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MLH1 Deficiency Enhances Radiosensitization with 5-Fluorodeoxyuridine by Increasing DNA Mismatches

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ABSTRACT

The antitumor drug 5-fluoro-2′-deoxyuridine (FdUrd) also sensitizes tumor cells to ionizing radiation in vitro and in vivo. Although radiosensitization with FdUrd requires dTTP depletion and S-phase arrest, the exact mechanism by which these events produce radiosensitization remains unknown. We hypothesized that the depletion of dTTP produces DNA mismatches that, if not repaired before irradiation, would result in radiosensitization. We evaluated this hypothesis in mismatch repair (MMR)-deficient HCT116 0-1 cells that lack the expression of the required MMR protein MLH1 (inactive MLH1), and in MMR-proficient (wild-type MLH1) HCT116 1-2 cells. Although HCT116 0-1 cells were less sensitive to FdUrd (IC_{50} = 3.5 μM) versus HCT116 1-2 cells (IC_{50} = 0.75 μM), when irradiation followed FdUrd (IC_{50}) the MLH1-inactivated cells exhibited greater radiosensitization compared with MMR-wild-type cells [radiation enhancement ratio (RER) = 1.8 ± 0.28 versus 1.1 ± 0.1, respectively] and an increase (≥8-fold) in nucleotide misincorporations. In SW620 cells and HCT116 1-2 MLH1-wild-type cells, FdUrd (IC_{50}) did not produce radiosensitization nor did it increase the mutation frequency, but after short hairpin RNA-directed suppression of MLH1 this concentration produced excellent radiosensitization (RER = 1.6 ± 0.10 and 1.5 ± 0.06, respectively) and an increase in nucleotide misincorporations (8-fold and 6-fold, respectively). Incubation with higher concentrations of FdUrd (IC_{90}) after suppression of MLH1 produced a further increase in ionizing radiation sensitivity in both SW620 and HCT116 1-2 cells (RER = 1.8 ± 0.03 and 1.7 ± 0.13, respectively) and nucleotide misincorporations (>10-fold in both cell lines). These results demonstrate an important role for MLH1 and implicate mismatches in radiosensitization by FdUrd.

The fluoropyrimidines (FPs) 5-fluorouracil and 5-fluoro-2′-deoxyuridine (FdUrd) form the mainstay of treatment of gastrointestinal cancers. In addition to their chemotherapeutic effects, they can sensitize tumor cells to ionizing radiation (IR), resulting in synergistic tumor cell killing, and they are some of the most widely used radiation sensitizers in patients (Sobrero et al., 1997; van Laar et al., 1998; Chu et al., 2003). Although these antimetabolites have been used commonly in conjunction with IR, the mechanism(s) underlying the radiosensitization effect remains to be fully elucidated.

The FPs exert their cytotoxic effects primarily through activation to 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), which is a potent inhibitor of thymidylate synthase (TS), resulting in depletion of dTTP and subsequently inhibition of DNA synthesis. Previous studies have determined that the radiosensitizing effect of FdUrd correlates with dTTP depletion but is not dependent on cytotoxicity (Davis et al., 1995). The ability of FdUrd to radiosensitize under minimally or noncytotoxic conditions was demonstrated to be sequence-dependent, occurring only when cells were exposed to drug before radiation (Bruso et al., 1990) and to correlate with the accumulation of cells in early to mid-S phase (Miller and Kinsella, 1992; McGinn et al., 1994). However, cell cycle redistribution alone or killing of radioresistant S-phase cells by FdUrd cannot sufficiently explain the effectiveness of drug and radiation because, compared with mid-S-phase control cells, mid-S-phase FdUrd-treated cells were markedly radiosensitized, and to the same degree as unsorted cells treated with the same concentration of FdUrd (Lawrence et al., 1996). In addition, aphidicolin blocked FdUrd radiosensitization when given to FdUrd-treated cells

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ABBREVIATIONS: FP, fluoropyrimidine; FdUrd, 5-fluoro-2′-deoxyuridine; IR, ionizing radiation; FdUMP, 5′-monophosphate; TS, thymidylate synthase; dsbs, DNA double-strand breaks; dFdCyd, gemcitabine; MMR, mismatch repair; BrdUrd, 5-bromo-2′-deoxyuridine; shRNA, short hairpin RNA; RER, radiation enhancement ratio.

before radiation, whereas the late G_1 p53-mediated checkpoint was determined not to be crucial to radiosensitization by FdUrd (Lawrence et al., 2000). Therefore, studies suggest that the ability of cells to traverse the G_1/S checkpoint followed by progression of DNA replication on a damaged template may be key elements of radiosensitization by FdUrd (Lawrence et al., 1996).

Ionizing radiation produces many types of DNA damage, and it is thought that the ineffective repair of DNA double-strand breaks (dsbs) contributes most strongly to cytotoxicity (Ward, 1990). Until recently, the most commonly proposed models for radiosensitization by antimetabolites included either an increase in DNA dsbs before or with radiation compared with radiation alone, or the inhibition of the repair of DNA dsbs after irradiation. Although FdUrd has been demonstrated to enhance cytotoxicity when administered with radiation by inhibiting the repair of radiation-induced DNA damage, radiosensitization can also occur in the absence of detectable DNA dsbs, suggesting another mechanism exists to explain the radiosensitizing effect of these drugs (Bruso et al., 1990; Davis et al., 1995). Our recent studies with another antimetabolite radiosensitizer, gemcitabine (dFdCyd), demonstrated that, at radiosensitizing concentrations, the dFdCyd-mediated depletion in dATP pools via inhibition of ribonucleotide reductase produced DNA mismatches that, if left unrepaired, resulted in radiosensitization (Flanagan et al., 2007). These studies also demonstrated a role for the mismatch repair (MMR) protein MLH1 whereby cells deficient in MLH1 were unable to repair drug-induced DNA mismatches and were more easily radiosensitized than MLH1-expressing, presumably MMR-proficient cells.

