DNA Mismatch Repair-dependent Response to Fluoropyrimidine-generated Damage*

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Mark Meyers‡, Mark W. Wagner‡, Anthony Mazurek§, Christoph Schmutte§, Richard Fishel§, and David A. Boothman‡‡

From the ‡Department of Radiation Oncology and Case Comprehensive Cancer Center, Laboratory of Molecular Stress Responses, Case Western Reserve University, Cleveland, Ohio 44106 and the §DNA Repair and Molecular Carcinogenesis Program, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Previous studies from our laboratory indicated that expression of the MLH1 DNA mismatch repair (MMR) gene was necessary to restore cytotoxicity and an efficient G2 arrest in HCT116 human colon cancer cells, as well as Mlh1<sup>−/−</sup> murine embryonic fibroblasts, after treatment with 5-fluoro-2-deoxyuridine (FdUrd). Here, we show that an identical phenomenon occurred when expression of MSH2, the other major MMR gene, was restored in HEC59 human endometrial carcinoma cells or was present in adenovirus E1A-immortalized Msh2<sup>−/−</sup> murine embryonic stem cells. Because MMR status had little effect on cellular responses (i.e. G2 arrest and lethality) to the thymidylate synthase inhibitor, Tomudex, and a greater level of [3H]FdUrd incorporation into DNA was found in MMR-deficient cells, we concluded that the differential FdUrd cytotoxicity between MMR-competent and MMR-deficient cells was mediated at the level of DNA incorporation. Analyses of ATPase activation suggested that the hMSH2-hMSH6 heterodimer only recognized FdUrd moieties (as the base 5-fluorouracil in DNA) when mispaired with guanine, but not paired with adenine. Furthermore, analyses of incorporated FdUrd using methyl-CpG-binding domain 4 glycosylase indicated that there was more misincorporated FU:Gua in the DNA of MMR-deficient HCT116 cells. Our data provide the first demonstration that MMR specifically detects FU:Gua (in the first round of DNA replication), signaling a sustained G2 arrest and lethality.

In addition to its roles in correcting DNA replication errors and editing recombination intermediates, DNA mismatch repair (MMR)<sup>1</sup> can process numerous DNA lesions (1–4). In fact, an intact MMR system is required for the lethality of specific DNA-damaging agents such as N-methyl-N’-nitro-N-nitrosoguanidine (MNNG), 6-thioguanine (6-TG), and cisplatin (5–7). MMR also mediates the lethality of fluoropyrimidines (FPs) such as 5-fluorouracil (FU) and 5-fluoro-2-deoxyuridine (FdUrd) (8, 9). Inactivation of MMR allows resistance to the cytotoxic effects of these agents, a phenomenon referred to as “damage tolerance” (10–13). Importantly, this enables cancer cells to uncouple persistent DNA damage from cell death, resulting in increased drug resistance (14–16).

The two major gene products that comprise MMR are MSH2 (which heterodimerizes with MSH3 or MSH6 to recognize mismatches and loops in DNA) and MLH1 (which heterodimerizes with PMS2 or MLH3 to act as a molecular matchmaker between the MSH2 complex and other DNA repair/replication and perhaps cell cycle factors) (17, 18). Defects in these two genes account for most cases of hereditary non-polyposis colorectal cancer, a familial condition with a predisposition to cancers of the colon, endometrium, stomach, ovary, and biliary tracts (19), as well as sporadic tumors of the colon (20), endometrium (21), stomach (22), head and neck (23), and prostate (24).

Others and we (8, 9) have demonstrated that cells deficient in MLH1 are resistant to the cytotoxic effects of FU and FdUrd. Because FPs are the agents of choice in the treatment of colorectal cancer, understanding potential resistance mechanisms is important. FPs exert cytotoxic effects through incorporation into RNA and/or DNA, as well as inhibition of thymidylate synthase (TS). The inhibition of TS, which is the central enzyme of de novo pyrimidine synthesis, leads to decreases in intracellular dTTP pools; this depletion results in immediate cytosolic effects (via inhibition of DNA synthesis) and alters dNTP pool sizes (thus increasing the error rate of DNA polymerase) (25). A hallmark of MMR deficiency is instability in the length of repetitive sequences in DNA, referred to as microsatellite instability (MSI). This reflects the inability of MMR-deficient cells to correct insertions and deletions in their DNA that result from polymerase slippage at these sequences (26). It is also an easily measured clinical marker. Due to the resistance of MMR-deficient (i.e. MSI<sup>−</sup>) cancer cells to FU and FdUrd, one would expect that FP treatment of these cells would be detrimental. Indeed, three recent clinical reports have found that standard, FU-based chemotherapy given to colon cancer patients with high levels of MSI did not result in a significant survival advantage (27–29). These studies concluded that FU therapy for MSI<sup>−</sup> cancers was not indicated. However, the mechanisms of MMR-dependent death after FP exposure remain unclear.

