Enhanced Radiosensitization with Gemcitabine in Mismatch Repair-Deficient HCT116 Cells

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ABSTRACT

Gemcitabine [2',2'-difluoro-2'-deoxycytidine (dFdCd)] is a potent ionizing radiation sensitizer in solid tumor cells in vitro and in vivo. Previously, we have demonstrated (Shewach et al., Cancer Res., 54: 3218–3223, 1994) a strong correlation between depletion of dATP (caused by dFdCyd diphosphate-mediated inhibition of ribonucleotide reductase) and radiosensitization. In addition, we and others (Latz et al., Int. J. Radiat. Oncol. Biol. Phys., 41: 875–882, 1998; Ostruszka and Shewach, Cancer Res., 60: 6080–6088, 2000) have shown that the accumulation of cells in S phase prior to irradiation is also important for radiosensitization with dFdCyd. This led us to hypothesize that the incorporation of incorrect nucleotides because of the dATP pool imbalance was important for radiosensitization with dFdCyd, and, therefore, cells deficient in mismatch repair (MMR) would exhibit greater radiosensitization. We tested this hypothesis by evaluating the ability of HCT116 colon carcinoma cell lines, which differ in MMR proficiency, to be radiosensitized by dFdCyd. The MMR-proficient cell line (HCT116 + ch3) was more sensitive to dFdCyd alone than were the MMR-deficient cell lines (HCT116, HCT116 + ch2, and HCT116 p53−/−). Interestingly, the MMR-proficient cells could not be radiosensitized at concentrations of dFdCyd ≤IC50, although extremely high concentrations of dFdCyd (>IC50) enhanced cell killing with radiation. In contrast, the MMR-deficient cells were radiosensitized at concentrations of dFdCyd ≤IC50 with radiation enhancement ratios of ~1.5. Cell cycle analysis, using dual parameter flow cytometry, demonstrated that all of the cell lines accumulated in S phase after dFdCyd treatment, and, shortly after irradiation, a prominent but transient G2-M block was observed. In the MMR-deficient cells, the IC50 for dFdCyd produced a ≥80% decrease in dATP within 4 h after drug addition, and this low dATP level was maintained for another 12–20 h. Although the IC50 of dFdCyd was unable to sustain a >80% decrease in the dATP level in the MMR-proficient cells, the IC50 did achieve this level of dATP depletion; however, it was unable to radiosensitize the MMR-proficient cells. Similar results were obtained with HCT116 cells, in which the MMR deficiency was corrected by transfection with a vector containing the hMLH1 cDNA. In addition, the deletion of p53 did not increase radiation enhancement ratios. These results demonstrate that MMR deficiency promotes radiosensitization with dFdCyd. We suggest that dATP depletion produces errors of replication in MMR-deficient cells, which, if left unrepaired, enhances cell death by ionizing radiation.

INTRODUCTION

Gemcitabine (dFdCyd) is a nucleoside analogue with broad-spectrum activity in the treatment of solid malignancies, particularly pancreatic (1, 2) and non-small cell lung cancer (3, 4). In vitro studies have demonstrated that nontoxic concentrations of dFdCyd can enhance cell killing by ionizing radiation (termed “radiosensitization”) in colorectal, breast, pancreatic, ovarian, and non-small cell lung cancer cell lines (5–8). In addition, dFdCyd, when combined with ionizing radiation, can increase tumor growth delay in animal models (9–12). Preliminary results from clinical trials suggest that dFdCyd functions as a radiosensitizer in patients as well (13–17).

To produce its cytotoxic and radiosensitizing effects, dFdCyd requires intracellular phosphorylation (18). The diphosphate, dFdCDP, is a potent inhibitor of ribonucleotide reductase (19). In solid tumor cells, this inhibition results in a decrease in dATP levels primarily. The triphosphate, dFdCTP, can incorporate into DNA and inhibit DNA synthesis through its competitive (with dCTP) inhibition of DNA polymerases (20, 21). Incorporation of dFdCTP into DNA has been correlated with cytotoxicity (20, 22, 23), whereas inhibition of dNTP synthesis (or dATP depletion) accounts primarily for the inhibition of DNA synthesis (24).