The FdUMP-mediated inhibition of TS and subsequent depletion in dTTP result in the inhibition of DNA synthesis (Martomo and Mathews, 2002; Meyers et al., 2003) and induce perturbations in the levels of the other deoxynucleotides (dATP, dGTP, and dCTP) through feedback mechanisms (Longley et al., 2003). dNTP imbalances (in particular, dATP/dTTP ratio) are thought to severely disrupt DNA synthesis and repair (Houghton et al., 1995) and can produce errors in DNA replication such as single-base substitutions, and insertions or deletions, resulting in frameshift mutagenesis and a damaged template (Bebenek et al., 1992; Martomo and Mathews, 2002). The depletion of dTTP pools in the presence of FdUrd may contribute to the decreased ability to perform DNA repair (Lawrence et al., 1993). The MMR system plays a role in correcting DNA mismatches during replication (Kunkel and Erie, 2005) and has been demonstrated to play a role in cytotoxicity of FdUrd whereby MMR-deficient cells are significantly more resistant to the drug than their MMR-proficient counterparts (Carethers et al., 1999; Meyers et al., 2001, 2005). However, a role for MMR in the radiosensitizing property of FdUrd has not been explored.

We hypothesize that MMR deficiency will enhance radiosensitization by FdUrd by preventing correction of misincorporated nucleotides in DNA produced by depleted dTTP. We have evaluated this hypothesis in two colorectal carcinoma cell lines, HCT116 and SW620 cells, that differ in their expression of MLH1, a required MMR protein. Although we postulate that dTTP depletion leads to nucleotide misincorporation in DNA, it has not been demonstrated that FdUrd can produce these lesions. The present study directly tests the hypothesis that the dNTP pool imbalances produced by FdUrd can produce mismatches in DNA and that these are the lesions that result in radiosensitization.

Materials and Methods

Cell Culture, Plasmid, and Drug Preparation. HCT116 colon carcinoma cells are MMR-deficient due to inactivation of MLH1. The HCT116 1-2 cell line was produced from the parental HCT116 colon carcinoma cell line and contains wild-type MLH1 cDNA, whereas the HCT116 0-1 cell line contains the vector without the MLH1 insert (Jacob et al., 2001). SW620 colon carcinoma cells are considered MMR-proficient because they express the two major MMR proteins, MLH1 and MSH2 (Taverna et al., 2000). All cells were maintained in Dulbecco's modified essential medium (Invitrogen, Carlsbad, CA), supplemented with 10% fetal calf serum (Invitrogen), and 2 mM L-glutamine (Thermo Fisher Scientific, Waltham, MA). FdUrd (Sigma-Aldrich, St. Louis, MO) was dissolved in phosphate-buffered saline. Cell cycle distribution was determined by dual parameter [propidium iodide/5-bromo-2′-deoxyuridine (BrdUrd)] flow cytometric analysis as described previously (Ostruszka and Shewach, 2000), and DNA synthesis was measured by BrdUrd incorporation (Ostruszka and Shewach, 2003).

Cell Survival and Radiosensitization Assay. Cells were left untreated or treated with FdUrd at various concentrations for 24 h before irradiation [Co^{60} (AECL Theratron 80) at 1–2 Gy/min]. After FdUrd and/or IR (0, 2, 5, 7.5, and 10 Gy), cells were assessed for clonogenic survival as described previously (Shewach et al., 1994). Radiation sensitivity is expressed in terms of the mean inactivation dose (D-bar), which represents the area under the cell survival curve (Fertil et al., 1984). Radiosensitization is expressed as an enhancement ratio, which is defined as the mean inactivation dose (control)/mean inactivation dose (drug).

Determination of Nucleotide Pools. Nucleotides were extracted from cells using 0.4 N perchloric acid, neutralized, and ribonucleotides were removed using a boronate affinity column (Shewach et al., 1994). Cellular dNTPs were separated and quantified using a strong anion exchange column (Whatman, Hillshore, OR) with a high-pressure liquid chromatography system (Waters, Milford, MA) equipped with a photodiode array detector and controlled by Millennium 2010 software. Nucleotides were eluted at 2 ml/min with a linear gradient of ammonium phosphate buffer (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 254, 281, or 292 nm by comparison with the absorbance of a known amount of authentic standard.

pSP189 Plasmid Mutation Assay. The pSP189 plasmid can replicate in either bacterial or mammalian cells, and it contains *supF* suppressor tRNA that corrects an amber mutation in MBM7070 *Escherichia coli*. A single mutation at nearly any site in the coding sequence for the *supF* gene sequence prevents the expression of β-galactosidase (Seidman et al., 1985). The assay was performed as described previously (Flanagan et al., 2007). In brief, cells were transfected with the pSP189 plasmid overnight, incubated with FdUrd for 24 h, and plasmid extracted 24 h later. Replicated plasmid DNA was electroporated into MBM7070 *E. coli*, and transformants were grown on agar plates with ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactoside. White and blue colonies were enumerated, and mutation frequencies were calculated as number white colonies/number (white + blue) colonies. DNA from some control and all mutant clones was isolated and sequenced at the University of Michigan DNA Sequencing Core (An Arbor, MI) using the 20-mer primer (5′-GGCGACACGGAAATGTTGAA).

Transfection with shRNA. Small interfering RNAs were expressed from short hairpin RNA (shRNA) lentiviral plasmids (pLKO.1-purp) containing MLH1 (GenBank accession no. NM_000249) target sequences (targeting nucleotides 2186–2206 of human MLH1; Sigma MISSION, SHGLY-NM_000249; Sigma-Aldrich). The control shRNA contains a hairpin insert that will generate small interfering RNAs, but

it does not target any known human gene. Human embryonic kidney 293FT cells in six-well plates were cotransfected with lentiviral plasmid, shRNA to MLH1 or control shRNA (1.0 μg), lentiviral packaging vector (pCMVΔdR8.91; 1.0 μg), and the vesicular stomatitis virus G glycoprotein expression vector pVSV-G (0.5 μg) using Superfect transfection reagent (QIAGEN, Valencia, CA). Viral supernatants were collected 48 h after transfection, isolated by centrifugation, and then purified by filter sterilization (0.45 μM). One microgram per microliter of polybrene (Thermo Fisher Scientific) was added to viral samples and SW620 cells were transduced at 37°C overnight. Virus-containing media were removed; stably expressing cells were selected with 2 μg/ml puromycin and harvested at the appropriate time for determination of MLH1 protein expression. Five shRNAs to MLH1 were tested for knockdown efficiency, and we chose to use the individual construct MISSION TRNCN0000040053-249.2.2358 shRNA to MLH1 containing the sequence CCGGGCTTCGCCAGAGCATCAGCTTCTCGAGAAGCTGATGCTCTGGCGAAGCTTTTT because it produced the strongest and longest suppression of MLH1.

