Cytotoxicity and DNA Fragmentation Associated with Sequential Gemcitabine and 5-Fluoro-2'-deoxyuridine in HT-29 Colon Cancer Cells

Qianfang Ren, Vivian Kao, and Jean L. Grem

Developmental Therapeutics Department, Medicine Branch, Division of Clinical Sciences, National Cancer Institute, National Naval Medical Center, Bethesda, Maryland 20889-5105

ABSTRACT

The combined cytotoxic effects of the antimitabolites gemcitabine (dFdCyd) and 5-fluoro-2'-deoxyuridine (FdUrd) were studied. Cytotoxicity, biochemical perturbations, and DNA damage seen with dFdCyd and FdUrd alone and in combination were evaluated in HT-29 human colon cancer cells. A 4-h exposure to dFdCyd followed by FdUrd for 24 h produced more than additive cytotoxicity and marked S-phase accumulation. Cells progressed through the cell cycle, however, after a 22-h drug-free interval. [3H]FdCyd was rapidly metabolized to the 5'-triphosphate and incorporated into DNA. [3H]FdUrd was anabolized exclusively to FdUrd monophosphate, and preexposure to dFdCyd did not affect FdUrd monophosphate formation. Thymidylate synthase catalytic activity was inhibited by 48% after a 4-h exposure to 10 mM FdUrd and by 80% after exposure to the combination. Sequential 4-h exposures to 15 mM dFdCyd and 10 mM FdUrd led to greater depletion of dTTP pools (29% of control) than with either drug alone. Greater effects on nascent DNA integrity were seen with sequential dFdCyd followed by FdUrd. Although parental DNA damage was not evident immediately after exposure to 15 mM dFdCyd for 4 h followed by 10 mM FdUrd for 24 h, high molecular mass DNA fragmentation was evident 72-96 h after drug removal. Sequential dFdCyd/FdUrd was associated with prominent disturbance of the cell cycle, dTTP pool depletion, dATP/dTTP imbalance, and nascent DNA damage. Induction of double-strand parental DNA damage and cell death was delayed, consistent with postmitotic apoptosis.

INTRODUCTION

dFdCyd2 (gemcitabine) is a pyrimidine analogue of deoxy-cytidine in which the deoxyribose moiety contains two fluorine atoms in place of the hydrogens at the 2' position. The drug enters cells by the facilitated nucleoside transport mechanism and undergoes phosphorylation to the 5'-monophosphate form (dFdCMP) by deoxycytidine kinase (1-3). The drug is subsequently phosphorylated to the 5'-diphosphate (dFdCDP) and 5'-triphosphate derivatives (dFdCTP), respectively. Inhibition of ribonucleotide reductase by dFdCDP decreases the physiological deoxyribonucleotide triphosphate pools (4, 5). Incorporation of dFdCTP into DNA appears to be a critical event for induction of programmed cell death (6-9). dFdCyd is converted to the inactive metabolite 2',2'-difluorodeoxyuridine by cytidine deaminase. In vitro primer extension studies indicate that dFdCTP competes with dCTP for incorporation into the C sites of the growing DNA strand by purified DNA polymerases α and ε (involved in DNA replication and repair; Ref. 6). After incorporation of the dFdCMP residue, the primer extends by one deoxynucleotide before a major pause in the polymerization process occurs. Incorporation of a dFdCdCyd molecule into a DNA template affects both the kinetics and fidelity of base insertion by the Klenow fragment of bacterial DNA polymerase I (10). Inhibition of DNA synthesis may therefore result from both perturbations of deoxynucleotide pools and interference by the incorporated dFdCyd residues with DNA chain elongation. Of particular interest is the clinical activity of dFdCyd in various human solid tumors (11-14).

FdUrd, a fluorinated analogue of uracil, is used in a variety of chemotherapeutic combinations in the treatment of solid tumors (15). Incorporation of FUTP into RNA affects normal RNA processing and function. Another active metabolite, FdUMP, competes with the normal substrate, dUMP, for binding to the nucleotide site of TS, thus resulting in enzyme inhibition and depletion of dTTP pools. Incorporation of the fraudulent nucleotides dUTP and FdUrd into DNA stimulates excision repair by uracil-DNA glycosylase. In the setting of decreased dTTP and increased dUTP, a futile cycle may ensue, accompanied by interference with nascent DNA synthesis and integrity.

