Radiosensitization by Gemcitabine in *p53* Wild-Type and Mutant MCF-7 Breast Carcinoma Cell Lines[1]

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ABSTRACT

The nucleoside analogue 2′,2′-difluoro-2′-deoxycytidine (dFdCyd) is a potent radiosensitizer in several solid tumor cell lines. Radiosensitization has correlated with the dFdCyd-mediated decrease in dATP levels and is S-phase specific. Previous studies suggested that a cell line that was unable to progress through S phase after dFdCyd and radiation was not radiosensitized apparently because of the expression of wild-type p53. We have extended these results by using the MCF-7 human breast carcinoma cell line (wild-type *p53*) and the MCF-7/Adr subline (mutant *p53*) to determine whether p53 status affected radiosensitization or cell cycle progression after dFdCyd and radiation treatment. Both cell lines were sensitive to nanomolar concentrations of dFdCyd and showed significant radiosensitization, with radiation enhancement ratios of 1.6–1.8 after a 24-h exposure to either the IC_{10} or IC_{50} for dFdCyd. Nucleotide pool analysis demonstrated a >85% reduction in dATP pools in both cell lines within 8 h after drug addition. Both cell lines accumulated in S phase after a 24-h incubation with dFdCyd. After subsequent irradiation, MCF-7/Adr cells continued to progress through the cell cycle for at least 72 h. MCF-7 cells progressed for at least 24 h, and then exhibited a G_1 block at 48 h after drug and radiation treatment. These results demonstrate that a wild-type *p53* cell line can be radiosensitized by dFdCyd, presumably because it was able to deplete dATP levels and progress through the cell cycle for at least 24 h after drug and radiation treatment.

INTRODUCTION

Gemcitabine (dFdCyd[3]) is a nucleoside analogue that is used clinically to treat patients with pancreatic (1–5) or non-small cell lung cancer (6–12). *In vitro* studies have demonstrated that dFdCyd, at nontoxic concentrations, can enhance cell killing by ionizing radiation (termed "radiosensitization") in colorectal, breast, pancreatic, ovarian, and non-small cell lung cancer cell lines (13–15). In addition, dFdCyd can increase tumor growth delay when combined with ionizing radiation in animal models (16–18). Preliminary results from clinical trials suggest that dFdCyd functions as a radiosensitizer in patients as well (19, 20).

In order for dFdCyd to produce its cytotoxic effects, it must first be phosphorylated by dCyd kinase to the 5′-monophosphate of dFdCyd (21). Additional phosphorylation produces dFdCDP and dFdCTP, both of which are the active forms of dFdCyd that can lead to cytotoxicity. The dFdCDP form can inhibit ribonucleotide reductase, decreasing synthesis of the necessary dNTPs for DNA synthesis (22). In solid tumor cells, this inhibition results in a decrease in dATP levels primarily. The dFdCTP form can incorporate into DNA and inhibit DNA synthesis through its competitive (with dCTP) inhibition of DNA polymerases (23, 24). The mechanism for radiosensitization with dFdCyd has yet to be fully characterized. Unlike the radiosensitizer BrdUrd, which can increase radiation-induced DNA damage (25–27) and decrease the rate of DNA repair (28, 29), dFdCyd neither increases double-strand breaks nor decreases the rate of their repair (30). Correlative studies have suggested that the dFdCDP-mediated decrease in dATP is important for radiosensitization (13, 15, 30, 31). In addition, cell cycle analysis has suggested that dFdCyd-treated cells must be in S phase at the time of irradiation in order for radiosensitization to occur (15, 32, 33).

Previous work has shown that dFdCyd is a potent radiosensitizer in several solid tumor cell lines (13, 14, 31), but in the D54 human glioblastoma cell line, dFdCyd was unable to produce radiosensitization regardless of dose or length of incubation (33). This cell line expressed wild-type p53; did not accumulate in S phase after dFdCyd treatment; and 24 h after ionizing radiation, a strong G_1 block was observed. In contrast, the U251 human glioblastoma cell line expressing a mutant p53 showed S-phase progression without a G_1 block after dFdCyd and radiation, and potent radiosensitization was observed. In addition, two other cell lines expressing wild-type p53 could not be radiosensitized at noncytotoxic concentrations of dFdCyd (34, 35). This led us to hypothesize that expression of wild-type p53 prevented radiosensitization in the D54 cell line. However, other factors may have contributed to the difference in radiosensitization, because these cell lines were derived from different patients.

