Delayed Radiosensitization of Human Colon Carcinoma Cells after a Brief Exposure to 2',2'-Difluoro-2'-deoxycytidine (Gemcitabine)"}

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ABSTRACT
We have shown that 2',2'-difluoro-2'-deoxycytidine (dFdCyd; Gemcitabine), a deoxycytidine analogue, is a potent radiation sensitizer when cells are exposed to it continuously for >16 h in low concentrations (in the range of 10 nm). However, the most common method of clinical administration is by short-term infusion (30–90 min). Therefore, we wished to determine under what conditions dFdCyd could produce radiosensitization after a relatively brief exposure to drug. We hypothesized that the long half-life of the phosphorylated metabolites of dFdCyd would produce long-lasting dNTP pool perturbation, particularly dATP pools, leading to radiosensitization hours or even days after the drug was removed from the medium. We tested this hypothesis by exposing HT29 human colon cancer cells for 2 h to clinically relevant concentrations of dFdCyd, removing the drug from the medium, and assessing radiosensitivity up to 72 h later. We found that 100 nm dFdCyd, which was noncytotoxic, radiosensitized HT29 cells up to 48 h after drug removal. During this period, there was an increase in the S phase population, whereas by 72 h after drug removal, the cell cycle distribution resembled that seen under control conditions. dATP pools remained depleted throughout the 72-h period after drug treatment. This study supports the hypothesis that radiosensitization occurs in cells that are replicating DNA in the presence of perturbed dNTP pools. Furthermore, they may be useful in the design of rational clinical trials using dFdCyd as a radiosensitizer.

INTRODUCTION
dFdCyd3 is an analogue of 1-β-D-arabinofuranosylcytosine that has clinical activity against solid tumors, particularly pancreatic (1, 2) and non-small cell lung cancer (3, 4). dFdCyd must be phosphorylated by deoxycytidine kinase to produce cytotoxicity (5). The phosphorylated metabolites produce multiple cellular effects. 2',2'-difluoro-dCDP can inhibit ribonucleotide reductase, resulting in perturbation of dNTP pools (6), particularly dATP pool depletion (7). dFdCTP blocks DNA polymerases necessary for replication (8, 9) by competing with dCTP. Furthermore, low levels of 2',2'-difluoro-dCMP can be misincorporated into DNA, which decreases the fidelity of DNA replication (10).

In addition to its cytotoxic effects, dFdCyd is a potent radiation sensitizer of EMT6 rodent tumor cells (11) and a variety of human tumor cell lines (7, 12, 13). We have found that continuous exposure to noncytotoxic concentrations of dFdCyd (10 nm) radiosensitizes HT29 human colon cancer cells after approximately 16–24 h (7). Radiosensitization appears to be associated not with the accumulation of the cytotoxic metabolite dFdCPT but with the depletion of dATP pools in cells that were redistributed throughout S phase (7, 12).

After investigation of several infusion schedules in Phase I/II trials, dFdCyd is now most commonly administered as a brief infusion once a week. Therefore, we wished to extend our initial studies using a long continuous exposure to determine whether radiosensitization could be obtained using a brief exposure to clinically relevant concentrations of dFdCyd. In view of the prolonged retention of the toxic metabolite dFdCPT (7, 14), we hypothesized that a brief exposure to dFdCyd could produce significant delayed radiosensitization, which would correlate with a prolonged suppression of dATP pools. We found that radiosensitization equivalent to or greater than that which resulted from a 24-h continuous incubation with a low concentration of dFdCyd was produced 24–48 h after a 2-h exposure to 100 nm or 3 μM dFdCyd. Increased sensitivity was seen in cell populations with decreased dATP pools and a higher S phase fraction. In addition, we assessed the effect of dFdCyd on the induction and repair of radiation-induced DNA damage.

MATERIALS AND METHODS
Cell Culture. HT29 human colon cancer cells were cultured as described previously (7). Cells were checked for mycoplasma every 3 months.