The combination of dFdCyd and FdUrd represents a reasonable therapeutic strategy for the treatment of malignancies in which both drugs are active because their mechanisms of action and spectra of clinical toxicity are different. We have demonstrated previously a striking sequence-dependent interaction be-
tween Ara-C and FUra in human colon cancer cells (17). Exposure to FUra for 24 h followed by Ara-C was markedly antagonistic. FUra-mediated inhibition of DNA synthesis prior to Ara-C exposure significantly decreased the amount of Ara-C incorporated into DNA, thus accounting for the antagonism. In contrast, initial exposure to Ara-C followed by FUra was accompanied by greater inhibition of DNA synthesis compared with either Ara-C or FUra alone and led to more than additive cytotoxicity. Because incorporation of both Ara-C and dFdCyd into DNA are essential prerequisites for induction of programmed cell death, it seemed reasonable to select sequential exposure to dFdCyd followed by FdUrd for the present studies to avoid potential interference with incorporation of dFdCyd into DNA. FdUrd was used in place of FUra because it produces more selective inhibition of TS and DNA-directed cytotoxic effects.

MATERIALS AND METHODS

Materials. dFdCyd and [3H]dFdCyd (18.6 Ci/mmol) were kindly provided by Lilly Research Laboratories (Indianapolis, IN). Other isotopes were obtained from Moravek Biochemicals (Brea, CA). Calcium- and magnesium-free PBS (pH 7.4) was purchased from Biofluids, Inc. (Rockville, MD). Unless otherwise stated, chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cytotoxicity. HT-29 cells were grown in RPMI 1640 supplemented with 10% dialyzed fetal bovine serum (both obtained from Life Technologies, Inc., Grand Island, NY) and 2 mM glutamine. For cell growth experiments, 20,000 cells were plated in replicate in 6-well plates. To assess survivorship, 500 cells were plated in replicate in 6-well plates. After 48 h, when the cells had entered exponential growth, either PBS (controls) or dFdCyd was added at concentrations ranging from 3 to 40 nM. After 4 h, the medium was gently aspirated, the cells were washed with 3 ml of cold PBS, and fresh medium was added. PBS or FdUrd at various concentrations ranging from 3 to 18 nM was then added. After an additional 24 h, the medium was aspirated, and the cells were washed with 3 ml of cold PBS and incubated in fresh, drug-free medium. The cell number was determined at 72 h in a Coulter Multisizer II (Miami, FL), at which time the control cell number was 319,330 ± 20,290. On day 10, colonies of 50 or more cells were stained and enumerated; the average control colony number was 100 ± 7. To assess any interaction between the two drugs, various combinations of sequential dFdCyd and FdUrd at fixed concentration ratios were evaluated. The fraction of affected cells for each drug alone and the combination were calculated, and the data were analyzed using CalcuSyn version 1.1 software (Biosoft, Ferguson, MO), assuming a mutually nonexclusive model. The combination index was used to signify antagonism (>1), additivity (=1), or synergism (<1).

dFdCyd and FdUrd Metabolism and Incorporation into DNA. Exponentially growing cells were exposed to [3H]-dFdCyd (15 nM; specific activity, 1 μCi/225 pmol) for 4 h, after which the medium was aspirated and the cells were washed with cold PBS. The cells were then extracted with 0.5 n perchloric acid immediately or after an additional 4-h incubation with either drug-free medium or 10 nM FdUrd. For FdUrd metabolism, HT-29 cells were preincubated with either drug-free medium or 15 nM FdUrd for 4 h; after the cells were washed, FdUrd (10 nM; specific activity, 1 μCi/150 pmol) was added for 4 h, and the cells were then extracted with 0.5 n perchloric acid. The acid-soluble fraction was isolated, neutralized, and lyophilized. The radioactivity in an aliquot of the reconstituted sample was determined, and the distribution of [3H]TdFdCyd and [3H]dFdCyd metabolites was measured by anion exchange and reverse-phase high-performance liquid chromatography methods, respectively, with in-line scintillation detection (Packard Instruments, Mount Prospect, IL; Refs. 16–18).