Therefore, in the studies presented, here we have examined the role of p53 on the ability of dFdCyd to produce radiosensitization using a pair of isogenic cell lines, the wild-type p53-expressing MCF-7 cells and the mutant p53-expressing MCF-7/Adr sub-line. We predicted that only the mutant p53-expressing MCF-7/Adr cells would be radiosensitized, whereas induction of wild-type p53 in MCF-7 cells would prevent S-phase accumulation. Instead, we found that both cell lines were radiosensitized equally by dFdCyd. Despite the induction of wild-type p53 after

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[3] The abbreviations used are: dFdCyd, 2′,2′-difluoro-2′-deoxycytidine; dNTP, deoxynucleoside triphosphate; dFdCDP, 5′-diphosphate of dFdCyd; dFdCTP, 5′-triphosphate of dFdCyd; Adr, Adriamycin; BrdUrd, 5-bromo-2′-deoxyuridine; S_{NI}, S-phase BrdUrd non-incorporating.

dFdCyd treatment, MCF-7 cells were able to accumulate in S-phase by the time of irradiation. Both dFdCyd and ionizing radiation produced a delayed G_1 block 24–48 h after treatment; however, this appeared to occur too late to affect radiosensitization. A preliminary account of a portion of these results has been reported (36).

MATERIALS AND METHODS

Cell Culture and Drug Preparation. The MCF-7 and MCF-7/Adr breast carcinoma cells were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY), supplemented with 10% calf serum (Life Technologies, Inc.), and 2 mM L-glutamine (Fisher Scientific, Fair Lawn, NJ). Cells were kept in logarithmic growth as a monolayer in T-75 culture flasks at 37°C in a humidified atmosphere containing 5% CO_2. Gemcitabine, a generous gift from Eli Lilly and Co. (Indianapolis, IN), was dissolved in PBS to obtain a stock solution of 10 mM and sterilized before diluting further with PBS to achieve final concentrations of 1, 10, and 100 μM.

Cell Survival Assay. After dFdCyd and/or radiation treatment, cells were assessed for clonogenic survival as described previously (15). 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 (37). 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 MCF-7 and MCF-7/Adr cells were irradiated using Co^{60} (AECL Theratron 80) at 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 cells were irradiated at room temperature.

Nucleotide Pool Analysis. Cellular nucleotides were assayed as described previously (15). Briefly, cells were harvested by trypsinization, and nucleotides were extracted using 0.4 N perchloric acid. Neutralized extracts were stored at −20°C until analysis. Ribonucleotides were removed from the extracts using a boronate affinity column (38). 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, Hillshore, 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 2.8). Nucleotides were identified on the basis of their UV absorbance spectrum and quantified at either 254 or 281 nm by comparison to the absorbance of a known amount of authentic standard. All nucleotide pool measurements represent the average of at least four determinations except for the 16-h point (duplicate determinations).

Flow Cytometry Analysis. Cells were incubated in the dark with 30 μM BrdUrd (Sigma Chemical Co., St. Louis, MO) for 15 min preceding the conclusion of the incubation period and processed as described (39). Briefly, cells were washed with PBS after BrdUrd incubation, trypsinized, and diluted with media. After the centrifugation and washing steps, cells were fixed with cold 70% ethanol to a final concentration of 1×10^6 cells/ml and stored at 4°C until analysis.

On the day of flow cytometry analysis, the cell samples were pelleted, washed with PBS, and resuspended in 1 ml of PBS containing 0.5 mg/ml RNase A (Boehringer Mannheim, Germany) and incubated at 37°C for 30 min. The cells were centrifuged and then resuspended in 1 ml 0.1 N HCl containing 0.7% Triton X-100 (Sigma Chemical Co.) on ice for 10 min. After centrifugation, the cell pellets were resuspended in 1 ml high-performance liquid chromatography water, heated in a water bath at 97°C for 15 min, and then placed on ice for 15 min.

After resuspension, the supernatant was removed, the sample was transferred to a 1.5-ml microfuge tube, and mouse anti-BrdUrd antibody (PharMingen, San Diego, CA) was added to each sample for 30 min. Samples were centrifuged and then FITC-conjugated goat antimouse antibody (Sigma Chemical Co.) was added to the cells for 30 min. After centrifugation, the supernatant was removed, and then the pellets were resuspended in 0.5 ml of propidium iodide [18 μg of propidium iodide (Sigma Chemical Co.) and 40 μg of RNase A/ml] in PBS before analysis with the Coulter EPICS Elite EPS flow cytometer.

Western Blot Analysis. After harvesting, cell pellets were incubated with 50 μl of lysis buffer, vortexed, and centrifuged. The supernatant was used for analysis after the determination of protein concentration with a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). For each cell sample, 50 μg of protein was loaded onto a 10% polyacrylamide gel. After electrophoresis for 2 h at 150 V, the protein was transferred onto Immobilon-P transfer membrane (Millipore Corp., Bedford, MA) for 2 h at 125 mA. Incubating the membrane with a 5% milk solution for 1 h at 37°C blocked nonspecific binding sites. The membrane was then incubated with a 1:500 dilution of p53 (Ab-6) monoclonal mouse IgG antibody (Calbiochem) for 2 h and then incubated with a 1:20,000 dilution of secondary antimouse IgG horseradish peroxidase linked antibody for 1 h at room temperature. After the primary and secondary monoclonal antibody incubations, the membrane was thoroughly washed in Tris-buffered saline with 0.1% Tween 20. Proteins that bound the antibodies were visualized using an enhanced chemiluminescence detection system (Pierce, Rockford, IL), and the relative band intensities were quantitated using the Kodak Digital Science IS440CF and 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY).