Although the radiation-sensitizing ability of dFdCyd has been well documented, the mechanism has yet to be fully characterized. Unlike the radiosensitizer BrdUrd, which can increase radiation-induced DNA damage (25, 26) and decrease the rate of DNA repair (27, 28), dFdCyd neither increases double-strand breaks nor decreases the rate of their repair (29). Correlative studies have suggested that the dFdCDP-mediated decrease in dATP is important for radiosensitization (6, 8, 29, 30). In addition, cell cycle analysis has suggested that dFdCyd-treated cells must be in S phase at the time of irradiation in order for maximal radiosensitization to occur (31, 32).

Little is known about DNA repair pathways that may alter cytotoxicity or radiosensitivity with dFdCyd. dFdCMP in template DNA has been shown to increase mismatches opposite it in the nascent strand (33). The decrease in the levels of the endogenous dNTPs mediated by dFdCDP may also lead to misincorporations in DNA, because dNTP pool imbalances have been demonstrated to produce errors in DNA replication (34–36). Therefore, MMR may play an important role in correcting errors in DNA induced by dFdCyd. Mutations in MMR genes have been associated with the development of hereditary nonpolyposis colorectal cancer, a common colon cancer with over 6000 cases reported each year (37–40). In addition, MMR deficiency may alter the response of some tumors to common therapies such as 6-thioguanine (41–43). The MMR system is a complex of proteins involved in the recognition and repair of mismatched bases in DNA (37) or the detection of DNA damage that can lead to an injury signal and apoptosis (44). At least six different proteins in mammalian cells have been identified that can participate in MMR (hMLH2, hMLH6, hMLH3, hMLL1, hPMS2, and hMLH3).
Inactivation of hMLH1 and hMSH2 have been most commonly identified in hereditary nonpolyposis colorectal cancer and sporadic colorectal tumors (37, 46). hMLH1 is a key protein involved in stabilization of the MMR complex (37, 47) and hMSH2 is involved in the recognition of DNA mismatches (41).

We hypothesize that MMR is important for dFdCyd radiosensitization. The depletion of dATP via dFdCDP-mediated inhibition of ribonucleotide reductase may lead to misincorporation of nucleotides in DNA. This lesion, if unrepaird, may result in greater cell killing in combination with ionizing radiation. Therefore, we would predict that cells deficient in MMR would be more sensitive to dFdCyd and radiation, because of the inability to recognize and repair damage from dFdCyd, compared with MMR-proficient cells. We have evaluated this hypothesis in matched HCT116 colorectal cell lines that differ in MMR proficiency. A preliminary account of a portion of these results has been reported (48, 49).

MATERIALS AND METHODS

Cell Culture and Drug Preparation. The HCT116, HCT116 + ch2, HCT116 + ch3, and HCT116 p53−/− cell lines were cultured in RPMI 1640 (Invitrogen, Grand Island, NY), supplemented with 10% calf serum (Invitrogen), and 2 mM L-glutamine (Fisher Scientific, Fair Lawn, NJ). The HCT116 1-2 cells (containing full-length wild-type hMLH1 cDNA) and the HCT116 0-1 cells (empty vector control) were maintained in DMEM (Invitrogen), supplemented with 10% FCS (Invitrogen), 2 mM L-glutamine (Fisher Scientific), 100 μg/ml hygromycin (Invitrogen), and penicillin/streptomycin (Invitrogen; Ref. 43). Cells were maintained in logarithmic growth as a monolayer in T-75 culture flasks at 37°C in a humidified atmosphere containing 5% CO2. Gencitabine, a generous gift from Eli Lilly and Co. (Indianapolis, IN), was dissolved in PBS to obtain a stock solution.

Cell Survival Assay. After dFdCyd and/or radiation treatment, cells were assessed for clonogenic survival as described previously (6). Radiation survival data from dFdCyd-treated cells were corrected for plating efficiency using an unirradiated plate treated with dFdCyd under the same conditions. Cell survival curves were fit using a linear-quadratic equation. Radiation sensitivity is expressed in terms of the mean inactivation dose, which represents the area under the cell survival curve (50). Radiosensitization is expressed as an enhancement ratio, which is defined as the mean inactivation dose (control): mean inactivation dose (dFdCyd).