Cell Survival Assays. Cell survival was assessed using a clonogenic assay, as described previously (7). All dFdCyd exposures were for 2 h. Radiation survival data from dFdCyd-treated cells were corrected for plating efficiency using an unirradiated plate treated with drug under the same conditions. Cell survival curves were fitted using the linear-quadratic equation, and the mean inactivation dose (the area under the cell survival curve) was calculated according to the method of Fertil et al. (15). The cell survival enhancement ratio was calculated as the ratio of the mean inactivation dose under control conditions
Delayed Radio sensitization by dFdCyd

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**Table 1** Influence of concentration of dFdCyd and length of time after dFdCyd exposure on cytotoxicity and radiosensitization of HT29 cells

<table>
<thead>
<tr>
<th>Time following Gemcitabine removal (h)</th>
<th>100 nM</th>
<th>3 µM</th>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Enhancement ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surviving fraction</td>
<td>1.12 ± 0.05</td>
<td>1.21 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>1.24 ± 0.06</td>
<td>0.71 ± 0.09</td>
</tr>
<tr>
<td>Enhancement ratio</td>
<td>1.17 ± 0.07</td>
<td>1.25 ± 0.18</td>
</tr>
<tr>
<td>Surviving fraction</td>
<td>1.61 ± 0.18</td>
<td>0.70 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>1.23 ± 0.10</td>
<td>1.38 ± 0.16</td>
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<tr>
<td></td>
<td>1.78 ± 0.22</td>
<td>0.66 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>1.62 ± 0.21</td>
<td>1.04 ± 0.30</td>
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<tr>
<td></td>
<td>2.95 ± 0.59</td>
<td>0.15 ± 0.05</td>
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<td></td>
<td>1.29 ± 0.26</td>
<td>0.41 ± 0.02</td>
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<tr>
<td></td>
<td>0.96 ± 0.07</td>
<td>0.43 ± 0.12</td>
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</tbody>
</table>

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RESULTS

We began by determining the cytotoxicity of dFdCyd when cells were exposed briefly, and the drug was then removed from the medium. In these experiments, HT29 cells were exposed for 2 h to 100 nM or 3 µM dFdCyd (concentrations of 20 µM are routinely attained in the plasma of patients receiving therapeutic dFdCyd; Refs. 19 and 20), the extracellular drug was washed away, and cells were returned to drug-free medium for various times before assessment of clonogenic survival. We found that there was no or modest cytotoxicity for up to 72 h after treatment with 100 nM dFdCyd. Cells that were treated with 3 µM dFdCyd showed decreased clonogenicity when they were incubated in drug-free medium for 24 h (Table 1) or longer (not shown). Therefore, for the experiments using 3 µM dFdCyd, the maximum elimination period was 24 h.

We could then study the effect of a drug elimination period on radiation sensitivity. Cells were exposed to either 100 nM or 3 µM dFdCyd and irradiated up to 72 h (for 100 nM dFdCyd) or 24 h (for 3 µM dFdCyd) later. Whereas radiosensitivity was minimally increased immediately after drug exposure, the enhancement ratio was 1.62 ± 0.21 and 2.95 ± 0.59 h after exposure to 100 nM or 3.0 µM dFdCyd, respectively (Table 1; Fig. 1). This degree of enhanced radiation sensitivity is similar to (or exceeds) that which we found after a 24-h exposure to 10 nM dFdCyd (7). Therefore, these data confirmed our hypothesis that a relatively brief exposure to a higher (but clinically achievable) concentration of dFdCyd can produce an increase in radiation sensitivity similar to that caused by a more prolonged exposure to a lower concentration.

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Fig. 1  dFdCyd radiosensitizes cells after a brief exposure. HT29 cells were exposed to 100 nM (•) or 3 µM (○) dFdCyd for 2 h. The medium was then removed, and the cells were placed in medium without drug. These cells and untreated cells (□) were irradiated 24 h later and assessed for clonogenic survival. The results of a single representative experiment are shown.

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delayed radiosensitization by dFdCyd countings. The pieces, which were then melted and analyzed by scintillation counting. The distribution was analyzed using a random breakage model (18), which permitted us to estimate the sensitivity of DNA to radiation-induced fragmentation (measured in terms of DNA dSBpGy⁻¹·bp⁻¹ delivered). Data are presented as the mean ± SE of at least four experiments.