Western Blot Analysis. Cell lysates were prepared in radioimmunoprecipitation assay lysis buffer (0.5 M Tris-HCl, 1.5 M NaCl, 2.5% deoxycholic acid, 10% Nonidet P-40, and 10 mM EDTA, pH 7.4), with the addition of protease and phosphatase inhibitors [complete mini protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN), 1 mM sodium orthovanadate, and 1 mM sodium fluoride]. Proteins were separated by SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels and transferred onto Immobilon-P transfer membrane (Millipore, Billerica, MA). Membranes were probed with MLH1 polyclonal rabbit IgG antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:100 and anti-rabbit IgG horseradish peroxidase linked antibodies at 1:20 000 dilutions. Proteins were detected and visualized using an enhanced chemiluminescence detection system (Pierce Chemical, Rockford, IL).

Results

Cytotoxicity and Radiosensitization with FdUrd in Matched HCT116 Cell Lines. The HCT116 MLH1-inactivated cell line was less sensitive to FdUrd than the matched HCT116 MLH1-wild-type cell line (Table 1; IC_{50} = 3.5 and 0.75 μM for HCT116 0-1 and HCT116 1-2 cells, respectively). The ability of FdUrd to radiosensitize HCT116 cell lines was examined by irradiating cells after a 24-h incubation with FdUrd at equitoxic concentrations (IC_{10} or IC_{50}). As illustrated in Table 1, MLH1-inactivated HCT116 cells were radiosensitized at a noncytotoxic concentration, whereby ≥IC_{10} FdUrd produced an excellent radiation enhancement ratio (RER = 1.8). In contrast, incubation with ≤IC_{50} of FdUrd did not significantly enhance the sensitivity of the HCT116 MLH1-wild-type cells to radiation-induced cytotoxicity (RER ≤ 1.2); however, significant radiosensitization was observed at the IC_{90} (RER = 1.4 ± 0.1).

Effect of FdUrd on dNTP Pools and Cell Cycle Distribution. Because dTTP depletion is necessary for radiosensitization with FdUrd (Davis et al., 1995), we wanted to determine whether the inability of the MLH1 proficient HCT116 1-2 cells to undergo radiosensitization by FdUrd was due to lesser depletion of dTTP compared with the MLH1 inactivated HCT116 0-1 cells. Equitoxic concentrations (IC_{50}) of FdUrd produced similar changes in dTTP and other dNTPs in each cell line at 4 h, with ~40% reduction in dTTP and >50% reduction in dGTP with a concomitant ≥35% increase in dATP (Table 2). This pattern of dNTP effects is typical after FP administration (Wadler et al., 1996). At 24 h, the HCT116 MLH1-inactivated cells displayed an increase in dTTP, dATP, and dGTP, whereas all four dNTPs were depressed in the MLH1-wild-type cells.

Previous studies have demonstrated that radiosensitization with FdUrd depends upon the ability of cells to enter S phase during drug exposure (Miller and Kinsella, 1992; McGinn et al., 1994). To determine whether MLH1 inactivation altered the cell cycle progression of HCT116 cells treated with FdUrd, cell cycle distribution was measured for 72 h after the end of drug exposure by dual parameter (BrdUrd and propidium iodide) flow cytometry. Both cell lines exhibited similar S-phase accumulation (>66%) after incubation for 24 h with FdUrd (IC_{50}) (Table 3). Thus, although IC_{50} FdUrd resulted in similar cell cycle distributions plus depressed dNTP pools to at least the same degree and for a longer period in the MLH1-wild-type compared with MLH1 inactivated cells, MLH1-expressing cells were not radiosensitized at this drug concentration.

Effect of FdUrd on Mutation Frequency in HCT116 Cell Lines. Because differential effects on dNTP pools or cell cycle distribution could not explain the inability of FdUrd to radiosensitize the MLH1-wild-type HCT116 cells, we evalu-

TABLE 1

Effect of FdUrd on the sensitivity of HCT 116 0-1, HCT116 1-2, and SW620 cells to ionizing radiation

Radiation enhancement ratios (mean ± S.E.) are shown for all cell lines after a 24-h drug incubation followed by irradiation. Sensitivity to radiation in control cells (no drug) is shown as the D-bar. Each value is an average of at least three separate (mean ± S.E.) or two separate (mean ± S.D.) experiments.

MLH1 Expression	Cell Line	[FdUrd]	Radiation Enhancement Ratio	D-Bar (No Drug)
		μM		
Inactivated	HCT116 0-1	0.25 (IC_{10})	1.8 ± 0.33*	
		3.5 (IC_{50})	1.8 ± 0.28*	3.1 ± 0.19
Wild type	HCT116 1-2	0.125 (IC_{10})	1.2 ± 0.03	
		0.75 (IC_{50})	1.1 ± 0.10	2.9 ± 0.14
		3.5 (IC_{90})	1.4 ± 0.10*[a]	
Suppressed	HCT116 1-2 + shRNA-MLH1	0.75	1.5 ± 0.06*[a]	2.8 ± 0.21[a]
		3.5	1.7 ± 0.13*[a]	
Wild type	HCT116 1-2 + shRNA-nonspecific	0.75	1.1 ± 0.10[a]	2.8 ± 0.13[a]
Wild type	SW620	0.35 (IC_{50})	1.2 ± 0.12	
		3.5 (IC_{90})	1.4 ± 0.04*	2.2 ± 0.14
Suppressed	SW620 + shRNA-MLH1	0.35	1.6 ± 0.10*	2.3 ± 0.24
		3.5	1.8 ± 0.03*[a]	
Wild type	SW620 + shRNA-nonspecific	0.35	1.2 ± 0.08	2.3 ± 0.17

* Significantly > 1 ($P < 0.05$).
[a] Mean ± S.E.

ated the functional effect of dNTP pool imbalances on producing errors in DNA replication using a plasmid mutation assay. Cells were transfected with pSP189 plasmid, and FdUrd or no drug (control) was added for 24 h. The mutation frequencies in plasmids replicated in the untreated control group of both cell lines were similar (0.11 ± 0.025 and 0.12 ± 0.025% in MLH1-inactivated or MLH1-wild-type cells, respectively; $P < 0.05$), as reported by others (Jeong et al., 1999; Tobi et al., 1999). Although FdUrd (IC_{50}) decreased dTTP by >40% for at least 24 h in MLH1-expressing HCT116 1-2 cells, there was no significant increase in the plasmid mutation frequency at this drug concentration. In contrast, in HCT116 0-1 MLH1-inactivated cells, FdUrd (IC_{50}) decreased dTTP by ~40% for <24 h, yet this concentration resulted in a significantly (nearly 8-fold) increased plasmid mutation frequency compared with control (0.83 ± 0.005 and 0.11 ± 0.025%, respectively; $P < 0.0001$; Fig. 1A).