Irradiation of Cells. Monolayer cultures of cells were irradiated using 60Co (AECL Theratron 80) at 0.1–2 Gy/min. Dosimetry was carried out using an ionizing chamber connected to an electrometer system that was directly traceable to a National Institute of Standards and Technology standard. All of the cells were irradiated at room temperature.

High-Performance Liquid Chromatography Analysis. Cellular nucleotides were assayed as described previously (6). Briefly, for the determination of dFdCTP, cells were incubated with [3H]dFdCyd (Moravek Biochemicals, Inc., Brea, CA), and nucleotides were harvested by trypsinization and were extracted using 0.4N perchloric acid. Neutralized extracts were stored at −20°C until analysis. Ribonucleotides were removed from the extracts using a boronate affinity column (51). Cellular dNTPs and dFdCTP were separated and quantified by strong anion exchange high-performance liquid chromatography using a Waters Alliance (Milford, MA) gradient system equipped with a photodiode array detector and controlled by Millennium 2010 software. Samples were loaded onto a Partisphere 4.6 × 250 mm strong anion exchange column (Whatman, Millipore, OR), and nucleotides were eluted at 2 ml/min with a linear gradient of ammonium phosphate buffer ranging in concentration from 0.15 M (pH 2.8) to 0.60 M (pH 2.9 or 3.4). Nucleotides were identified based on their UV absorbance spectrum and quantified at either 254 or 281 nm by comparison to the absorbance of a known amount of authentic standard. [3H]dFdCTP was identified by collecting 0.5 minute fractions using an automated fraction collector and adding 9 ml of ScintVerse liquid scintillation mixture (Fisher Scientific). Radioactivity was measured using a LS6000 series liquid scintillation system from Beckman Instruments, Inc. (Fullerton, CA).

Flow Cytometry Analysis. Cells were incubated in the dark with 30 μM BrdUrd (Sigma Chemical Co., St. Louis, MO) for 15 min before the conclusion of the incubation period and processed as previously described using a purified mouse anti-BrdUrd primary antibody (1:100) and a goat antimouse FITC-conjugate antibody (1:20; Refs. 52 and 53). Total DNA was stained with propidium iodide, and samples were analyzed for BrdUrd and propidium iodide content using a Coulter EPICS Elite EPS flow cytometer.

Western Blot Analysis. An analysis of protein expression by Western blot analysis was performed essentially as described previously (53). Commercially available antibodies were used for expression of p53 (Ab-6 monoclonal mouse IgG antibody; Calbioche, hMLH1 (polyclonal rabbit IgG antibody; Santa Cruz Biotechnology), and hMSH2 (polyclonal rabbit IgG antibody; Santa Cruz Biotechnology). Proteins that bound the antibodies were visualized using an enhanced chemiluminescence detection system (Pierce, Rockford, IL).

dFdCMP Incorporation into DNA. Cells were incubated for up to 24 h with [3H]dFdCyd, and DNA was harvested periodically using a DNeasy kit (Qiagen, Valencia, CA). The amount of DNA in each sample was determined by UV, an aliquot of DNA was added to 5 ml of scintillation fluid, and radioactivity was measured by scintillation spectrometry.