Analysis of Nucleotide Pools. Cellular nucleotide pools were analyzed as described previously (7). Briefly, cells were harvested by trypsinization, extracted with 0.4 M perchloric acid, neutralized, and analyzed by anion-exchange HPLC. The nucleotides were eluted from the column using a linear gradient of ammonium phosphate buffer ranging in concentration from 0.15 to 0.60 M, pH 3.7. dNTPs were quantified by HPLC with a limit of detection of approximately 20 pmol. Nucleotide pool measurements represent the mean ± SE of two or more determinations from at least two experiments.

RESULTS

We began by determining the cytotoxicity of dFdCyd when cells were exposed briefly, and the drug was then removed from the medium. In these experiments, HT29 cells were exposed for 2 h to 100 nM or 3 µM dFdCyd (concentrations of 20 µM are routinely attained in the plasma of patients receiving therapeutic dFdCyd; Refs. 19 and 20), the extracellular drug was washed away, and cells were returned to drug-free medium for various times before assessment of clonogenic survival. We found that there was no or modest cytotoxicity for up to 72 h after treatment with 100 nM dFdCyd. Cells that were treated with 3 µM dFdCyd showed decreased clonogenicity when they were incubated in drug-free medium for 24 h (Table 1) or longer (not shown). Therefore, for the experiments using 3 µM dFdCyd, the maximum elimination period was 24 h.

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Fig. 2  Effect of a 2-h exposure to dFdCyd on cell cycle distribution and BrdUrd incorporation. HT29 cells were exposed for 2 h to control conditions (A), 100 nM dFdCyd (B), or 3 μM dFdCyd (C). During the last 15 min of the incubation, 30 μM BrdUrd was added. Cells were then assessed for DNA and BrdUrd content using two-parameter flow cytometry as described in “Materials and Methods.” Line at the seventh tick mark on the BrdUrd content axis indicates the maximum background signal. The results of a single representative experiment are shown.

Because a 24-h exposure to 10 nM dFdCyd produced substantial effects on cell cycle distribution, it was of interest to determine whether similar cell cycle changes resulted from the conditions used in the current study. We began by determining that a 2-h exposure to either 100 nM or 3 μM dFdCyd produced little effect on cell cycle distribution assessed by one-parameter flow cytometry (not shown). However, two-parameter flow cytometry demonstrated that treatment with both 100 nM and 3 μM dFdCyd blocked BrdUrd incorporation in cells with S phase DNA content (Fig. 2).

We then assessed the impact of the drug elimination period on cell cycle distribution and the ability of cells to synthesize DNA (BrdUrd incorporation). We found that 100 nM dFdCyd caused redistribution of cells into S phase. The predominant phase at 24 h after exposure was early to mid-S phase, whereas at 48 h after exposure there was a greater late S phase component. This suggested that 100 nM dFdCyd treatment was slowing S phase transit. Under these latter conditions, a substantial fraction of cells that by DNA content alone could not be distinguished from G2-M incorporated BrdUrd and were, therefore, actually in late S phase. The cell cycle distribution resembled that of control cells 72 h after treatment with 100 nM dFdCyd (Fig. 3, A–C). Twenty-four (Fig. 3D) to 48 (not shown) h after exposure to 3 μM dFdCyd, cells arrested with G1-S boundary and, to a lesser extent, with S phase DNA content. In contrast to the results with 100 nM dFdCyd, 3 μM dFdCyd continued to inhibit BrdUrd incorporation, suggesting that DNA synthesis was permanently arrested even in cells with S phase DNA content. (Note that this treatment condition produced substantial cytotoxicity; Table 1). The finding that radiosensitization occurs under conditions that produce a variety of cell cycle distributions suggests that cell cycle redistribution alone does not account for radiosensitization.