Radiosensitization by FdUrd in MMR-Proficient Cells after shRNA-Mediated Suppression of MLH1 Expression. After demonstrating that MLH1 inactivation was associated with radiosensitization by FdUrd and an increased plasmid mutation frequency, we used shRNA technology to decrease expression of wild-type MLH1 in the HCT116 1-2 cells and examined the ability of FdUrd to enhance IR sensitivity. In addition, we examined another cell line, SW620, to determine whether the effect of MLH1 on radiosensitization and cytotoxicity by FdUrd was unique to the HCT116 cell lines or could also be reproduced in other

TABLE 2

Endogenous dNTP pools in HCT116 and SW620 cells after incubation FdUrd

Exponentially growing HCT116 MLH1-wild-type and MLH1-inactivated cells were incubated with an IC_{50} for FdUrd or left untreated (control). SW620 cells were left untreated or transduced with shRNA targeted to MLH1 mRNA or nonspecific (NS) shRNA. Four days after shRNA treatment, exponentially growing cells were incubated with an IC_{50} of FdUrd or left untreated (control). dNTP pools were extracted and analyzed as described under *Materials and Methods*. The data are presented as a percentage of the corresponding control value and represent the mean ± S.D. from duplicate determinations. Control values (picomoles of dNTP per 10^6 cells): HCT116 0-1 cells, dCTP: 3.43 ± 1.0, dTTP: 29.7 ± 3.0, dATP: 6.1 ± 1.1, dGTP: 2.05 ± 0.6. HCT116 1-2 cells, dCTP: 4.0 ± 1.7, dTTP: 32.9 ± 2.8, dATP: 6.7 ± 0.8, dGTP: 1.97 ± 0.4. SW620, dCTP: 3.67 ± 0.5, dTTP: 20.05 ± 1.3, dATP: 3.55 ± 0.2, dGTP: 0.85 ± 0.04. SW620 + NSshRNA, dCTP: 7.06 ± 0.6, dTTP: 37.6 ± 3.1, dATP: 8.1 ± 0.2, dGTP: 1.84 ± 0.1. SW620 + MLH1shRNA, dCTP: 5.73 ± 1.3, dTTP: 21.42 ± 0.8, dATP: 4.93 ± 0.4, dGTP: 1.54 ± 0.5.

Cell Line	MLH1 Status	Time	Control Value			
			dCTP	dTTP	dATP	dGTP
		h		%		
HCT116 0-1	Inactivated	4	101.5 ± 17.3	62.0 ± 9.5	136.1 ± 18.5	42.2 ± 6.6
		24	95.6 ± 46.2	233.7 ± 49.9	338.5 ± 91.6	122.0 ± 48.3
HCT116 1-2	Wild type	4	85.0 ± 7.1	56.5 ± 12.6	174.4 ± 14.9	41.6 ± 0.7
		24	33.4 ± 12.6	51.5 ± 0.5	71.2 ± 2.0	18.5 ± 0.4
SW620	Wild type	4	91.0 ± 1.9	10.14 ± 0.5	143.1 ± 9.6	18.2 ± 2.5
		24	283.4 ± 96.3	17.06 ± 1.5	524.6 ± 92.6	41.8 ± 7.5
SW620 + nonspecific shRNA	Suppressed	4	91.4 ± 12.1	8.9 ± 0.5	161.3 ± 7.0	18.2 ± 4.2
		24	560.2 ± 77.1	24.9 ± 7.5	790.5 ± 38.9	89.1 ± 27.7
SW620 + MLH1 targeted shRNA	Wild type	4	87.1 ± 0.5	11.3 ± 0.8	152.1 ± 8.6	26.62 ± 9.2
		24	410.0 ± 17.0	22.27 ± 0.07	781.9 ± 76.0	46.75 ± 22.0

TABLE 3

Effect of FdUrd on cell cycle distribution of HCT116 and SW620 cells

Zero hour represents the time at drug washout after a 24-h incubation with IC_{50} FdUrd. Cells were harvested at the time indicated, and cell cycle distribution was analyzed by dual flow cytometry as described under *Materials and Methods*.

Cell Line	MLH1 Status	FdUrd IC_{50}	Time	G_1	S	G_2/M	Cells
			h				× 10^6
HCT116 0-1	Inactivated	Control	0	71.8	21.7	6.5	4.7
		FdUrd	0	11.7	84.1	4.2	3.6
			24	33.3	28.7	37.9	2.9
			48	72.6	14.0	13.4	5.7
			72	42.4	39.4	18.1	6.7
HCT116 1-2	Wild type	Control	0	75.1	18.8	6.1	2.7
		FdUrd	0	18.9	66.8	14.2	1.9
			24	59.8	18.5	21.7	3.8
			48	78.6	14.3	7.0	5.7
			72	78.4	15.2	6.4	5.9
SW620	Wild type	Control	0	55.6	44.4	9.1	2.6
		FdUrd	0	8.9	89.4	1.7	1.8
			24	6.1	79.2	14.8	2
			48	40.8	35.8	23.4	2.2
			72	41.7	39.3	19.0	2.3
SW620 + NS-shRNA	Suppressed	Control	0	72.1	20.3	7.6	2.6
		FdUrd	0	67.8	24.1	8.1	2.0
			24	5.1	90.5	4.4	1.8
			48	49.8	33.8	16.4	2.0
			72	53.0	28.1	18.9	2.1
SW620 + MLH1-shRNA	Wild type	Control	0	68.0	24.3	7.7	2.5
		FdUrd	0	43.3	51.7	5.1	2.1
			24	6.7	88.8	4.5	1.8
			48	41.3	38.1	20.6	2.1
			72	57.6	23.1	19.3	2.2