RESULTS

Cytotoxicity of dFdCyd. The sensitivity to dFdCyd of the MMR-deficient HCT116, HCT116 + ch2, and HCT116 p53−/− cell lines and the MMR-proficient HCT116 + ch3 cell line was studied to determine a range of nontoxic and toxic drug concentrations. Western blot analysis (data not shown) demonstrated that only the HCT116 + ch3 cell line expressed the hMLH1 protein, in which MMR proficiency was restored by transferring chromosome 3 containing a functional copy of the hMLH1 gene (54). Transfer of chromosome 2 (HCT116 + ch2 cell line) controlled for the chromosome transfer procedure. All of the cell lines expressed hMSH2, and p53 was expressed in all but the HCT116 p53−/− cell line. As illustrated in Fig. 1, both the HCT116 and HCT116 + ch2 cell lines showed similar sensitivity over a range of 1 to 100 nM dFdCyd after a 24-h drug incubation. The HCT116 + ch3 cells were more sensitive to dFdCyd treatment, whereas the HCT116 p53−/− cells were the least sensitive to dFdCyd. From these data, nontoxic concentrations of dFdCyd (≤10% decrease in cell survival) were estimated as 30 nm for the HCT116 and HCT116 + ch2 cell lines, and 15 and 300 nm for the HCT116 + ch3 and HCT116 p53−/− cell lines, respectively. Cytotoxic concentrations of dFdCyd used for radiosensitization studies, representing ~50% cell kill, were 40 nm, 90 nm, 100 nm, and 800 nm for the HCT116 + ch3, HCT116 + ch2, HCT116, and HCT116 p53−/− cell lines, respectively.
Radiosensitization by dFdCyd. The ability of dFdCyd to radiosensitize HCT116, HCT116 + ch2, HCT116 + ch3, and HCT116 p53−/− cells was examined by irradiating cells after a 24-h incubation with dFdCyd. As illustrated in Table 1, the nontoxic concentrations of dFdCyd did not significantly enhance the sensitivity of the HCT116 + ch2 and HCT116 + ch3 cell lines to radiation-induced cytotoxicity, but the parental HCT116 cells were radiosensitized at 30 nm with a radiation-enhancement ratio of 1.3. Incubation with the IC₅₀ for dFdCyd produced radiosensitization in the HCT116, HCT116 + ch2, and HCT116 p53−/− cells, with radiation enhancement ratios of ~1.5. In contrast, in the HCT116 + ch3 cells, incubation with ≤IC₅₀ of dFdCyd did not significantly enhance cell death from radiation. Although the low radiation enhancement ratio of 1.2 with the 40 nM dFdCyd (IC₅₀) was significantly greater than 1.0 according to statistical analysis, it does not appear to be of biological significance because the higher concentration of 100 nM dFdCyd (IC₉₀) did not produce significant radiosensitization. Radiosensitization in the HCT116 + ch3 cells was observed only at excessively high concentrations of dFdCyd of 300 nm, which by itself killed >96% of the cells.

Because the MMR deficiency was corrected by the insertion of an entire chromosome, which may have altered cytotoxicity and/or radiosensitization because of other genes on chromosome 3, we also determined dFdCyd sensitivity and radiosensitization in HCT116 cells transfected with hMLH1 cDNA. Similar to the results with the HCT116 + ch3 cells, the HCT116 1-2 (hMLH1 cDNA) cells were approximately three times more sensitive to dFdCyd compared with the empty vector control HCT116 0-1 cells, based on relative IC₅₀ values. Furthermore, only the MMR-deficient HCT116 0-1 cells exhibited significant radiosensitization with dFdCyd (Table 1).

Effect of dFdCyd on Nucleotide Pools. In other solid tumor cell lines, radiosensitization with dFdCyd has correlated with a >80% decrease in the endogenous dATP levels because of dFdCDP-mediated inhibition of ribonucleotide reductase (8, 30, 53). Therefore, effects on dFdCTP accumulation and the endogenous dNTP pool levels were examined in the HCT116, HCT116 + ch2, HCT116 + ch3, and HCT116 p53−/− cells during incubation with dFdCyd for 24 h. All of the cell lines achieved their peak dFdCTP levels between 2 and 8 h after drug addition as shown in Fig. 2 (range of 0.15 to 1.00 nmol dFdCTP per 10⁷ cells). Most cell lines retained >70% of the peak level of dFdCTP for the remainder of the incubation period. However, the HCT116 p53−/− cell line showed a rapid decrease in dFdCTP after achieving a peak level at 2 h after incubation with 500 or 800 nm dFdCyd (Fig. 2B). By the end of the drug incubation, <0.03 nmol dFdCTP per 10⁷ cells remained in this cell line. The low level of dFdCTP in the HCT116 p53−/− cell line appeared to be attributable to a greater deamination of the drug. We observed that at least twice as much of the deamination product, 2′,2′-difluoro-2′-deoxyuridine, was generated throughout the 24-h incubation period in the p53−/− cells compared with the other HCT116 strains (data not shown). This differential metabolism also appears to explain the difference in IC₉₀ values between the cell lines.