To assess incorporation of dFdCyd into DNA, HT-29 cells were exposed to [3H]dFdCyd (15 nM; 1 μCi/300 pmol) for 4 h, following which the cells were either harvested immediately or washed and incubated in fresh medium with either 10 nM FdUrd or no drug for 24 h. Alternatively, cells were exposed to [3H]-FdUrd (10 nM; 1 μCi/200 pmol) for 24 h with or without a 4-h preexposure to 15 nM dFdCyd. At the desired times, the DNA was extracted using the DNA Stat-30 kit (Tel-Test "B", Inc., Friendswood, TX) as recommended by the manufacturer, and trichloroacetic acid-precipitable counts retained on 0.45 μm HA filters were determined.

Measurement of Deoxynucleotide Pools. Exponentially growing cells were exposed to 15 nM dFdCyd (4 h) and 10 nM FdUrd (24 h) individually or sequentially. The cells were extracted with 0.5 n perchloric acid, neutralized, and lyophilized, and the samples were stored at −70°C until the time of analysis. Deoxynucleoside triphosphate pools were determined by a DNA polymerase (Escherichia coli, Klenow fragment) assay using synthetic oligonucleotides as template and primers (19).

TS Assays. TS catalytic activity in cellular lysates was determined by tritium release from [5-3H]dUMP as described previously (20). The proportion of the total cellular lysate used in the assay was 16 ± 2%. Protein was quantified by the Bio-Rad protein assay kit. For analysis of total TS content, equal amounts of protein were resolved by PAGE using TS106 monoclonal antibody as the primary antibody (21, 22). The protein bands were developed with an enhanced chemiluminescence kit (ECL kit; Amersham, Buckinghamshire, UK). The relative quantities of TS in the bound and unbound state were determined by densitometry using Immobilon membranes (Pall Scientific, San Rafael, CA). The position of the TS protein band (35 kDa) was identified both by protein standard markers and by inclusion of lysate from NCI-H630/R10 cells, a line that highly overexpresses TS protein (21).

Cell Cycle Analysis. After the desired drug exposure, cell nuclei were isolated, incubated in propidium iodide and DNase-free RNase for 60 min, and then gently filtered through 35-μm strainer caps into 12 × 75 mm polystyrene tubes. DNA histogram data were collected using a FACScan (Becton Dickinson, San Jose, CA). The list mode files were analyzed with "ModFit LT for Win32" version 2.0 software (Verity Software House, Inc., Topsham, ME).

Detection of Single-Strand and Double-Strand Breaks in DNA. Induction of single-strand breaks in DNA was determined by alkaline elution at a fixed pH of 12.1 as described previously (22, 23). The cells were either labeled with [14C]ethidium (0.05 μCi/ml) for the final 4 h of drug exposure (to assess nascent DNA) or prelabeled for 24 h prior to drug exposure.
Fig. 1  Cytotoxicity of dFdCyd and FdUrd in HT-29 cells. Exponentially growing cells were exposed to either dFdCyd or FdUrd at the indicated concentrations for 4 or 24 h, respectively, after which the cells were washed and incubated in drug-free medium. Cell number and colony formation were determined at 72 h and 10 days, respectively. The data are the means; bars, SE. The cell growth data are from five to seven separate experiments done in duplicate, and colony formation data are from three to four separate experiments done in duplicate.

Fig. 2  Median effect analysis of dFdCyd followed by FdUrd. Exponentially growing cells were exposed to no drugs, dFdCyd from h 0 to 4, FdUrd from h 4 to 28, or the combination at five different concentrations with a fixed ratio of either 1:1 (growth) or 1:1.67 (colony formation) in three separate experiments, each done in duplicate. The results for each combination for all three experiments were averaged and subjected to median effect analysis using a mutually nonexclusive model; the results are presented as the combination index versus fraction affected. ○, observed data points for the five drug combinations; ——, the combination index plot predicted by the software. A combination index < 1.0 indicates synergism.

exposure (to assess parental DNA). The total radioactivity was defined as the sum of the dpm in each elution fraction plus that retained on the filter, minus the background. Double-strand breaks were determined by a filter binding assay at a nondenaturing pH (23). The total radioactivity was determined by combining the counts from the eluting fractions (loading, wash, lysis, and EDTA wash) plus the dpm retained on the filter. DNA fragmentation was calculated by dividing the dpm in the eluting fractions by the total dpm.