NS, nonspecific.

cells. Previous studies demonstrated that SW620 cells were only minimally radiosensitized by FdUrd at concentrations up to IC_{50} (Davis et al., 1995). In our studies, SW620 cells did not exhibit significant radiosensitization until a highly toxic concentration of FdUrd (3.5 μM, IC_{90}) was used (RER = 1.4 $\pm$ 0.04), similar to the HCT116 MLH1-wild-type cells (Table 1). After transduction of SW620 and HCT116 1-2 cells with lentivirus-delivered MLH1 shRNA and selection for transduced cells, nearly complete depletion of MLH1 protein was observed in both cell lines by 5 days, and it remained depressed through at least 9 days after transduction (Fig. 1, B and C). The ability of FdUrd to radiosensitize SW620 and HCT116 1-2 cells was examined by irradiating cells after a 24-h incubation with FdUrd. As illustrated in Table 1, SW620 MLH1-expressing cells did not exhibit significant radiosensitization at 0.35 μM (IC_{50}), but when MLH1 expression was suppressed with shRNA, the same concentration of FdUrd produced excellent radiosensitization (RER = 1.6 $\pm$ 0.10 in SW620 + shRNA-MLH1 cells). Likewise, incubation with 0.75 μM FdUrd (IC_{50}) did not significantly enhance the sensitivity of HCT116 1-2 MLH1-wild-type cells, but when MLH1 expression was suppressed with shRNA the same concentration of FdUrd produced enhanced IR sensitivity (RER = 1.5 $\pm$ 0.06 in HCT116 1-2 + shRNA-MLH1 cells). Incubation with higher concentrations of FdUrd (IC_{90}) after suppression of MLH1 produced a further increase in IR sensitivity in both SW620 and HCT116 1-2 cells (RER = 1.8 $\pm$ 0.03 and 1.7 $\pm$ 0.13, respectively). Control SW620 and HCT116 cells treated with nonspecific shRNA and then incubated with FdUrd (IC_{50}) and radiation did not exhibit