RESULTS

Cytotoxicity of dFdCyd. The sensitivity of MCF-7 and MCF-7/Adr cells to dFdCyd was studied to determine a range of nontoxic and toxic drug concentrations for subsequent studies. Cells were treated with a range of concentrations from 1 nM to 300 nM of dFdCyd to determine these values. Both cell lines showed similar sensitivities to dFdCyd at equimolar concentrations after a 24-h drug incubation (approximately one cell-population doubling time; data not shown). The IC_{10} and IC_{50} values were similar for each cell line, with 10 and 80 nM for the

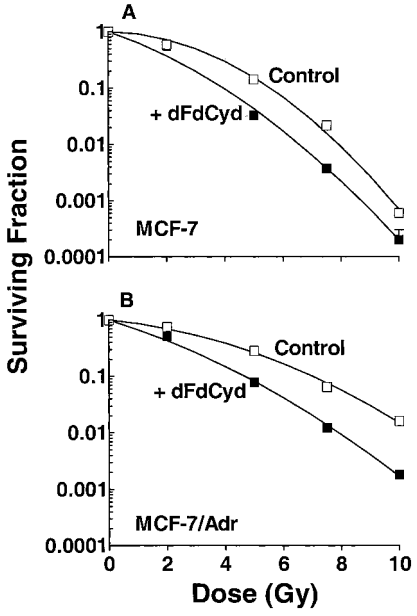

Fig. 1 Effect of dFdCyd on the sensitivity of MCF-7 (*A*) and MCF-7/Adr (*B*) cells to ionizing radiation. Cells were incubated with no drug (□) or IC_{10} dFdCyd (■) for 24 h and then irradiated. Cell survival was assessed using a colony-formation assay. The surviving fraction was corrected for cell survival in the absence of radiation. Values represent the mean of triplicate determinations; *bars*, SE. Results are shown from a representative experiment. Standard errors are <7% of the mean, except for data points at a radiation dose of 7.5 or 10 Gy, where the surviving fraction is extremely low.

MCF-7 cells, and 25 and 60 nM for the MCF-7/Adr cells, respectively. Cell survival at the IC_{10} was not significantly different from survival of untreated control cells for each cell line. Therefore, the IC_{10} values for dFdCyd were used to standardize a nontoxic dose of dFdCyd.

Radiosensitization by dFdCyd. The ability of dFdCyd to radiosensitize MCF-7 and MCF-7/Adr cells was examined by irradiating cells after a 24-h incubation with dFdCyd. As illustrated in Fig. 1, the IC_{10} of dFdCyd enhanced the sensitivity of both the MCF-7 and MCF-7/Adr cell lines to radiation-induced cytotoxicity. A similar degree of radiosensitization was observed with dFdCyd at the IC_{50} value, with radiation enhancement ratios of 1.7 for the MCF-7 cells and 1.6 for the MCF-7/Adr cells (Table 1). The MCF-7 cells were more sensitive than the MCF-7/Adr cells to radiation alone, as evidenced by a lower D-bar value (Table 1; $P = 0.002$). These data demonstrate that the extent of radiosensitization by dFdCyd was similar in both cell lines.

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 attributable to dFdCDP-mediated inhibition of ribonucleotide reductase. Therefore, the effects on dFdCTP and the endogenous dNTP pool levels were examined in the MCF-7 and MCF-7/Adr cells during incubation with dFdCyd for 24 h. In both cell lines, dFdCTP increased during the first 4 h after dFdCyd addition at the IC_{10} (Fig. 2). By 16 h after drug addition, the amount of

Table 1 Effect of dFdCyd on the sensitivity of MCF-7 and MCF-7/Adr cells to ionizing radiation

Radiation enhancement ratios (mean ± SE) are shown for both cell lines after a 24-h drug incubation with dFdCyd IC_{10} or IC_{50}. Sensitivity to radiation is shown as a D-bar (mean ± SE) as well as the fraction of cells surviving the drug treatments (mean ± SE). Each calculation is an average of at least three separate experiments.