Detection of High Molecular Weight DNA Fragmentation. After the desired drug exposure, intact cells were embedded in 0.5% low melting point agarose plugs (100,000 cells/plug), digested for 48 h with five volumes of a buffer containing 0.5 M EDTA (pH 9.0), 1% sodium lauroyl sarcosine, and 0.5 mg/ml proteinase K at 50°C, and then washed and stored at 4°C as recommended by the manufacturer (Bio-Rad Laboratories; Refs. 18 and 23). Individual plugs were placed on the teeth of a gel comb, and a 1.0% chromosomal grade agarose gel (160 ml) was cast. A previously described Chef Mapper (Bio-Rad Laboratories) program was run for 17.5 h, during which Tris-borate-EDTA buffer (0.5×, 4 liters) was recirculated at 14°C (23). The gels were subsequently stained with ethidium bromide.

RESULTS
dFdCyd- and FdUrd-mediated Growth Inhibition and Lethality. In the clinical setting, dFdCyd is most commonly administered as a short infusion, and extending the duration of infusion leads to a marked reduction in the maximum tolerated dose (24, 25). In preliminary experiments, we compared growth inhibition following either 4- or 24-h exposure to dFdCyd. The IC_{50} for a 4-h dFdCyd exposure was 15–20 nm. Extending the duration of exposure to 24 h led to a marked increase in cytotoxicity (IC_{50} < 1 nm). Therefore, a 4-h exposure to dFdCyd was selected for subsequent exper-
Cytotoxicity with Sequential Gemcitabine and FdUrd

Exponentially growing cells were exposed to dFdCyd and FdUrd as indicated, and the distribution of radiolabeled metabolites was determined by high-performance liquid chromatography analysis with in-line scintillation detection. The data are presented as the mean ± SE and are from six ([3H]dFdCyd) and eight ([3H]FdUrd) separate experiments, each done in duplicate. FdUMP was the only tritiated nucleotide metabolite of FdUrd formed under the experimental conditions.

**Table 1**  dFdCyd and FdUrd metabolism

<table>
<thead>
<tr>
<th>Condition</th>
<th>dFdCDP*</th>
<th>dFdCTP</th>
<th>FdUMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 nM [3H]dFdCyd × 4 h</td>
<td>0.36 ± 0.10</td>
<td>8.08 ± 1.32</td>
<td>NA*</td>
</tr>
<tr>
<td>15 nM [3H]dFdCyd × 4 h to 4 h no drug</td>
<td>0.18 ± 0.05</td>
<td>4.07 ± 0.99</td>
<td>NA</td>
</tr>
<tr>
<td>15 nM [3H]dFdCyd × 4 h to 10 nM FdUrd × 4 h</td>
<td>0.14 ± 0.36</td>
<td>3.70 ± 0.77</td>
<td>NA</td>
</tr>
<tr>
<td>10 nM [3H]FdUrd × 4 h</td>
<td>NA</td>
<td>NA</td>
<td>1.56 ± 0.02</td>
</tr>
<tr>
<td>15 nm dFdCyd × 4 h to 10 nm [3H]FdUrd × 4 h</td>
<td>NA</td>
<td>NA</td>
<td>1.81 ± 0.03</td>
</tr>
</tbody>
</table>

* dFdCDP, deoxycytidine diphosphate; dFdCTP, deoxycytidine triphosphate; FdUMP, 5-fluoro-2'-deoxyuridine monophosphate.

Table 2  dFdCyd and FdUrd DNA incorporation

<table>
<thead>
<tr>
<th>Condition</th>
<th>dFdCyd-DNA (fmol/μg DNA)</th>
<th>FdUrd-DNA (fmol/μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 nM [3H]dFdCyd × 4 h</td>
<td>1.12 ± 0.44</td>
<td>NA*</td>
</tr>
<tr>
<td>15 nM [3H]dFdCyd × 4 h to no drug × 24 h</td>
<td>2.52 ± 1.06</td>
<td>NA</td>
</tr>
<tr>
<td>15 nM [3H]dFdCyd × 4 h to 10 nm FdUrd × 24 h</td>
<td>2.52 ± 1.32</td>
<td>NA</td>
</tr>
<tr>
<td>No drug to 10 nm [3H]FdUrd × 24 h</td>
<td>NA</td>
<td>1.60 ± 0.58</td>
</tr>
<tr>
<td>15 nm dFdCyd × 4 h to 10 nm [3H]FdUrd × 24 h</td>
<td>NA</td>
<td>1.16 ± 0.46</td>
</tr>
</tbody>
</table>

* NA, not applicable.