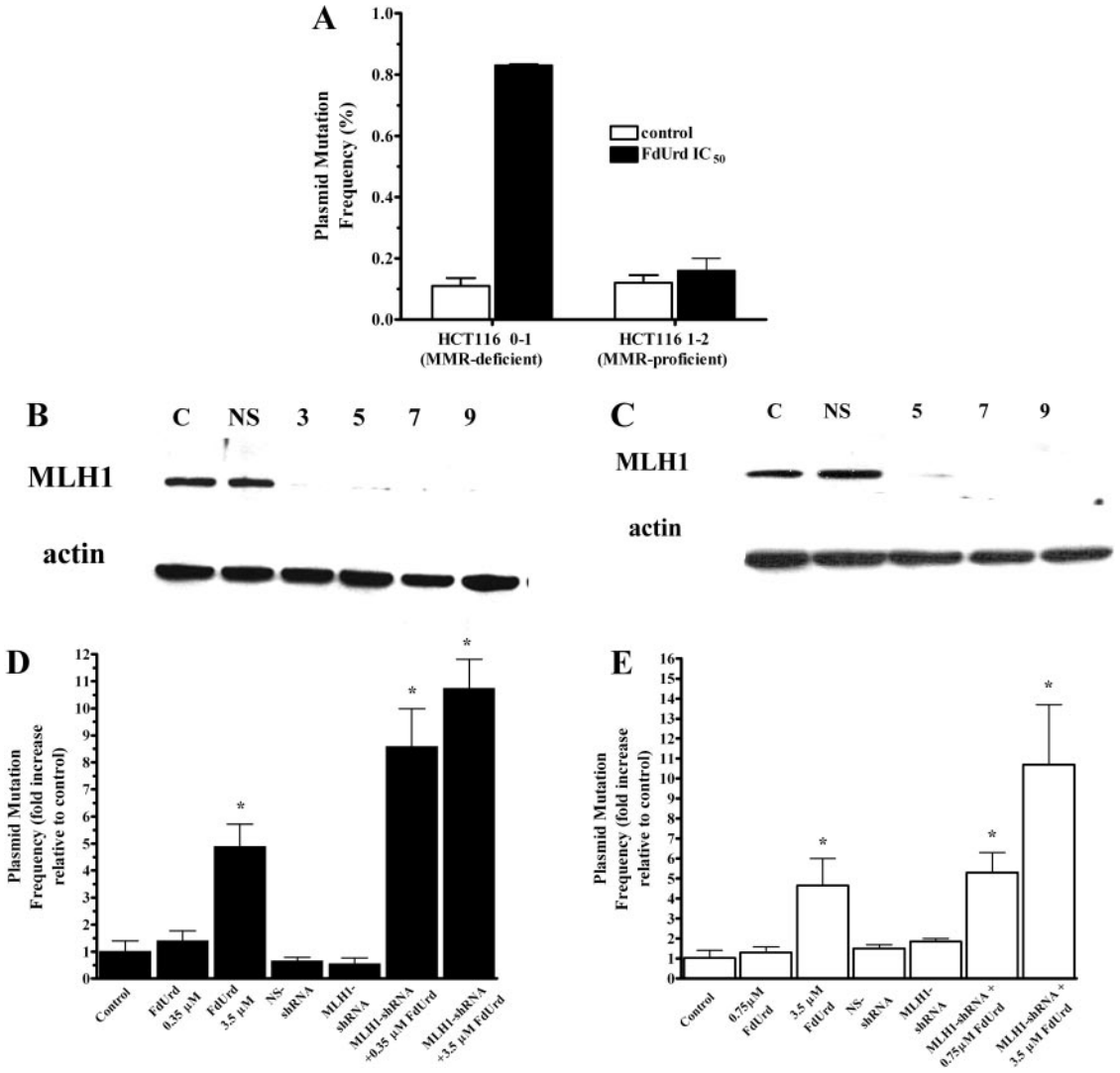

Fig. 1. MLH1 deficiency promotes an increase in FdUrd-mediated DNA mismatches and potentiates radiosensitization by FdUrd. A, pSP189 plasmid mutation frequency in HCT116 MLH1-inactivated and HCT116 MLH1-wild-type cells. Cells were transfected with pSP189 plasmid overnight, washed, and incubated with FUrd (IC_{50}) for 24 h. Plasmids were harvested 24 h after drug washout. DNA was harvested from replicated plasmids, electroporated into *E. coli*, and mutation frequency was calculated as number of white colonies/number of (white + blue) colonies. The *supF* sequence of a portion of the control plasmids and all of the mutant plasmids were confirmed by DNA sequencing. The data presented are the means $\pm$ S.D. $*$, $P < 0.05$, significantly more than control, within each cell line. B and C, whole cell lysates were analyzed by Western blotting for MLH1. Expression of β-actin is shown as a loading control. A representative blot from a minimum of three separate experiments is shown. D and E, pSP189 plasmid mutation frequency in SW620 cells (D) and HCT116 1-2 cells (E). Both cell lines were left untreated or transduced with MLH1-shRNA or NS-shRNA. Five days after the absence or presence of shRNA treatment, cells were incubated with IC_{50} or IC_{90} for FdUrd or left untreated (control). Results are expressed as the -fold increase relative to the untreated control cells within each treatment group. The data presented are the means $\pm$ S.E. of at least three separate experiments. $*$, $P < 0.05$, significantly more than control.

radiosensitization (RER = 1.2 ± 0.08 and 1.1 ± 0.1, respectively), and treatment of either cell line with shRNA for MLH1 alone or a nonspecific shRNA alone did not increase radiation sensitivity according to D-bar values ($P > 0.05$). Further examination of the SW620 cell line revealed that these differences in radiosensitization were not mediated by differences in dNTP pool effects, because an equimolar concentration of FdUrd (0.35 μM) produced similar depletion of dTTP (≥90% reduction in dTTP) and comparable changes in the other dNTP pools in SW620 control cells or after treatment with shRNA (Table 2). In addition, similar S-phase accumulation and subsequent cell cycle progression was observed after 24-h FdUrd (0.35 μM) in all SW620 cell lines, regardless of MLH1 status (Table 3). Thus, despite similar effects on cytotoxicity, dNTP pools and cell cycle distribution, 0.35 μM FdUrd produced radiosensitization in the SW620 + shRNA-MLH1 cells but not in the SW620 control or SW620 + NS-shRNA cells (Table 1), suggesting an important role for MLH1 in radiosensitization with this drug.

Effect of shRNA-Mediated Suppression of MLH1 on Plasmid Mutation Frequency with FdUrd in HCT116 MLH1-Wild-Type and SW620 Cells. Because shRNA-mediated suppression of MLH1 in HCT116 MLH1-wild-type cells and SW620 cells resulted in increased sensitivity of these cells to FdUrd-mediated radiosensitization, we wished to determine whether this corresponded to an increase in the frequency of misincorporation in DNA using the plasmid mutation assay. Compared with control HCT116 MLH1-wild-type cells and control SW620 cells expressing MLH1 (mutation frequency = 0.12 ± 0.02 and 0.09 ± 0.02%, respectively), FdUrd did not induce a significant increase in plasmid mutation frequency at IC_{50} (0.16 ± 0.04 and 0.12 ± 0.05%, respectively; $P > 0.05$), but at a highly toxic (IC_{90}) radiosensitizing concentration, a significant increase (>5-fold in HCT116 1-2 MLH1-wild-type cells and >4.5-fold in SW620 cells, respectively) was observed (Fig. 1, D and E). After shRNA-induced suppression of MLH1 and incubation with FdUrd (IC_{50}), conditions that induced radiosensitization, the mutation frequency increased approximately 6-fold in HCT116 1-2 MLH1-wild-type cells and 8-fold in SW620 cells compared with control cells treated only with shRNA. Incubation with higher concentrations of FdUrd (IC_{90}) after suppression of MLH1 produced a further increase (>10-fold) in mutation frequency in both cell lines. Compared with untreated control cells, plasmid mutation frequency was not significantly different with addition of MLH1 shRNA alone, nonspecific shRNA alone, or FdUrd in either cell line. Thus, only a radiosensitizing concentration of FdUrd produced an increase in plasmid mutation frequency.

Type and Frequency of Mutations in pSP189. DNA sequencing results from mutant colonies demonstrated that the majority of plasmid mutations generated in MMR-proficient and MMR-deficient cells were single-base substitutions in the control (no drug) or FdUrd-treated cells (≥90 and ≥70% of total plasmid mutations HCT116 and SW620 cells, respectively) (Fig. 2). Insertions and deletions accounted for the remainder of mutations observed within each group. The most common mutations observed with FdUrd in both HCT116 MLH1-wild-type and MLH1-inactivated cell lines were transversions with the largest increase in base substitution events in all cells observed at A:T sites, as expected for a drug that elicits a decrease in dTTP. After a moderate FdUrd (IC_{50}) concentration in HCT116 or SW620 cells, the relative contribution of base substitutions to total replication errors did not change from control (no drug). However, plasmids replicated in HCT116 1-2 MLH1-wild-type cells and SW620 cells after a high FdUrd (IC_{90}) or after the suppression of MLH1 protein revealed small changes in the relative contribution of base substitutions to total replication errors compared with control, but transversions remained largely dominant regardless of treatment.

Discussion

It is generally accepted that depletion of dTTP, due to FdUMP-mediated inhibition of TS, is the primary effect that produces radiosensitization with FPs. Although it has been suggested that dTTP depletion may slow the rate of repair of radiation-induced dsbs, that radiosensitization can occur in the absence of detectable DNA dsbs suggests other mechanism(s) exist to explain the radiosensitizing effect of these drugs (Bruso et al., 1990). Here, we provide evidence that the FdUrd-mediated decrease in dTTP produces mismatches in DNA that, although not required for cytotoxicity, are associated with radiosensitization. This is the first demonstration of a lesion in DNA that leads to radiosensitization with FdUrd. Furthermore, demonstration that MLH1 deficiency induced both mismatches in DNA and radiosensitization suggests that errors of replication play an integral role in radiosensitization with FdUrd.