All of the cell lines incubated with radiosensitizing concentrations of dFdCyd exhibited a significant decrease in dATP levels between 0 and 4 h to below 20% of control levels as shown in Fig. 3A. In the HCT116, HCT116 + ch2, and HCT116 p53−/− cell lines, the dATP levels remained low throughout the rest of the 24 h incubation period (Fig. 3A). Although the addition of chromosome 2 should have provided an extra copy of the gene for the R2 subunit of ribonucleotide reductase in the HCT116 + ch2 cells, that apparently did not interfere with the ability of dFdCDP to inhibit ribonucleotide reductase, because >90% depletion of dATP depletion was observed in these cells during dFdCyd incubation. Fig. 3B shows the HCT116 + ch3 cells at the IC₉₀ for dFdCyd, in which the dATP levels decreased between 0 and 4 h but then increased and returned to control levels by the end of the drug incubation. At the IC₉₀ for dFdCyd, the dATP levels in these cells remained below 20% of control for the majority of the incubation (Fig. 3B).

Table 1. Effect of dFdCyd on the sensitivity of HCT116, HCT116 + ch2, HCT116 0-1, HCT116 + ch3, HCT116 1-2, and HCT116 p53−/− cells to ionizing radiation

<table>
<thead>
<tr>
<th>MMR status</th>
<th>Cell line</th>
<th>[dFdCyd]</th>
<th>Radiation enhancement ratio</th>
<th>D-bar (no drug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMR-deficient</td>
<td>HCT116</td>
<td>30 nm</td>
<td>1.3 ± 0.1a</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 nm</td>
<td>1.5 ± 1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HCT116 + ch2</td>
<td>30 nm</td>
<td>1.1 ± 0.1</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>90 nm</td>
<td>1.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HCT116 0-1</td>
<td>10 nm</td>
<td>1.3 ± 0.1a</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 nm</td>
<td>1.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>MMR-proficient</td>
<td>HCT116 + ch3</td>
<td>15 nm</td>
<td>1.1 ± 0.1</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40 nm</td>
<td>1.2 ± 0.1a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 nm (IC₅₀)</td>
<td>1.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>300 nm (IC₉₀)</td>
<td>1.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HCT116 1-2</td>
<td>1 nm</td>
<td>1.1 ± 0.1</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 nm</td>
<td>1.1 ± 0.1</td>
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</tr>
<tr>
<td></td>
<td>MMR-deficient</td>
<td>HCT116 p53−/−</td>
<td>500 nm (IC₅₀)</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800 nm</td>
<td>1.5 ± 0.2a</td>
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</table>

* Significantly >1.0 (P < 0.05).

Fig. 2. Accumulation of dFdCTP in HCT116 cells. Exponentially growing cells were incubated with dFdCyd for 24 h. Cells were harvested periodically and analyzed for dFdCTP content as described in Materials and Methods. Each point represents an average of at least duplicate determinations; bars, SE. A, HCT116 (100 nm); B, HCT116 + ch2 (90 nm); C, HCT116 + ch3 (40 nm); D, HCT116 + ch3 (100 nm). B, 500 nm HCT116 p53−/−; C, 800 nm HCT116 p53−/−.
Effect of dFdCyd and/or Radiation on Cell Cycle Distribution. Because previous studies suggested that progression of cells into S phase is important for radiosensitization by dFdCyd (31, 32), the cell cycle distribution of the HCT116, HCT116 + ch2, HCT116 + ch3, and HCT116 p53−/− cells was examined after treatment with dFdCyd and/or ionizing radiation (Tables 2 and 3). After a 24-h incubation with dFdCyd at the IC50, more than 75% of the cells had accumulated in S phase in all four of the cell lines. During the 72 h after drug washout, the percentage of cells in S phase decreased in all of the cell lines to near control levels. In contrast, 24 h after a 5-Gy dose of ionizing radiation, more than 30% of the cells were accumulated in G2-M in all of the cell lines. These cells appeared to be blocked in G2-M for up to 48 h after irradiation, because high G2-M and low S-phase percentages persisted throughout this time, and the cell numbers did not change significantly (data not shown). The combination of dFdCyd and radiation initially produced an early S-phase accumulation, followed by a G2-M block at 24–48 h after drug washout in all of the cell lines. The G2-M block was sustained for up to 72 h after drug washout/irradiation. Similar results were observed initially with the IC10 of dFdCyd in the HCT116 + ch2 and HCT116 + ch3 cells, in which a 24-h incubation with dFdCyd produced an accumulation of cells in S phase, but cells showed a normal cell cycle distribution by 24 h after drug washout (data not shown). The percentage of apoptotic cells was not increased in any of the cell lines above 8.5% (control values, 0.2–3.3%) after any of the drug and/or radiation treatments. Increased levels (∼12–18%) of SNI (represents dying cells) were apparent in the HCT116 and HCT116 p53−/− cells treated with dFdCyd alone or with radiation at 48 and 72 h after drug washout. The other cell lines did not show a significant increase in SNI.