Cell line	dFdCyd	Radiation enhancement ratio	Surviving fraction (drug alone)	D-bar (no drug)
MCF-7	10 nM	1.9 ± 0.1	88.0 ± 8.5	2.6 ± 0.2
	80 nM	1.7 ± 0.3	53.9 ± 17.6	
MCF-7/Adr	25 nM	1.6 ± 0.1	99.3 ± 9.2	3.6 ± 0.2
	60 nM	1.6 ± 0.1	71.3 ± 6.7	

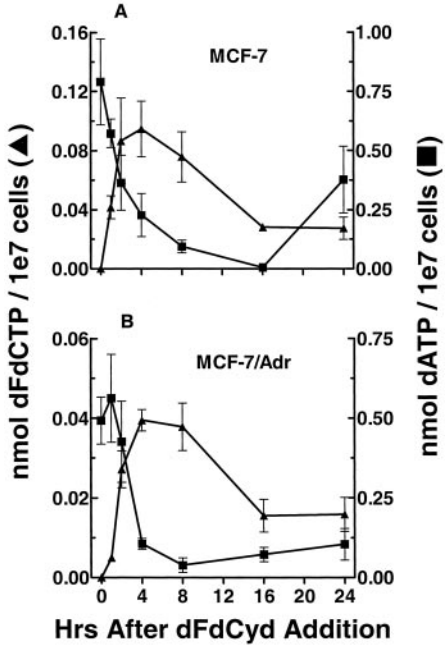

Fig. 2 Accumulation of dFdCTP and decrease in dATP pool levels in MCF-7 (*A*) and MCF-7/Adr (*B*) cells. Exponentially growing cells were incubated with the IC_{10} of dFdCyd for the indicated times. Cells were harvested periodically and analyzed for nucleotide pool content as described in "Materials and Methods." Each point represents an average of at least four determinations, except for the 16-h point (duplicate determinations); *bars*, SE.

dFdCTP had decreased in both cell lines and remained at the lower level for the next 8 h. Whereas the dFdCTP levels during the 24-h incubation followed a similar pattern in both cell lines, MCF-7 cells had a higher peak level (0.09 nmol/10^7 cells) compared with MCF-7/Adr cells (0.04 nmol/10^7 cells). In both cell lines, the dATP pools began to decrease within 2 h after dFdCyd addition (IC_{10}), with additional decreases to <30% and <15% of control after 4 and 8 h, respectively (Fig. 2). The dATP levels increased to nearly 50% of control levels between 16 and 24 h after drug addition in the MCF-7 cells (Fig. 2*A*) with a smaller increase to 21% of control levels in the MCF-7/Adr cells (Fig. 2*B*). Continuous incubation with the IC_{50} of dFdCyd

decreased dATP to <13% of control levels in both cell lines within 4 h after drug addition, and that low dATP level was maintained until the conclusion of the incubation period (data not shown). There were no significant differences in dCTP, dGTP, and dTTP during dFdCyd incubation in the two cell lines at either the IC_{10} or IC_{50} (data not shown).

Effect of dFdCyd and/or Radiation on Cell Cycle Distribution. Because previous studies suggested that the progression of cells into S phase was important for radiosensitization by dFdCyd (32, 33), the cell cycle distribution of the MCF-7 and MCF-7/Adr cells was examined after treatment with dFdCyd and/or ionizing radiation (Fig. 3). After a 24-h incubation with dFdCyd at the IC_{50}, >75% of the MCF-7 cells had accumulated in S phase (Fig. 3*A*; Table 2). The percentage of cells in S phase decreased during the 72 h after drug washout. In contrast, 24 h after a 5-Gy dose of ionizing radiation, >75% of the MCF-7 cells were in G_1. These cells appeared to be blocked in G_1 for ≥48 h after irradiation because high G_1-low S-phase percentages were maintained, and the cell number did not double. The drug and radiation combination in the MCF-7 cells initially produced an early S-phase accumulation and, 48 h later, a G_1 block with a decrease in cell number between 48–72 h. Similar results were initially observed with the IC_{10} of dFdCyd in the MCF-7 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 (Table 3).

Similar to the results in the MCF-7 cells, a 24-h drug incubation with the IC_{50} of dFdCyd in the MCF-7/Adr cells resulted in accumulation of >85% of the cells in S phase. The percentage of cells in S phase remained higher than the percentage of cells in G_1 for at least 72 h after drug washout (Fig. 3*B*; Table 2). After 5 Gy alone, the cell cycle distribution of the MCF-7/Adr cells resembled that of control untreated cells except for a modest increase in the G_2-M population. The combination of drug and radiation treatment produced similar effects to that of the drug alone for the first 24 h after drug washout. At 48 and 72 h after drug washout, the percentage of cells in S phase decreased, whereas the percentage of cells in G_2-M increased. Similar results were observed with the IC_{10} of dFdCyd (Table 3).

Both the MCF-7 and MCF-7/Adr cells exhibited an increased number of cells in S_{NI}, which represents dying cells (40), at 48 and 72 h after dFdCyd alone (IC_{50}) or with radiation, whereas radiation alone did not increase S_{NI} (Table 2). The percentage of apoptotic cells was not increased in the MCF-7 cells after drug and/or radiation treatment compared with control cells. In the MCF-7/Adr cells, apoptosis increased to 18% at 48 and 72 h after the addition of dFdCyd, however the combination of dFdCyd and radiation did not increase the apoptotic fraction further (10–13%).

