normalized to 100%). C, growth inhibition in thymidine synthase-proficient (C-18/TS; closed symbols) and thymidine synthase–deficient (C-18/TS−; open symbols) cells by FdUMP[10] (● and ○, respectively) or FdU (● and ○, respectively). Growth inhibition was measured by MTT assays after 72 hours drug exposure. Experiments were done in triplicate. Points, average values; bars, SDs.

Discussion

The COMPARE analysis in the NCI DTP screen (Table 1) indicates that FdUMP[10] and several other FdU derivatives are most closely related to camptothecin derivatives, which are highly specific Top1 inhibitors (13, 16, 17), and to ara-C, which can also trap Top1 cleavage complexes (25, 41). FU is, however, not closely correlated with FdUMP[10] in the COMPARE analysis inasmuch as FU is ranked 237th with a Pearson correlation coefficient of 0.557. FU is also markedly less cytotoxic than FdUMP[10]. Thus, FdUMP[10] is markedly more potent than FU and presents a spectrum of activity suggestive of a different mechanism of action. The cross-resistance of P388/CPT45 cells lacking detectable Top1 (24, 25) to FdUMP[10] (Fig. 2) provided the basis for investigating Top1 as a potential target of FdUMP[10].

The present study shows induction of Top1-DNA complexes by FdUMP[10] in the three cell lines tested (murine leukemia P388, human leukemia CEM, and human colon carcinoma HCT116) (Fig. 4). By contrast to camptothecin (36), the formation of Top1-DNA complexes by FdUMP[10] took several hours in P388 cells and at least one day in CEM and HCT116 cells. Apoptotic Top1 cleavage complexes (20–22) are unlikely to be the primary mechanism for the FdUMP[10]-induced Top1-DNA complexes because the pan-caspase inhibitor z-VAD-fmk failed to inhibit the Top1-DNA complexes in all the three cell lines examined (Fig. 4). Thus, our results favor the possibility that the FdUMP[10] induces Top1-DNA cleavage complexes at pyrimidine misincorporation sites (18, 34, 42) as a result of thymidylate synthase inhibition by FdUMP.

FdUMP[10] had no direct effect on either Top1-mediated cleavage or camptothecin-induced trapping of Top1-DNA cleavage complexes when using recombinant Top1 in a model DNA oligonucleotide substrate (data not shown). By contrast, incorporated FdU induced Top1 cleavage complexes with efficiency comparable to camptothecin (Fig. 7, compare lanes 2 and 3). At the DNA level, FdU substitution has been reported to enhance base pair opening (43) and to form stable ionized and wobble base pairs with dG in duplex DNA (44). The nuclear magnetic resonance structure of an FdU-substituted DNA duplex shows alteration in base roll at the site of FdU substitution (45). The structural distortions resulting from FdU:dG base pairs probably account for the enhancement of Top1-mediated DNA cleavage. It is therefore likely that the Top1-DNA complexes induced in cells by FdUMP[10] in cells are due at least in part to the incorporation of FdU (Fig. 7) or 2′-deoxyuridine (34) into replicating DNA as
a result of thymidine depletion after thymidylate synthase inhibition.

Thymineless death seems to be a prevalent mechanism for FdUMP[10]-mediated cytotoxicity, and thymidylate synthase inhibition the prevalent mechanism for the induction of Top1-DNA complexes by FdUMP[10]. Indeed, the antiproliferative activity of FdUMP[10] was blocked by addition excess thymidylate (Fig. 5A). In addition, we have observed a dramatic enhancement of thymidine incorporation in FdUMP[10]-treated cells (Fig. 5B), which is typical of thymidylate synthase inhibitors. Moreover, thymidylate synthase– and thymidine kinase–deficient cells are highly resistant to FdUMP[10] (Fig. 5C and D). It is therefore likely that FdUMP is the active metabolite of FdUMP[10]. A previous study indicated a much greater stability of FdUMP[10] over FdU or FU in plasma (11). FdUMP[10] could be degraded progressively into FdUMP and FdUMP[10] polymers containing n units (<10) of FdUMP by enzymes such as Trex1 and Trex2 (38). Because FdUMP is a potent inhibitor of thymidylate synthase, we propose the FdUMP[10]-induced Top1-DNA complexes to be related to thymidylate synthase inhibition. Accordingly, FdUMP, FdU, and raltitrexed also produced Top1-DNA complexes (Fig. 6) and showed cross-resistance in the Top1-deficient P388/CPT45 cells (Fig. 2).