**dFdCMP Incorporation into DNA.** As shown in Fig. 4, the amount of dFdCMP in the DNA for each cell line increased during a 24-h drug incubation. When incubated with dFdCyd for 24 h at their respective IC50 values, the HCT116 parent, HCT116 + ch2, and the HCT116 p53−/− cell lines all incorporated similar levels of dFdCMP (31, 28, and 27 fmol dFdCMP per μg DNA after 24 h). In the HCT116 + ch3 cells, increasing the amount of dFdCyd from 40 nM to 100 nM dFdCyd (IC50) resulted in approximately a 2-fold increase in the incorporation of dFdCMP in DNA. The HCT116 + ch2 cell line exhibited substantially higher incorporation when incubated with dFdCyd at its IC50 value (∼82 fmol dFdCMP per μg of DNA after 24 h).

### Table 2. Effect of dFdCyd and/or radiation on cell cycle distribution

<table>
<thead>
<tr>
<th></th>
<th>.h TCT116 + ch3</th>
<th></th>
<th>HCT116 + ch2</th>
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</thead>
<tbody>
<tr>
<td>IC50 dFdCyd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>61.3</td>
<td>26.7</td>
<td>7.3</td>
</tr>
<tr>
<td>24</td>
<td>36.0</td>
<td>43.0</td>
<td>17.4</td>
</tr>
<tr>
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<td>60.8</td>
<td>23.3</td>
<td>11.5</td>
</tr>
<tr>
<td>72</td>
<td>50.0</td>
<td>39.5</td>
<td>7.9</td>
</tr>
<tr>
<td>5 Gy</td>
<td>63.6</td>
<td>24.6</td>
<td>8.9</td>
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<tr>
<td>72</td>
<td>42.5</td>
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<td>22.9</td>
</tr>
<tr>
<td>IC50 dFdCyd + 5 Gy</td>
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<tr>
<td>0</td>
<td>17.4</td>
<td>76.4</td>
<td>3.3</td>
</tr>
<tr>
<td>24</td>
<td>36.0</td>
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<tr>
<td>72</td>
<td>38.5</td>
<td>20.0</td>
<td>34.0</td>
</tr>
</tbody>
</table>

Zero hour represents the time at drug washout after a 24-h incubation with dFdCyd and/or irradiation. Cells were harvested periodically as indicated and cell cycle distribution was analyzed by dual parameter flow cytometry as described in “Materials and Methods.”
DISCUSSION

Although the potent radiosensitizing ability of dFdCyd has been well documented both in vitro and in vivo, the mechanism by which this occurs has not been elucidated. On the basis of previous findings by us and others that radiosensitization with dFdCyd correlated with dATP depletion and S-phase accumulation (31, 53), but not with an alteration in DNA double-strand breaks or their repair (29, 55), we have hypothesized that DNA mismatches result from the dATP pool imbalance and that these are the lesions that produce radiosensitization. By comparing cells that differed in their ability to repair DNA mismatches, we have demonstrated that the MMR-deficient cells are better radiosensitized by dFdCyd than are the corresponding MMR-proficient cells. These results suggest that DNA mismatches occur with dFdCyd exposure and that they are an important determinant of radiosensitization with dFdCyd.