Effect of dFdCyd or Radiation on p53 Protein Levels in MCF-7 Cells. To determine whether dFdCyd and/or ionizing radiation increased wild-type p53 protein levels, p53 expression was examined in the wild-type *p53* MCF-7 cells and the mutant *p53* MCF-7/Adr cells by Western blot analysis using an antibody that bound to denatured wild-type or mutant p53. As expected, MCF-7/Adr cells constitutively overexpressed mutant p53, and there was no apparent change after dFdCyd treatment or ionizing radiation (data not shown). During a 24-h incubation with the IC_{10} of dFdCyd in the wild-type *p53* MCF-7 cells, p53 expression did not change substantially, with <2-fold variation in the amount of protein compared with untreated cells (Fig. 4*A*). A greater increase in p53 expression was observed after incubation with the IC_{50} of dFdCyd, which increased p53 protein levels 4-fold compared with untreated cells by 8 h (Fig. 4*B*) and remained elevated for at least 72 h after drug washout (data not shown). Ionizing radiation (5 Gy) alone produced an increase in protein levels of p53 by 10-fold as compared with untreated cells within 2 h (Fig. 4*C*), which was sustained for at least 72 h after ionizing radiation treatment (data not shown).

DISCUSSION

The wild-type p53 protein is a tumor suppressor protein that is highly expressed in cells subsequent to DNA damage (41, 42). Typically, exposure of cells with wild-type *p53* to DNA-damaging agents increases the amount of p53 protein, resulting in a cascade of events that may lead to a G_1 or G_2 cell cycle block or promote apoptosis (41, 42). Mutation of p53 is a common finding in human tumors, and this has been causally linked to the carcinogenic process (41). In addition, cancer cells that express only mutated p53 protein have shown altered sensitivity to therapeutic modalities such as chemotherapeutic drugs or ionizing radiation (43). This is presumably mediated by the inability of the mutant p53 to recognize DNA damage and initiate events to either correct the damage or promote apoptosis. Because at least half of all tumors express mutant p53 (44–47), it is important to understand the effect of this mutation on sensitivity to therapeutic regimens.

In previous studies in this laboratory comparing a wild-type *p53* with a mutant *p53* cell line, only the cell line expressing mutant p53 was radiosensitized by dFdCyd (33). However, these studies were limited by the fact that the two cell lines were derived from different patients, and therefore it was difficult to ascribe the observed difference in radiosensitivity solely to the effect of wild-type p53, because other genetic differences existed as well. Here we have extended these results by evaluating the ability of dFdCyd to radiosensitize cells using the wild-type p53-expressing MCF-7 cell line and its mutant *p53* subline developed after exposure to Adr (MCF-7/Adr). Whereas the MCF-7/Adr cells have been reported to have increased levels of P-glycoprotein, glutathione *S*-transferase (48, 49), and glutathione peroxidase (50), these activities should not affect sensitivity to either dFdCyd or ionizing radiation.

The difference in p53 status did not appear to affect cytotoxicity at the concentrations of dFdCyd examined in the MCF-7 and MCF-7/Adr cell lines. Consequently, metabolism of dFdCyd and effects on dNTP pools were similar. These results are consistent with a previous report that indicated that dFdCyd sensitivity did not differ significantly in a variety of glioblastoma cell lines with either mutant or deleted p53 (51). However, RKO cells showed increased sensitivity to dFdCyd when p53 function was abrogated by expression of the human papillomavirus E6 protein (34). Other reports have suggested that a loss of p53 function increases sensitivity to other chemotherapeutic agents, such as cisplatin, pentoxifylline, and camptothecin, in MCF-7 cells (52, 53). It is likely that the relationship between