To evaluate the role of MLH1 in radiosensitization with FdUrd, we initially measured the cytotoxicity of FdUrd ± ionizing radiation in the HCT116 0-1 (inactivated MLH1) and HCT116 1-2 (wild-type MLH1) cells. As reported by others, the HCT116 1-2 cells were more sensitive to FdUrd (Meyers et al., 2003, 2005); however, innate sensitivity to ionizing radiation was similar. Therefore, to evaluate both a noncytotoxic (IC_{10}) and a cytotoxic (IC_{50}) concentration of FdUrd on radiosensitization in the cell lines, we used equitoxic rather than equimolar concentrations of FdUrd. Under these conditions, only the MLH1-inactivated HCT116 0-1 cells exhibited radiosensitization. Both cell lines exhibited similar effects on the two parameters required for radiosensitization with FPs, dTTP depletion and accumulation in S phase. Indeed, dTTP depletion was more prolonged in the HCT116 1-2 cells, yet no radiosensitization was observed at ≤IC_{50}.

To further support our hypothesis that MLH1 plays a role in FdUrd-mediated radiosensitization, we used shRNA technology to return the HCT116 1-2 MLH1-wild-type cells to the MLH1 deficiency status of the parental cell line. After MLH1 suppression, we were able to radiosensitize these cells at a concentration of FdUrd shown here to be unable to increase cytotoxicity by ionizing radiation (Table 1). Likewise, the suppression of MLH1 expression in the SW620 cell line induced radiosensitization and, as in the HCT116 matched cell lines, insufficient dTTP depletion or lack of S-phase accumulation could not explain the lack of radiosensitization in parental SW620 cells. Because it has been demonstrated previously that radiosensitization requires the addition of FdUrd at least 8 h before irradiation (Bruso et al., 1990), we reasoned that radiosensitization requires an effect of dTTP depletion on DNA replication and not simply dNTP pool imbalance. Therefore, we have hypothesized that radiosensi-

tization with FdUrd is due to the ability of dTTP depletion to produce mismatches in DNA that, if not repaired before irradiation, will result in radiosensitization. Our results demonstrate that, in the HCT116 and SW620 cells, radiosensitization with FdUrd occurred only under conditions that produced mismatches in DNA. Thus, only cells with MLH1 inactivated (HCT116 0-1 cells) or suppressed with shRNA (HCT116 1-2 and SW620 cells) exhibited mismatches and radiosensitization at concentrations $\leq IC_{50}$. Radiosensitization was observed in the wild-type MLH1-expressing HCT116 1-2 and SW620 cells only at the corresponding IC_{90} for FdUrd, a concentration that also significantly increased mismatches.

Compared with the matched HCT116 cell lines, cultured separately over many years, suppression of MLH1 expression with lentivirus-delivered shRNA in the SW620 cells should allow a more accurate determination of the effect of MLH1 on radiosensitization and plasmid mutation frequency with FdUrd. Indeed, the demonstration that MLH1 suppression induced both mismatches and radiosensitization with FdUrd demonstrates a causal role for MLH1 deficiency in these processes.

Both HCT116 1-2 and SW620 cells express high levels of at least one MMR protein (Taverna et al., 2000) and are resistant to radiosensitization at moderate concentrations of FdUrd. However, MMR-proficient cells expressing reduced levels of MMR proteins can be radiosensitized at lower concentrations of FdUrd. For example, HT29 colon carcinoma cells have about 2-fold less MLH1 protein and 40-fold less MSH2 than SW620 cells (Taverna et al., 2000), but they are more susceptible to the combination of FdUrd and ionizing radiation (Davis et al., 1995). In our studies, only a toxic concentration of FdUrd (IC_{90}) in SW620 cells and HCT116 1-2 MLH1-wild-type cells produced radiosensitization and an increase in plasmid mutations. We hypothesize that, at low levels of FdUrd, the existing MMR capability is sufficient to correct errors of replication resulting from an imbalance in dNTP pools, but it can be overcome at sufficiently high concentrations of FdUrd that induce an increase in misincorporated nucleotides.