MMR deficiency in tumors is attributable to germ-line mutations and the inactivation of MMR proteins, most commonly hMLH1 and hMSH2 (37, 40). Tumor cell lines that are deficient in MMR are resistant to many chemotherapeutic agents such as alkylating agents (42), cisplatin (56), and doxorubicin (57). It has been reported previously that the HCT116 MMR-deficient cell line (wild-type p53) is less sensitive to dFdCyd compared with the MMR-proficient subline (56), and the results reported here confirm this finding. Our results further suggest that the higher sensitivity of the HCT116 + ch3 cell line compared with the parental HCT116 cell line is mediated by increased incorporation of dFdCMP into DNA at equimolar doses of dFdCyd. This increased incorporation could not be explained by higher levels of dFdCTP. We note that the relationship between dFdCMP incorporation and cell death does not appear to differ between the cell lines, because equitoxic doses showed similar levels of incorporation in the HCT116, HCT116 p53−/−, and the HCT116 + ch3 cells. Although we do not know the mechanism responsible for the difference in incorporation, it is possible that, in the MMR-proficient cells, the activation of MMR with the subsequent resynthesis of large tracts (58) of DNA results in increased incorporation of dFdCMP. The reason for the higher incorporation of dFdCMP in DNA in the HCT116 + ch2 cells at equitoxic doses is not clear but may occur as a consequence of an additional gene(s) donated by the extra copy of chromosome 2. For example, this chromosome also contains the genes for the R2 subunit of ribonucleotide reductase, as well as the genes for hMSH6 and hPMS1, any of which may alter incorporation or repair of dFdCMP in DNA. Regardless of the mechanism, the data indicate that the HCT116 + ch2 cells are more tolerant of dFdCMP in DNA.

It has been reported by others that a deficiency of mlh1 results in increased survival of fibroblast cells exposed to ionizing radiation (59). Results presented here demonstrate higher survival for the parental HCT116 cells exposed to ionizing radiation compared with the HCT116 + ch3 cells, as shown by a higher D-bar value (Table 1). However, the HCT116 + ch2 cells showed similar sensitivity to ionizing radiation as the HCT116 + ch3 cell line, and there was only a small difference in D-bar values in the cDNA-transfected cell lines. These results suggest that hMLH1 deficiency alone results in marginal differences in sensitivity to ionizing radiation.

As hypothesized, the MMR-deficient cell lines (HCT116,
HCT116 p53\(^{−/−}\) were radiosensitized at \(<IC_{50}\) for dFdCyd, whereas the MMR-proficient cell lines (HCT116 + ch3, HCT116 1-2) were not. The innate sensitivity of each cell line to ionizing radiation does not appear to affect radiosensitization with dFdCyd because cell lines with high D-bar values (HCT116 cells) or low D-bar values (HCT116 0-1 cells) exhibited similar radiation enhancement ratios. In addition, all of the cell lines showed similar S-phase accumulation with dFdCyd, demonstrating that cell cycle redistribution to a more radiosensitive phase cannot explain the difference in radiosensitization. These results further emphasize that dFdCMP incorporation into DNA is not associated with radiosensitization because, for similar levels of dFdCMP incorporation in the HCT116 and HCT116 + ch3 cell lines, only the MMR-deficient cell line was radiosensitized. In the HCT116 + ch3 cell line, lack of radiosensitization at the \(IC_{50}\) for dFdCyd may be attributable to the inability to decrease dATP by at least 80% for a sustained period of time. However, dFdCyd at its \(IC_{50}\) (100 nM) depleted dATP by \(>90\%\) for at least 22 h; yet these conditions did not result in radiosensitization. These results were further substantiated by studies in HCT116 cell lines in which the MMR defect was corrected by radiosensitization. These results were further substantiated by studies in HCT116 cell lines in which the MMR defect was corrected by radiosensitization. In the HCT116 + ch3 cell line, only a highly toxic concentration of dFdCyd (300 nM), which by itself killed \(>96\%\) of the cells, produced radiosensitization, suggesting that exposure to high concentrations of dFdCyd can overwhelm MMR and result in radiosensitization. Taken together, these results demonstrate that MMR status is an important factor in radiosensitization with dFdCyd.