Figure 8 summarizes the proposed mechanism of induction of cellular Top1-DNA complexes and DNA damage by FdUMP[10] and its active metabolite FdUMP. Although FdUMP[10] can enter some cells in multimeric form and be metabolized intracellularly to release FdUMP, it is likely that in the present studies FdUMP is produced from FdU released from FdUMP[10] because thymidine kinase–deficient cells are highly resistant to FdUMP[10] (Fig. 5D). FdUMP can induce Top1-DNA complexes in cells by at least three different routes. First, FdUMP can be converted to FdUTP, which in turn can be misincorporated into replicating DNA. The other two effects of FdUMP[10] are the accumulation of dUMP and depletion of dTMP both lead to dUTP accumulation. Depletion of dTMP and subsequently of dTTP produces an imbalance in the deoxynucleotide pool leading to FdUTP and dUTP incorporation instead of dTTP into replicating DNA by DNA polymerase. FdU incorporation could be degraded progressively into FdUMP and FdUMP[10] polymers containing n units (<10) of FdUMP by enzymes such as Trex1 and Trex2 (38). Because FdUMP is a potent inhibitor of thymidylate synthase, we propose the FdUMP[10]-induced Top1-DNA complexes to be related to thymidylate synthase inhibition. Accordingly, FdUMP, FdU, and raltitrexed also produced Top1-DNA complexes (Fig. 6) and showed cross-resistance in the Top1-deficient P388/CPT45 cells (Fig. 2).

Figure 7. Enhancement of Top1-mediated DNA cleavage by FdU mismatches. A, sequence of the control and mismatched oligonucleotides containing FdU at the +1 or +2 position or at both positions on the DNA strand opposite from the Top1 cleavage site (caret). Oligonucleotides were labeled at the 3’ end of the upper strand with [32P]cordycepin (A), and Top1-mediated DNA cleavage generates a 13mer cleavage product. B, gel pictures of Top1 reactions in the absence or presence of 1 μmol/L camptothecin at 25°C for 30 minutes. Reaction products were resolved by 20% sequencing gels. Imaging was done with a PhosphorImager. Right, migration position of the cleavage product. For each panel corresponding to one oligonucleotide, the lanes correspond to the following: lane 1, DNA alone; lane 2, + Top1; lane 3, + Top1 + camptothecin; lane 4, + Top1 + camptothecin followed by salt-induced reversal.

3 W.H. Gmeiner, unpublished data.
FdU and 2'-deoxyuridine are re-misincorporated because the cells lack dTTP as a nucleotide source. Moreover, unreppaired abasic sites can lead to irreversible trapping of Top1 (34) and can be converted to DNA nicks, which also produce irreversible Top1 cleavage complexes (42). Thus, FdUMP[10] produces Top1 cleavage complexes by altering nucleotide pools, which leads to pyrimidine misincorporation, abasic site formation, and Top1 inhibition. In addition, FdUMP[10] may induce Top1 cleavage complexes via thymineless death, which is associated with fragmentation of DNA and apoptosis (4, 5). Because apoptotic Top1 cleavage complexes seem to be a general response to a wide range of stimuli including staurosporine, arsenic trioxide, oxygen radicals (20–22), Fas, Trail, and Top2 inhibitors,4 thymineless death may also induce Top1 cleavage complexes during apoptotic DNA fragmentation.