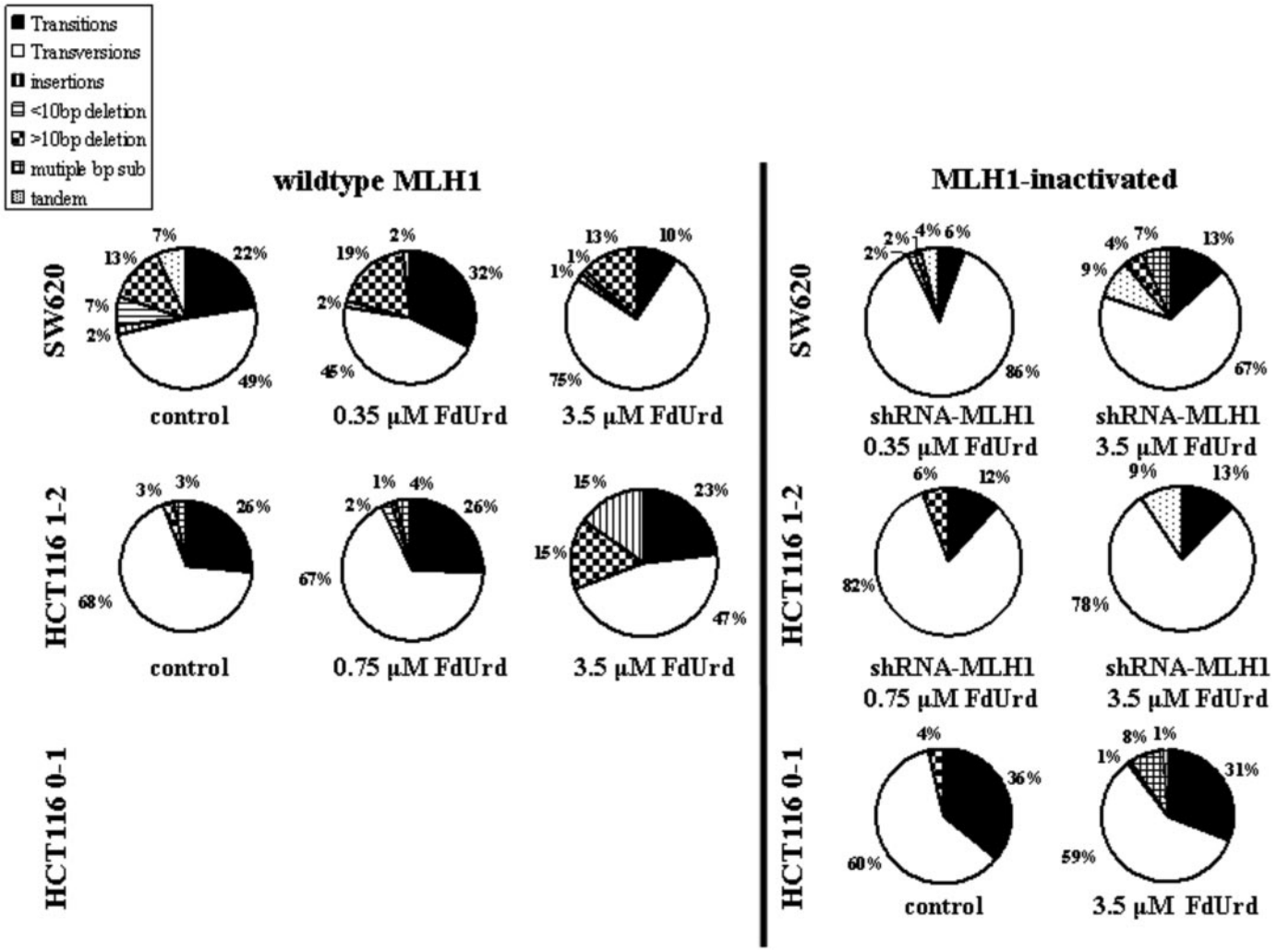

Fig. 2. Type and frequency of mutations in the *supF* sequence in pSP189 plasmids replicated in HCT116 cells and SW620 cells. HCT116 0-1 MLH1-inactivated, HCT116 1-2 MLH1-wild-type, and HCT116 MLH1-inactivated, and SW620 MLH1-wild-type and SW620 MLH1-inactivated cells were transfected with pSP189 plasmid overnight and exposed to no drug (control) or to 3.5 μM (IC_{50}) (HCT116 0-1 cells) or 0.75 μM (IC_{50}) and 3.5 μM (IC_{90}) (HCT116 1-2 cells), or 0.35 μM (IC_{50}) and 3.5 μM (IC_{90}) (SW620 cells) FdUrd for 24 h. Mutant colonies were picked and grown in Luria-Bertani broth followed by plasmid extraction and DNA sequencing. Mutation frequency was calculated as number of white colonies/number of (white + blue) colonies. At least 26 mutant colonies were counted within each group.

Because FdUrd can exert its cytotoxic effects through incorporation into DNA as well as through the inhibition of TS (Mader et al., 1998), it is difficult to eliminate the contribution of DNA incorporation to radiosensitization. However, because FdUMP incorporation into DNA is associated with cytotoxicity (Ingraham et al., 1982), the decreased cytotoxicity of FdUrd in MLH1-deficient cells compared with MLH1-wild-type cells under radiosensitizing conditions suggests that radiosensitization cannot be attributed to an increase in FdUMP incorporation into DNA. Furthermore, previous studies have determined that the radiosensitizing effect of FdUrd is not dependent on cytotoxicity (Davis et al., 1995). TS inhibition also produces an accumulation of dUMP, which may lead to increased levels of dUTP (Mitrovski et al., 1994), and a possible increase in dUTP incorporation into DNA (Ingraham et al., 1982), thus supplying another possible contributor to radiosensitization. We did not observe appreciable levels of dUTP in the HCT116 cell lines after FdUrd exposure. SW620 cells displayed a small amount of dUTP (data not shown), although the amount was similar regardless of MLH1 status. Therefore, this metabolite does not provide an explanation for the radiosensitization that occurred when MLH1 was suppressed or inactivated.

After FdUrd exposure, all of the cells used in our studies, regardless of MLH1 status, exhibited S-phase cell cycle arrest, a response strongly correlated with radiosensitization by FdUrd (Miller and Kinsella, 1992; McGinn et al., 1994; Lawrence et al., 1996). Some studies have described a shorter G_2 arrest after FP treatment in HCT116 MMR-deficient versus their MMR-proficient counterparts (Meyers et al., 2001), whereas others did not (Carethers et al., 1999), although drug concentration and exposure time varied. We used a moderate and equitoxic concentration of FdUrd for a moderate exposure time and did not observe a difference in G_2 response between MMR-proficient and MMR-deficient cells. Thus, differential effects on cell cycle progression cannot explain FdUrd radiosensitization observed in MLH1-inactivated but not MLH1-wild-type cells. Although the HCT116 MLH1-wild-type and MLH1-inactivated cells continued to progress through the cell cycle after FdUrd washout, whereas the SW620 cells progressed little (Table 3), within a cell line progression was similar regardless of MLH1 status. Furthermore, because a similar rate of DNA synthesis (as determined by the incorporation of BrdUrd; data not shown) was observed after drug washout within each cell line, regardless of MLH1 status, differences in mutation frequency cannot be attributed to DNA synthesis inhibition.

Despite similar effects on cytotoxicity, dNTP pools, and cell cycle distribution, only a radiosensitizing concentration of FdUrd produced an increase in plasmid mutation frequency in two different cell lines. These studies support our previous findings with dFdCyd and hydroxyurea, radiosensitizers that produce an imbalance in dNTP pools (primarily a decrease in dATP) due to inhibition of ribonucleotide reductase, where mismatches in DNA occurred only under radiosensitizing conditions, and MLH1 deficiency enhanced radiosensitization (Flanagan et al., 2007). Together, these studies strongly support errors of replication as a general mechanism of radiosensitization for drugs that produce imbalances in dNTPs. We have demonstrated that a decrease in dTTP produces different replication errors than drugs that produce decreases primarily in dATP (Flanagan et al., 2007), yet a strong relationship between DNA errors and radiosensitization still exists. These results demonstrate an important role for MLH1 and further implicate insufficient MMR in radiosensitization with drugs that produce dNTP imbalances.

Finally, these data suggest that tumors with innate or acquired deficiency in MLH1 would be most sensitive to radiosensitization with FdUrd but that higher doses of drug could be used in MLH1-expressing tumors to increase their radiosensitivity. Furthermore, the dependence of radiosensitization on DNA mismatches and not cytotoxicity suggests that, if clinical treatment with FdUrd and IR could be titrated to maximize DNA mismatches in tumors rather than cytotoxicity, normal tissue toxicity may be lessened. An understanding of the lesions and repair pathways leading to radiosensitization will aid us in optimizing chemoradiotherapy with the clinically important fluoropyrimidines.

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