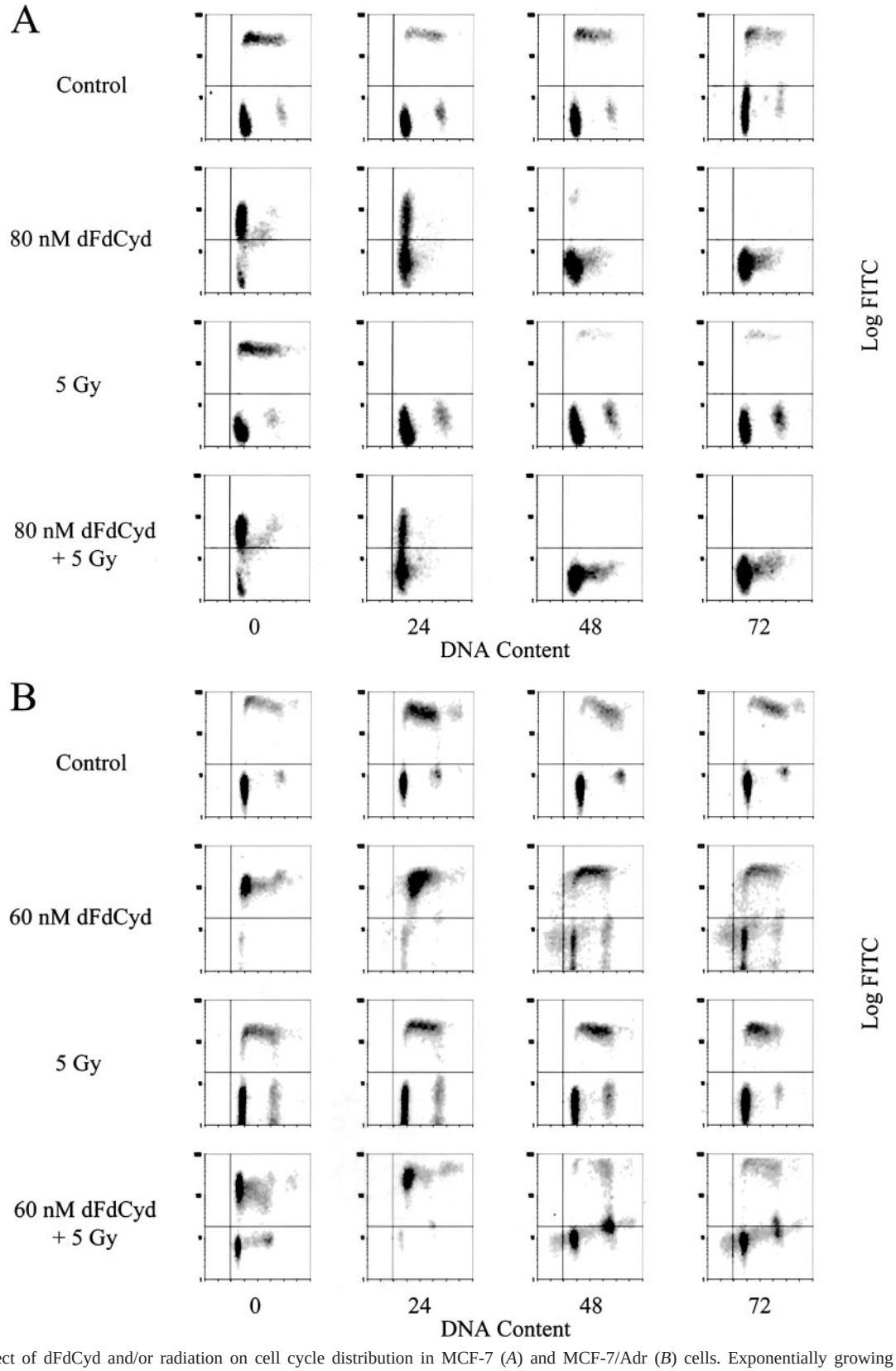

Fig. 3 Effect of dFdCyd and/or radiation on cell cycle distribution in MCF-7 (*A*) and MCF-7/Adr (*B*) cells. Exponentially growing cells were harvested after a 24-h IC_{50} drug incubation and/or a 5-Gy dose of ionizing radiation. Cells harvested at the indicated times after treatment were analyzed for cell cycle distribution as described in "Materials and Methods." Results are shown from a representative experiment (repeated at least three times).

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

Zero-h represents the time at drug washout after a 24-h incubation with dFdCyd and/or irradiation. Cells were harvested every 24 h for the controls and after drug washout and irradiation. The percentage of cells in G_1, S, G_2-M, S_{NI}, and apoptosis are shown. Results in the table are from the experiments shown in Fig. 3.

		MCF-7					MCF-7/Adr				
		G_1	S	G_2-M	S_{NI}	Apoptosis	G_1	S	G_2-M	S_{NI}	Apoptosis
Control	0	52.8	33.8	8.4	1.5	3.6	73.9	20.0	3.4	1.8	0.9
	24	68.2	16.4	9.0	1.7	4.7	47.4	45.2	4.8	1.1	1.5
	48	62.6	24.5	6.4	1.4	5.1	65.1	25.1	7.0	1.4	1.4
	72	46.3	35.8	4.2	2.4	11.3	67.7	23.1	5.1	2.0	2.0
IC_{50} dFdCyd	0	13.7	78.5	0.8	3.4	3.6	6.0	88.0	0.6	1.5	3.9
	24	35.8	50.4	2.1	8.9	2.8	6.5	85.8	1.1	2.0	4.6
	48	65.1	12.2	4.1	13.6	5.0	20.0	45.5	9.3	6.7	18.4
	72	66.2	8.5	3.9	15.3	6.1	26.5	42.2	6.2	7.4	17.7
5 Gy	0	46.0	40.7	7.6	3.8	1.8	56.1	28.1	11.1	2.5	2.1
	24	78.6	3.5	10.6	4.9	2.4	51.4	31.8	13.4	2.1	1.3
	48	75.3	8.2	10.9	3.6	2.0	48.7	33.3	12.2	4.0	1.7
	72	67.9	10.2	12.9	1.7	7.3	58.8	30.9	4.2	3.9	2.2
IC_{50} dFdCyd + 5 Gy	0	15.0	78.1	0.8	3.3	2.9	18.7	69.9	4.9	3.5	3.0
	24	41.2	42.1	1.4	7.4	7.9	2.5	93.9	0.3	0.6	2.7
	48	70.6	3.1	7.0	15.1	4.1	38.0	19.5	26.1	6.6	9.7
	72	68.2	7.3	6.2	15.1	3.2	40.2	21.7	15.8	9.6	12.7