On the basis of our previous finding that dFdCTP (and, presumably, 2',2'-difluoro-dCDP) had a long half-life in HT29 cells (7), we had hypothesized that a relatively brief exposure to...
Delayed Radiosensitization by dFdCyd

The repair of radiation-induced DNA damage was not well described by a random breakage model with a calculated mean decrease of 0.0087 ± 0.0031 per nM of dFdCyd after radiation (Table 2). Linear regression showed that FAR DNA damage 24 h after treatment with 3 pM these data using a random breakage model (18). We found that by a 24-h exposure to 10 nM dFdCyd (Table 3).

Table 2 Effect of dFdCyd* on radiation-induced DNA damage

<table>
<thead>
<tr>
<th>dFdCyd concentration (nM)</th>
<th>FAR Control</th>
<th>60 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.11 ± 0.04</td>
<td>0.68 ± 0.04</td>
</tr>
<tr>
<td>3</td>
<td>0.08 ± 0.03</td>
<td>0.49 ± 0.11</td>
</tr>
<tr>
<td>10</td>
<td>0.08 ± 0.03</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>30</td>
<td>0.08 ± 0.01</td>
<td>0.35 ± 0.04</td>
</tr>
</tbody>
</table>

* 24-h continuous exposure.

Table 3 Effect of dFdCyd* on the repair of radiation-induced DNA damage

<table>
<thead>
<tr>
<th>Time after irradiation (min)</th>
<th>Control</th>
<th>dFdCyd</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.04 ± 0.05</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>30</td>
<td>0.34 ± 0.01</td>
<td>0.36 ± 0.09</td>
</tr>
<tr>
<td>60</td>
<td>0.48 ± 0.03</td>
<td>0.50 ± 0.01</td>
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<tr>
<td>120</td>
<td>0.63 ± 0.02</td>
<td>0.57 ± 0.03</td>
</tr>
<tr>
<td>240</td>
<td>0.68 ± 0.07</td>
<td>0.61 ± 0.08</td>
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</table>

* 10 nM dFdCyd, 24-h continuous exposure.

dFdCyd could also produce relatively long-lasting changes in dNTP pools, particularly dATP. To test this hypothesis, cells were assessed for dNTP pools up to 72 h after exposure to 100 nM dFdCyd for 2 h. We found that dATP pools decreased to < 15% of the control values within 2 h after exposure (Fig. 4). A brief increase in dATP occurred approximately 10 h after dFdCyd washout; this increase appeared to be due to salvage of deoxyadenosine, because washing cells into medium with dialyzed serum diminished this transient increase in dATP (not shown). The dATP pools remained at ≈ 15% of the control value between 24 and 72 h after the removal of dFdCyd from the medium. After a 2-h exposure to 3 μM dFdCyd, the dATP pool was not detectable beginning from the time of exposure and continuing up to 72 h after drug treatment (not shown).

To try to gain insight into the mechanism of radiosensitization, we wanted to determine the effect of dFdCyd on the induction and repair of radiation-induced DNA damage. We began by measuring the effect of dFdCyd on radiation-induced DNA damage and repair under the conditions that we used in our initial study (7), which were, as described above, a 24-h continuous exposure to drug. We found that increasing concentrations of dFdCyd produced a significant decrease in the FAR after radiation (Table 2). Linear regression showed that FAR decreased by 0.0087 ± 0.0031 per nM of dFdCyd (P = 0.019). The repair of radiation-induced DNA damage was not affected by a 24-h exposure to 10 nM dFdCyd (Table 3).

We thought it was also important to determine the effect of the conditions used in this study on the induction and repair of radiation damage. There was no immediate effect of a 2-h exposure to 3 μM dFdCyd on radiation-induced DNA damage. However, cells that were irradiated 24 h after the 3 μM dFdCyd was removed showed significantly less DNA migration than control irradiated cells (Fig. 5). To quantify the decrease, we fit these data using a random breakage model (18). We found that DNA damage 24 h after treatment with 3 μM dFdCyd could be well described by a random breakage model with a calculated value of 7.6 ± 1.8 × 10^{-10} DSBS Gy^{-1} bp^{-1}, which was significantly lower than the control value of 2.0 ± 0.13 × 10^{-9} DSBS Gy^{-1} bp^{-1} (P < 0.05). We found that 3 μM dFdCyd had no effect on the repair of DNA damage assessed either at the end of a 2-h exposure or 24 h after the exposure was completed (Fig. 6).