In summary, similar to the nucleoside analogue ara-C (25, 41) and gemcitabine (37), Top1 trapping might also contribute to the cytotoxicity of FdUMP[10] and other thymidylate synthase inhibitors. The molecular mechanisms of action of FdUMP[10] are different from those of FU. FU exerts its anticancer effects by several mechanisms including (1) incorporation of FUTP into RNA, (2) incorporation of dUTP into DNA, and (3) inhibition of thymidylate synthase by its mononucleotide metabolites. The potential advantage of FdUMP[10] over FU as a thymidylate synthase inhibitor is an increased efficiency in being converted to FdUMP with fewer required steps of enzymatic activation. Thus, FdUMP[10] may selectively intervene in the thymidylate synthase/DNA-directed fluoropyrimidine pathways that are important for clinical efficacy (49), and, in contrast to FU, have a negligible effect on RNA.

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4 O. Sordet and Y. Pommier, unpublished data.

**References**


3. Van Triest B, Pinedo HM, Giaccone G, Peters GJ. Downstream molecular determinants of response to 5-fluorouracil and 2'-deoxyuridine (dU) can be removed by uracil-DNA glycosylase (UDG) generating abasic sites (*). However, because of dTTP depletion resulting from thymidylate synthase inhibition, a futile DNA repair process takes place as more FdU and 2'-deoxyuridine are misincorporated in the place of thymidine. Top1-DNA complexes are trapped in the immediate vicinity of misincorporated FdU (see Fig. 7) or 2'-deoxyuridine (34), near abasic sites (34) or gaps (42).

Figure 8. Hypothetical model for the mechanism of action of FdUMP[10] and induction of Top1-DNA complexes. FdUMP[10] slowly releases FdU outside and possibly inside the cells. Thymine kinase (TK) activates FdU to FdUMP. Three consequences may result from the accumulation of FdUMP in cells. One is the accumulation of FdUTP. The other two effects, increase in FdUMP and depletion of dTTP, result from thymidylate synthase (TS) inhibition by FdUMP.

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FDU and 2'-deoxyuridine are re-misincorporated because the cells lack dTTP as a nucleotide source. Moreover, unreppaired abasic sites can lead to irreversible trapping of Top1 (34) and can be converted to DNA nicks, which also produce irreversible Top1 cleavage complexes (42). Thus, FdUMP[10] produces Top1 cleavage complexes by altering nucleotide pools, which leads to pyrimidine misincorporation, abasic site formation, and Top1 inhibition. In addition, FdUMP[10] may induce Top1 cleavage complexes via thymineless death, which is associated with fragmentation of DNA and apoptosis (4, 5). Because apoptotic Top1 cleavage complexes seem to be a general response to a wide range of stimuli including staurosporine, arsenic trioxide, oxygen radicals (20–22), Fas, Trail, and Top2 inhibitors, thymineless death may also induce Top1 cleavage complexes during apoptotic DNA fragmentation.

In summary, similar to the nucleoside analogue ara-C (25, 41) and gemcitabine (37), Top1 trapping might also contribute to the cytotoxicity of FdUMP[10] and other thymidylate synthase inhibitors. The molecular mechanisms of action of FdUMP[10] are different from those of FU. FU exerts its anticancer effects by several mechanisms including (1) incorporation of FUTP into RNA, (2) incorporation of dUTP into DNA, and (3) inhibition of thymidylate synthase by its mononucleotide metabolites. The potential advantage of FdUMP[10] over FU as a thymidylate synthase inhibitor is an increased efficiency in being converted to FdUMP with fewer required steps of enzymatic activation. Thus, FdUMP[10] may selectively intervene in the thymidylate synthase/DNA-directed fluoropyrimidine pathways that are important for clinical efficacy (49), and, in contrast to FU, have a negligible effect on RNA.

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into DNA after thymidylate synthase inhibition is well documented (6, 46–48). Misincorporated FdU and 2'-deoxyuridine can be removed by uracil-DNA glycosylase. However, in the process of futile DNA repair catalyzed by uracil-DNA glycosylase, additional


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Zhi-Yong Liao, Olivier Sordet, Hong-Liang Zhang, et al.


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