Exponentially growing cells were exposed to the conditions indicated in the left column. The DNA was extracted as outlined in "Materials and Methods." The data, shown as the mean ± SE, are from four to seven separate experiments done in duplicate.

We next evaluated the effects of sequential exposure to dFdCyd (4 h) followed by FdUrd (24 h). As shown in Fig. 2, sequential dFdCyd/FdUrd led to more than additive cytotoxic effects in both cell growth and colony formation assays. Protection from 5-fluoropyrimidine-mediated toxicity by thymidine is generally taken as evidence of rescue from DNA-directed cytotoxic effects. We found that although 10 μM thymidine (4–28 h) had no effect on growth inhibition associated with an initial 4-h exposure to 15 nM dFdCyd, it provided partial protection against growth inhibition from both 10 nM FdUrd alone (h 4–28: 86 ± 17% versus 61 ± 14%, mean ± SD, n = 3) and dFdCyd →FdUrd (84 ± 11% versus 45 ± 10%). These results suggest that DNA-directed effects of FdUrd are important for the cytotoxicity seen with the combination.

**dFdCyd and FdUrd Metabolism and Incorporation into DNA.** The 5’-triphosphate derivative dFdCTP, the predominant [3H]FdUrd metabolite, was ~22-fold higher than the diphosphate pool size (dFdCDP; Table 1). After drug removal, the dFdCTP pool size decreased by one-half at 4 h, and subsequent exposure to FdUrd did not appreciably alter this. With [3H]FdUrd, FdUMP was the only phosphorylated metabolite identified. [3H]dFdCyd incorporation into DNA after a 4-h exposure to 15 nM was 1.1 fmol/μg (Table 2). Interestingly, [3H]dFdCyd content in DNA was ~2.2-fold higher 24 h after drug removal, with or without subsequent exposure to FdUrd, suggesting continued incorporation of dFdCTP into DNA. [3H]FdUrd incorporation into DNA reached 1.6 fmol/μg after a 24-h exposure to 10 nM. Although the amount was somewhat lower when cells where preexposed to dFdCyd (72% of FdUrd alone), the differences were not significant (P = 0.397).

**Biological and Cell Cycle Effects of dFdCyd and FdUrd.** Immediately after a 4-h exposure to 15 nm dFdCyd, an 82% reduction in dATP pools (to 18% of control) was evident (Table 3). dGTP pools were also decreased by 32% (to 68% of control), whereas dCTP and dTTP pools were unchanged. With a 4-h exposure to 10 nM FdUrd, dTTP and dGTP pools were diminished, whereas dATP and dCTP pools were somewhat higher than control. Sequential exposure to dFdCyd and FdUrd led to greater dTTP depletion than seen with FdUrd alone, whereas dGTP pools were similar to those seen with either drug alone. In contrast, dATP and dCTP pools were close to control values, suggesting that sequential drug exposure led to offsetting effects on these two deoxynucleotide pools. An increase in the dATP:dTTP ratio pools provides an index of deoxynucleotide imbalance (26–28). In cells treated with FdUrd alone or with preexposure to dFdCyd, the dATP:dTTP ratio was 2.9- and 3.4-fold higher than control, respectively.

TS catalytic activity in control cells averaged 40 pmol/min/mg (Fig. 3). TS activity was 1.6-fold higher after a 4-h exposure to dFdCyd, but this difference was not significant (P = 0.17, t test). Apparent TS activity was reduced by 48% after a 4-h exposure to 10 nM FdUrd, whereas sequential dFdCyd followed by a 4-h exposure to FdUrd was associated with 80% TS inhibition. When the duration of FdUrd exposure was extended to 24 h, TS catalytic activity was reduced by 67 and 74% without and with dFdCyd preexposure, respectively. TS activity
Table 3  Deoxyribonucleotide triphosphate pools

Exponentially growing cells were exposed to no drug, dFdCyd, FdUrd, or sequential dFdCyd plus FdUrd as indicated. Deoxyribonucleotide levels were determined by an enzymatic DNA polymerase assay. The assay mixture was incubated for 15 min at 37°C, and the calibration curve for each dRTP was linear between 1 and 40 pmol of substrate. The data are presented as the mean ± SE and are from at least four separate experiments, each done in duplicate.