**DISCUSSION**

These data demonstrate that exposure to dFdCyd at clinically relevant concentrations and durations can produce delayed radiosensitization up to 48 h after removal of drug from the medium. The finding that radiosensitization occurs after exposure to 100 nM dFdCyd, which is not cytotoxic, suggests that dFdCyd is a true radiosensitizer, although it is interesting, and perhaps clinically relevant, that an even greater enhancement ratio was observed after exposure to cytotoxic conditions. These
data clearly distinguish the radiosensitizing and cytotoxic effects of dFdCyd. In particular, although (noncytotoxic) 100 nM dFdCyd slows transit through S phase, there is continuing DNA synthesis, whereas (cytotoxic) 3 μM dFdCyd inhibits synthesis almost completely.

Our findings are consistent with our previous studies, which suggest that sensitization is associated with a decrease in dATP pools in S phase cells. In our previous studies of HT29 colon cancer cells (7) and Panc-1 and BxPC-3 pancreatic cancer cells (12), we used a variety of exposure durations and dFdCyd concentrations. Although some sensitization and minimal cell cycle redistribution were obtained at the end of a 4-h exposure to 3 μM dFdCyd in HT29 cells (enhancement ratio, 1.4 ± 0.1), maximal sensitization required longer exposures, during which there was substantial cell cycle redistribution into S phase.

Similar results have been obtained using MCF-7 breast cancer and SCC-1 and SCC-6 squamous cells (13) treated for up to 24 h with 10–100 nM dFdCyd. However, the absence of radiosensitization 72 h after the washout of 100 nM dFdCyd, despite the continued depletion of dATP pools, demonstrates that dATP pool depletion alone is not sufficient to radiosensitize.

We were surprised that dFdCyd produced neither an increase in the induction nor a decrease in the repair of radiation damage. In fact, dFdCyd appeared to retard the migration of irradiated DNA from the plug into the gel. This was probably due to the fact that dFdCyd redistributed the cells into S phase, which tend to release less DNA into the gel for the same deposited dose (21). It remains possible that perturbation of nucleotide pools produced by dFdCyd causes misrepair of radiation damage that is not apparent at the level of 0.2–5 Mbp fragments, which are assessed by the method of PFGE used in this study. These data show that the mechanism of dFdCyd sensitization is distinct from that of the thymidine analogues, which both increase the induction and decrease the repair of radiation damage, and the fluoropyrimidines, which decrease repair (for a recent review, see Ref. 13).

These data may be useful in the design of clinical trials using dFdCyd as a radiation sensitizer. First, the finding that radiosensitization can be achieved under noncytotoxic conditions suggests that dFdCyd doses substantially below those used for chemotherapeutic purposes might be sufficient to achieve radiosensitization. Second, they suggest that whereas the standard once-weekly treatment might sensitize two or three of the five radiation treatments typically given each week, a twice-a-week schedule (i.e., Monday and Wednesday) might permit sensitization of all radiation fractions. Third, they indicate that it might be relevant to simultaneously assess intracellular dATP pools and cell cycle distribution in tumor biopsies to determine whether dFdCyd is being administered to patients under conditions that might produce radiosensitization.

We have used some of these principles in the design of protocols using dFdCyd as a radiation sensitizer in the treatment of patients with unresectable head and neck cancers and pancreatic cancers. However, it is important to note that clinical trial design depends on both preclinical laboratory studies and clinical estimates of normal tissue toxicities, which are difficult to predict from preclinical models. Indeed, our early experience with low doses of dFdCyd and radiation for the treatment of patients with unresectable head and neck cancers has demonstrated significant radiosensitization of the normal tissues.

Therefore, it will be important to proceed cautiously in the development of combined radiation and dFdCyd in the clinic.

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Delayed radiosensitization of human colon carcinoma cells after a brief exposure to 2',2'-difluoro-2'-deoxycytidine (Gemcitabine).

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