Table 3 Effect of a 24 hour IC_{10} dFdCyd incubation on cell cycle distribution

Zero-h represents the time of drug washout after a 24-h incubation with dFdCyd. Percentage of cells in G_1, S, G_2-M, S_{NI}, and apoptosis are shown.

		MCF-7					MCF-7/Adr				
		G_1	S	G_2-M	S_{NI}	Apoptosis	G_1	S	G_2-M	S_{NI}	Apoptosis
Control	0	52.8	33.8	8.4	1.5	3.6	73.9	20.0	3.4	1.8	0.9
	24	68.2	16.4	9.0	1.7	4.7	47.4	45.2	4.8	1.1	1.5
	48	62.6	24.5	6.4	1.4	5.1	65.1	25.1	7.0	1.4	1.4
	72	46.3	35.8	4.2	2.4	11.3	67.7	23.1	5.1	2.0	2.0
IC_{10} dFdCyd	0	37.6	56.9	1.1	1.1	3.4	10.2	83.7	0.7	0.8	4.6
	24	65.0	20.8	6.4	3.4	4.5	4.3	89.0	0.5	0.4	5.9
	48	61.0	22.4	5.5	3.7	7.3	5.2	80.3	1.2	0.7	12.6
	72	54.5	22.3	7.0	4.0	12.2	26.2	42.3	6.0	7.7	17.9

p53 function and cytotoxicity is complex and dependent upon the particular cytotoxic agent, the cell line, and the cell death pathways that are used.

The studies presented here differ from previous reports in which wild-type p53-expressing cells were not radiosensitized by dFdCyd (33–35). Recently, we demonstrated that U251 glioblastoma cells harboring a mutant *p53* were radiosensitized by dFdCyd, whereas the wild-type p53-expressing D54 glioblastoma cells were not. There were several differences between these studies that may explain the results. Whereas equitoxic concentrations of dFdCyd produced similar levels of dFdCTP in the two glioblastoma cell lines, at dFdCyd concentrations $\leq IC_{50}$, dATP was depleted by $\geq$80% in the U251 cells compared with $\leq$30% depletion in the D54 cells. In addition, only the U251 cells accumulated in S phase after dFdCyd exposure. In contrast, here we have observed that the MCF-7 and MCF-7/Adr cells were able to deplete dATP to similar levels at equitoxic dFdCyd concentrations, and in both cell lines, >80% of the cells were in S phase after dFdCyd treatment and before irradiation. Thus, both of the MCF-7 cell lines displayed the factors we believe to be important in radiosensitization by dFdCyd, that is, high dATP depletion and S-phase accumulation; and, therefore, we would have expected radiosensitization to occur regardless of their difference in p53 function. Previous studies in isogenic RKO cell lines demonstrated that nontoxic doses of dFdCyd did not radiosensitize the cells, and abrogation of p53 function did not affect radiosensitization (34). Taken together, these reports demonstrate that p53 function alone is not a determinant of radiosensitization for dFdCyd.

These studies implicate the period immediately after irradiation as critical for radiosensitization by dFdCyd. The MCF-7 cells exhibited a G_1 block 48 h after the treatment with the IC_{50} of dFdCyd and ionizing radiation, compared with a normal cell cycle distribution 24 h after the IC_{10} of dFdCyd plus radiation, yet both conditions radiosensitized equally. The nonradiosensitized D54 cells also exhibited a G_1 block 24 h after dFdCyd and radiation. The major unifying feature in these studies is that, under radiosensitizing conditions with dFdCyd, a high percentage of cells (>70%) are in S phase after dFdCyd exposure and at the time of irradiation (15, 30, 33). This is consistent with a previous report using synchronized cell populations, which demonstrated that radiosensitization with dFdCyd was highest