<table>
<thead>
<tr>
<th>Condition</th>
<th>dATP (pmol/10⁶ cells)</th>
<th>dCTP (pmol/10⁶ cells)</th>
<th>dGTP (pmol/10⁶ cells)</th>
<th>dTTP (pmol/10⁶ cells)</th>
<th>dATP/dTTP ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>44.7 ± 3.0</td>
<td>48.8 ± 6.0</td>
<td>27.2 ± 4.2</td>
<td>82.4 ± 10.2</td>
<td>0.54</td>
</tr>
<tr>
<td>dFdCyd 15 nM h 0–4</td>
<td>7.9 ± 2.0</td>
<td>(0.18)</td>
<td>18.4 ± 3.0</td>
<td>80.1 ± 5.8</td>
<td>(0.19)</td>
</tr>
<tr>
<td>FdUrd 10 nM × h 4–8</td>
<td>59.6 ± 14.1</td>
<td>70.8 ± 12.0</td>
<td>15.3 ± 3.2</td>
<td>37.6 ± 11.8</td>
<td>1.59</td>
</tr>
<tr>
<td>dFdCyd → FdUrd</td>
<td>43.7 ± 14.5</td>
<td>(1.33)</td>
<td>18.6 ± 3.6</td>
<td>24.1 ± 7.8</td>
<td>(2.94)</td>
</tr>
</tbody>
</table>

* Pool size as a fraction of control.

Fig. 3  FdUrd-mediated inhibition of TS. TS catalytic activity in cellular lysates was determined by tritium release from [5-3H]dUMP. The concentrations of dFdCyd (dFdC) and FdUrd were 15 and 10 nM, respectively, and the exposure times are shown. The data, presented as the means, are from five or more separate experiments; bars, SE. TS catalytic activity was significantly different from control for both sequential exposures of dFdCyd followed by FdUrd h 4–8 (P = 0.036) and h 4–28 (P = 0.034, t test).

This was significantly lower than control with sequential dFdCyd and FdUrd (4- and 24-h exposures; P < 0.04, t test). Because enzyme activity in the catalytic assay is assessed by the release of tritium from [5-3H]dUMP, potential expansion of endogenous dUMP pools due to TS inhibition might lead to overestimation of the extent of TS inhibition. Therefore, Western immunoblot analysis was also used to assess TS ternary complex formation. A single band migrating at 35 kDa was evident in control cells and cells treated with dFdCyd alone, reflecting free TS. In contrast, a slightly higher molecular weight band that represented bound TS was seen in addition to the 35-kDa band in cells treated with FdUrd (data not shown). After a 4-h exposure to FdUrd alone or with preexposure to dFdCyd, densitometric analysis showed that the percentage of bound TS was 54 ± 6% and 75 ± 2% (mean ± range/2, n = 2 experiments). After a 24-h exposure to FdUrd, bound TS accounted for 67 ± 1% with FdUrd alone (mean ± SD, n = 3) and 79 ± 2% with dFdCyd followed by FdUrd. These results are consistent with the catalytic assay.

The effects of drug exposure on cell cycle distribution over time are shown in Table 4. For these experiments, cells were exposed to either diluent or to 15 nM dFdCyd for the initial 4 h. After drug removal, FdUrd or diluent was added for either an additional 4 h or 24 h. The cells were then harvested at the indicated times. No major effects were evident at 8 h; however, S-phase accumulation was evident at 28 h with dFdCyd and FdUrd given individually. The most striking effects were observed with the two drugs given sequentially. The S-phase accumulation had largely dissipated at h 50 (46 h and 22 h after removal of dFdCyd and FdUrd, respectively), indicating that the cells had progressed through the cell cycle.