Of interest was whether the deletion of p53 would increase radiosensitization in the HCT116 MMR-deficient cell line. Results with the HCT116 p53\(^{−/−}\) cells demonstrated that deletion of p53 did not improve radiation enhancement ratios, but these cells did exhibit decreased sensitivity to dFdCyd. The HCT116 p53\(^{−/−}\) cell line was \(>8\)-fold more resistant to dFdCyd than were the HCT116 and HCT116 + ch2 cell lines based on \(IC_{50}\) values, similar to a previous report (56). This decreased sensitivity appeared to be mediated by altered metabolism of dFdCyd. Whereas the HCT116 parent, HCT116 + ch2, and HCT116 + ch3 cells maintained at least 70% of their peak level of dFdCTP for the duration of the incubation, the HCT116 p53\(^{−/−}\) cells showed a \(>80\%\) decrease in the peak dFdCTP between 2 and 24 h after drug addition. In addition, the HCT116 p53\(^{−/−}\) cells required eight times as much dFdCyd to incorporate similar levels of dFdCMP into DNA compared with the HCT116 and HCT116 + ch3 cells. Thus, the difficulty in maintaining dFdCTP in the HCT116 p53\(^{−/−}\) cells resulted in lower dFdCMP incorporation into DNA and decreased cell death at low dFdCyd concentrations.

Recent reports have demonstrated that IdUr and BrdUr selectively radiosensitized MMR-deficient cells (60, 61). The authors attributed this difference to the lower incorporation of the thymidine analogues into DNA of MMR-proficient cells at equimolar concentrations. Presumably, MMR was able to recognize analogue incorporation and excise these lesions. In contrast, we observed higher incorporation of dFdCyd in DNA of MMR-proficient HCT116 cells. The presence of an iodine or bromine residue on C-5 of the pyrimidine base may form a bulky lesion recognizable by MMR, compared with the smaller fluorines on the sugar of dFdCyd. Because DNA incorporation is thought to be an important determinant of radiosensitization for IdUr and BrdUr, this difference between MMR-deficient and -proficient cells could significantly alter radiosensitization for these analogues. However, it is also possible that differences in DNA incorporation were mediated by lower levels of the 5'-triphosphates for IdUr and BrdUr, which were not measured in the studies. Because these analogue triphosphates inhibit pyrimidine reduction by ribonucleotide reductase (62), lower triphosphate levels would alter the effects on dCTP and dTTP pools. If, as proposed here and previously (63), dNTP pool imbalances contribute to radiosensitization, such effects may also explain, in part, the observed differences in radiosensitization with IdUr and BrdUr.

The results presented here demonstrate that MMR deficiency promotes radiosensitization by dFdCyd in HCT116 colon carcinoma cells. We propose that this occurs through the inability of this cell line to correct errors of replication resulting from dFdCyd-induced imbalance in dNTP pools. Most cell lines do not have a complete deficiency in MMR, but rather express some level of MMR proteins resulting in variable degrees of MMR proficiency (64). We hypothesize that, at low levels of dFdCyd, the existing MMR capability is sufficient to correct mismatches in DNA induced by dFdCyd, but, at higher concentrations, the MMR capability is exceeded and mismatches will persist, resulting in radiosensitization. The data presented here support this hypothesis by demonstrating that, in the MMR-proficient cells, we were able to produce radiosensitization only at highly toxic concentrations of dFdCyd. Studies are currently underway to determine whether or not radiosensitizing concentrations of dFdCyd produce the proposed mismatches in DNA. These results further highlight the observation that MMR-deficient cells are better radiosensitized by nucleoside analogues than are MMR-proficient cells, which may be an important therapeutic strategy.

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**REFERENCES**

Enhanced Radiosensitization with Gemcitabine in Mismatch Repair-Deficient HCT116 Cells

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