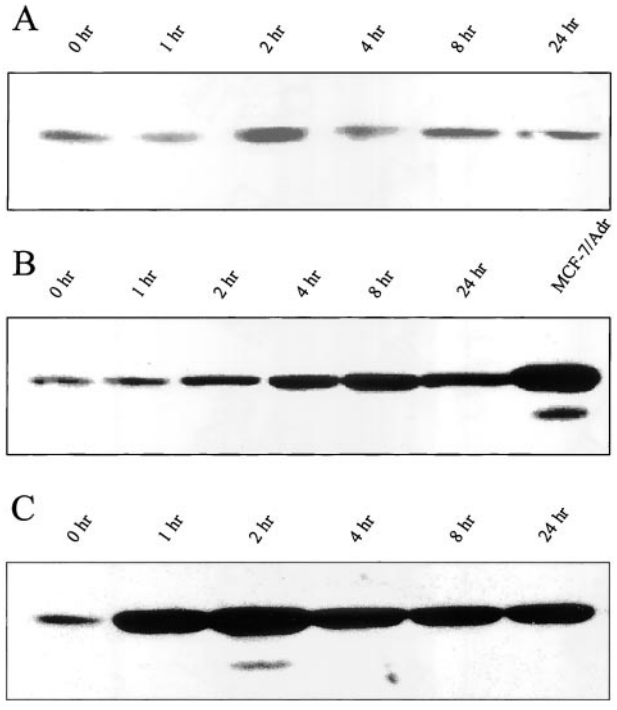

Fig. 4 Effect of dFdCyd or radiation on p53 protein levels. MCF-7 cells were exposed to IC_{10} dFdCyd (*A*), IC_{50} dFdCyd (*B*), or 5-Gy ionizing radiation (*C*). Cells were harvested at the indicated times for Western blot analysis, as described in "Materials and Methods." *B,* MCF-7/Adr served as a positive control, showing overexpression of mutant p53.

when cells were in S phase (32). Combining these with our previous findings that radiosensitization correlated with dATP depletion, and that dFdCTP did not increase DNA double-strand breaks or inhibit their repair after irradiation, we propose the following hypothesis. As cells accumulate in S phase during exposure to dFdCyd, they acquire a specific lesion, perhaps a misincorporation event for the depleted dATP, that may be repairable but, after the additional damage from ionizing radiation, repair is either overwhelmed or the dFdCyd-induced lesions are no longer recognized as needing repair. Cells that do not accumulate in S phase with dFdCyd treatment may not acquire this lesion, or they may be able to repair it before allowing cells to enter S phase. To substantiate this hypothesis, it will be important to identify a specific lesion in DNA that correlates with radiosensitization by dFdCyd.

The results demonstrated that dFdCyd at the IC_{10} did not induce p53 expression in the MCF-7 cells, whereas dFdCyd at the IC_{50} and ionizing radiation were able to induce p53. Cell cycle results were consistent with this expression pattern, where only the IC_{50} of dFdCyd and ionizing radiation were able to induce a G_1 block 24–48 h later. Thus, the MCF-7 cells respond to DNA damaging agents as expected for a wild-type *p53* cell line (43). We believe that their ability to accumulate in S-phase during the slow increase in p53 expression after dFdCyd addition permits radiosensitization.

Previously it was observed that the mutant p53 U251 glioblastoma cells that were radiosensitized by dFdCyd had increased levels of S_{NI} cells compared with the nonradiosensitized D54 cells with wild-type p53 (33). In the MCF-7 and MCF-7/Adr cell lines, the outcome was different; the IC_{50} of dFdCyd alone increased S_{NI}, but the addition of ionizing radiation did not increase further S_{NI} cell populations. Thus, increasing the number of S_{NI} cells may not be important for radiosensitization in the MCF-7 and MCF-7/Adr cells. Similar to our previous findings in the glioblastoma cell lines, the percentage of apoptotic cells did not change in the MCF-7 cells after drug and/or radiation treatment, and the MCF-7/Adr cells only had increased levels of apoptosis after drug treatment alone without additional increases from the addition of radiation. These studies indicate that cell death in S phase or through apoptosis is not required for radiosensitization with dFdCyd.

Our studies have demonstrated that dFdCyd and ionizing radiation can be combined to achieve more-than-additive cytotoxicity in human breast cancer cells *in vitro* regardless of p53 function. In view of the fact that many but not all human solid tumors express a mutant p53 (46), the ability of dFdCyd to radiosensitize cells expressing either mutant or wild-type p53 is clinically important. We recently reported on our clinical trial of dFdCyd with concurrent radiotherapy in patients with unresectable head and neck cancer, a tumor that historically exhibits a high percentage of cells with mutant p53 (54, 55). Indeed, most patients experienced a complete regression of their tumor, suggesting that radiosensitization with dFdCyd *in vivo* also does not depend on p53 function (56). Clinical trials are under way with dFdCyd and radiotherapy in several different malignancies. Additional studies to understand the critical events required for radiosensitization with dFdCyd may lead to improvements in the administration of this clinically promising therapy.

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