**DNA Damage.** The effect of drug exposure on the fragility of newly synthesized DNA was determined by fixed pH alkaline elution. Increasing single-strand breaks are reflected by a decreasing proportion of the total labeled DNA species retained on the filter relative to control. When the cells were labeled and harvested immediately after a 4-h exposure to dFdCyd at concentrations ranging from 10 to 40 nM, the percentage retained on the filter decreased with increasing drug concentration from 79% of control to 39% of control, respectively (r² = 0.91; data not shown). In addition, the extent of nascent DNA damage was inversely related to colony formation (r² = 0.78; data not shown). With a 4-h exposure to 15 nM dFdCyd followed by a 24-h drug-free period, however, the extent of single-strand breaks in nascent DNA was not as great (88% of nascent DNA retained versus control; Fig. 4). This observation suggests that interference with nascent DNA synthesis at this concentration of dFdCyd is greatest during drug exposure, consistent with prior reports (8). After a 24-h exposure to FdUrd, with the cells labeled for the final 4 h, an inverse correlation was also noted between nascent DNA retained and FdUrd concentration (r² = 0.83; data not shown). After exposure to 10 nM FdUrd for 24 h, only 78% of the nascent DNA was retained compared with control, whereas nascent DNA damage was greatest after sequential 10 nM dFdCyd at h 0–4 to 10 nM FdUrd at h 4–28. The alkaline-labile DNA strand breaks in newly synthesized DNA are presumably due to both deoxyribonucleotide triphosphate imbalance, which may interfere with
Cytotoxicity with Sequential Gemcitabine and FdUrd

The cell cycle distribution was determined at the indicated times following exposure to either no drug, 15 nm dFdCyd for the initial 4 h, and 10 nm FdUrd for either h 4–8 or h 4–28. The data for the 8-h and 50-h time points are from a single experiment. The results for the 24-h time point, shown as the mean ± range/2, are from two separate experiments. The data for controls are presented as the mean and SD and are pooled results from these four separate experiments. Information on 10,000–20,000 cells was collected for each sample.

<table>
<thead>
<tr>
<th>First exposure h 0–4</th>
<th>Second exposure</th>
<th>Time of analysis</th>
<th>% in G1</th>
<th>% in G2/M</th>
<th>% in S</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>No drug</td>
<td>8, 28, and 50 h</td>
<td>54 ± 8</td>
<td>13 ± 2</td>
<td>33 ± 9</td>
</tr>
<tr>
<td>dFdCyd</td>
<td>No drug</td>
<td>8 h</td>
<td>60</td>
<td>6</td>
<td>34</td>
</tr>
<tr>
<td>No drug</td>
<td>FdUrd h 4–8</td>
<td>8 h</td>
<td>48</td>
<td>6</td>
<td>45</td>
</tr>
<tr>
<td>dFdCyd</td>
<td>FdUrd h 4–8</td>
<td>8 h</td>
<td>58</td>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>dFdCyd</td>
<td>No drug h 4–28</td>
<td>28 h</td>
<td>9 ± 7</td>
<td>27 ± 1</td>
<td>63 ± 7</td>
</tr>
<tr>
<td>No drug</td>
<td>FdUrd h 4–28</td>
<td>28 h</td>
<td>8 ± 8</td>
<td>33 ± 6</td>
<td>59 ± 2</td>
</tr>
<tr>
<td>dFdCyd</td>
<td>FdUrd h 4–28</td>
<td>28 h</td>
<td>6 ± 4</td>
<td>13 ± 2</td>
<td>81 ± 3</td>
</tr>
<tr>
<td>dFdCyd</td>
<td>No drug h 4–50</td>
<td>50 h</td>
<td>38</td>
<td>27</td>
<td>25</td>
</tr>
<tr>
<td>No drug</td>
<td>FdUrd h 4–28</td>
<td>50 h</td>
<td>49</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>dFdCyd</td>
<td>FdUrd h 4–28</td>
<td>50 h</td>
<td>38</td>
<td>35</td>
<td>27</td>
</tr>
</tbody>
</table>

Fig. 4 Induction of single-strand breaks in nascent DNA. Exponentially growing cells were exposed to either no drug or to 15 nm dFdCyd (dFdC) and/or 10 nm FdUrd as indicated. The cells were incubated with [14C]thymidine (0.05 μCi/ml) from h 24 to 28 and then were harvested and subjected to alkaline elution (pH 12.1) for 15 h. For each condition, the counts retained on the filter were determined relative to the total amount of counts on the filter and in the eluting fractions. The fraction retained on the filter for drug-treated cells was then expressed as a percentage of control for each experiment. The data are presented as the means and are from five or more separate experiments done in duplicate; bars, SE. In control cells, the fraction of [14C]thymidine-labeled DNA retained on the filter was 78.9 ± 4.0%.

Oligonucleosomal DNA laddering was not detected using conventional agarose electrophoresis for up to 72 h after drug removal (data not shown). Because traditional DNA purification techniques result in extensive shearing of DNA, induction of high molecular mass DNA fragments might be overlooked. DNA was therefore purified from intact cells, embedded in agarose plugs and analyzed by pulsed-field gel electrophoresis. Delayed appearance of parental DNA fragmentation of <1-Mb fragments was observed in cells treated with sequential dFdCyd→FdUrd 72 and 96 h after drug exposure (Fig. 5).

**DISCUSSION**

We found that sequential exposure to dFdCyd for 4 h followed by FdUrd for 24 h led to more than additive growth inhibition and interference with colony formation. To simplify the analyses, the IC50 concentrations were used to study possible mechanism(s) of interaction between the drugs. Our results indicate that giving dFdCyd first allowed its metabolism and incorporation of dFdCTP into DNA to proceed without interference. Furthermore, preexposure to dFdCyd did not perturb FdUrd metabolism. The opposite sequence was not studied because we established previously that fluoropyrimidine administration prior to Ara-C decreased Ara-C DNA incorporation and was associated with marked antagonism in cytotoxicity assays (16). Because incorporation of both Ara-C and dFdCyd are essential prerequisites for induction of programmed cell death, it seemed logical to avoid an antagonistic sequence in the present studies. The present results are consistent with our prior study in that sequential administration of a deoxycytidine analogue followed by a 24-h exposure to a 5-fluoropyrimidine resulted in more than additive cytotoxicity. Exposure to FdUrd after dFdCyd avoided any potential interference with dFdCyd metabolism and DNA incorporation. Whereas preexposure to dFdCyd did not affect freeFdUMP formation, the extent of TS inhibition early during FdUrd exposure was greater than that observed with FdUrd alone. The basis for this observation is not clear, but the dTTP pool data corroborated this phenomenon.

Acute biochemical and metabolic derangements seen with the combination included pronounced effects on the cell cycle with S-phase accumulation, depletion of dTTP, an increase in the dATP:dTTP ratio, and induction of single-strand breaks in both DNA synthesis and repair, and incorporation of fraudulent nucleotides into DNA either directly (as with dFdCyd) or indirectly (as with FdUrd by stimulating excision/repair).

There was no evidence of parental DNA damage as measured by either single-strand or double-strand breaks immediately after exposure to dFdCyd alone, FdUrd alone, or the combination. Induction of double-strand breaks in parental DNA is a hallmark of programmed cell death. We therefore monitored the cells for delayed induction of double-strand parental DNA damage at 24-h intervals after exposure to sequential 15 nm dFdCyd at h 0–4 to 10 nm FdUrd at h 4–28.
cells are representative of the many cell types, particularly of epithelial cancer cells. Previous investigators have reported that cytotoxic agents occurring when certain epithelial cancer cells are exposed to experimental conditions used. Furthermore, the delayed appearance and persistence of DNA fragmentation in the HT-29 cells in the present report may signify that exposure of epithelial cancer cell lines to various DNA-damaging agents, including FdUrd, produced high molecular mass DNA fragmentation. These results provide a rationale for clinical evaluation of this drug combination. Whether any potential sequence-dependent interactions will be observed in the clinical setting with 5-fluorouracil given either by bolus injection or by infusion either immediately before or after a conventional 30-min dFdCyd infusion remain to be determined.

REFERENCES


25. Pollera, C. F., Ceribelli, A., Crecco, M., Oliva, C., and Calabresi, F. Prolonged infusion gemcitabine, a clinical phase I study at low- (300 mg/m2) and high-dose (875 mg/m2) levels. Invest. New Drugs, 15: 115–121, 1997.
Cytotoxicity and DNA fragmentation associated with sequential gemcitabine and 5-fluoro-2'-deoxyuridine in HT-29 colon cancer cells.

Q Ren, V Kao and J L Grem


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/4/11/2811

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.