In this report, we used two different genetically matched systems to show that a deficiency in MSH2, as previously noted for MLH1, resulted in resistance to FdUrd, with an accompanying defect in G2 arrest. We show that these responses oc-
curred within one replication cycle in the presence ofFdUrd, suggesting that MMR directly detects FdUrd lesions in DNA rather than some secondary signal. Determination of overall \(^{3}H\)FdUrd incorporation into DNA indicated that higher levels of FdUrd were present in the DNA of MMR-deficient cells, suggesting direct detection and repair of FdUrd lesions in DNA by MMR. Consistent with these data, we show that the hMSH2-hMSH6 complex recognized FdUrd in DNA when paired with Gua (i.e. as an FU:Gua base pair); however, it did not recognize FU:Ade lesions. We believe that MMR directly detects FU:Gua in DNA and that the MMR-mediated response to this lesion appears to be absent. The exact mechanism of this lethality is not yet known.

MATERIALS AND METHODS

Reagents and Chemicals—FdUrd and 6-TG were purchased from Sigma. Tomudex (i.e. ICI D1694, ZD1694, or Raltitrexed) was generously provided by AstraZeneca (Cheshire, UK). Radiolabeled FdUrd (6-\(^{3}H\)FdUrd, 13.9 Ci/mmol) and GdU (8-\(^{3}H\)GdU, 5.1 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). Oligonucleotides with various mispairings were prepared as described previously (30).

Cell and Culture Conditions—HCT116 and HCT116 3-6 cells were generously provided by Dr. C. Richard Boland (University of California, San Diego), in which a normal human chromosome 3 (which contains the MLH1 gene) was introduced by microcell fusion into HCT116 (an MLH1-deficient human colon cancer cell line) to generate HCT116 3-6 cells (10). HEC59 and HEC59 2-4 cells were kindly provided to us by Dr. Thomas A. Kunkel (NIEHS, National Institutes of Health, Research Triangle Park, NC), in which chromosome 2 (containing wild-type MSH2) was introduced into MSH2-deficient HEC59 human endometrial carcinoma cells to create HEC59 2-4 cells (12). Msh2\(^{−/−}\) and Msh2\(^{+/−}\) murine embryonic stem (ES) cells were generously provided by Dr. Niels de Wind (Leiden University, The Netherlands), in which the Msh2\(^{−/−}\) knock-out ES dMsh2-2 and isogenic Msh2\(^{+/−}\) wt-2 cell lines were each infected with a retroviral vector containing the adenovirus EIA gene (31). Human testis cDNA (Clontech, Palo Alto, CA) was used to generate full-length oligonucleotides were analyzed following electrophoresis on 20% DNA sequencing gels. This assay demonstrated the specificity of MBD4 for FU:Gua.

Cytotoxicity Assays—Cells were assayed by colony-forming ability as described (9). However, treatment of HEC59 and HEC59 2-4 cells with Tomudex was performed in medium containing 1% fetal bovine serum (FBS; HyClone, Logan, UT) plus 10% dialyzed HEC59 2-4 cells with Tomudex was performed in medium containing 1% fetal bovine serum (FBS; HyClone, Logan, UT) plus 10% dialyzed dithiothreitol, and 0.1 mg/ml bovine serum albumin at 37 °C for 16 h after release, corresponding to a time just prior to entry into S phase but after the p53-mediated G1-S cell cycle checkpoint (9). Murine Msh2\(^{−/−}\) and Msh2\(^{+/−}\) ES cells were treated in asynchronous, log-phase growth with continuous exposure to drug. Cell cycle populations were analyzed using ModFit LT version 3.1 software (Verity Software House, Topsham, ME).

ATPase Assays—AMFR activity assays, were performed as described previously (32). The ATPase assay was performed by annealing the upper strand, 5′-GCT TAG CAT CGA GGA TCU GAC TGG TCN CAA TTC AGC GG-3′, to its complement, where U represents Ura or FU opposite Ade or Gua, and Gua (in the UpG context) is preceded by an unmethylated Cyt (i.e. mCGp). MBD4, and methylated Cyt (i.e. mCpG), were prepared by end-labeling with T4 polynucleotide kinase and \(^{32}P\)ATP, and then annealed. Labeled duplex (20 nM) was incubated with 100 ng of purified UDG or MBD4 in a buffer containing 10 mM Tris, pH 8.0, 5 mM EDTA, 1 mM dithiothreitol, and 0.1 mg/ml bovine serum albumin at 37 °C for 1 h. The reaction product was then heated to 95 °C for 5 min in the presence of 1% NaOH and then neutralized. Upon addition of dye (80% formamide, 10 mM EDTA, 1 mg/ml xylene cyanol, and 1 mg/ml bromophenol blue) was added to each sample. Sizes of cleaved compared with full-length oligonucleotides were analyzed following electrophoresis on 20% DNA sequencing gels. This assay demonstrated the specificity of MBD4 for FU:Gua.

FU-binding Partner Analyses in Genomic DNA from MMR-deficient Cells—HCT116 or HCT116 3-6 cells (2 \(\times\) 10\(^5\)) were incubated continuously with 2.5 \(\mu\)M (278 \(\mu\)Ci) \(^{3}H\)FdUrd for 3 or 10 days, and genomic DNA was extracted as above. Parallel exposures for 3 or 10 days were done using 12 \(\mu\)Ci of \(^{3}H\)Gua in the presence of unlabeled FdUrd (2.5 \(\mu\)M) to account for any differences in DNA replication between HCT116 and HCT116 3-6 cells; concomitant FdUrd exposure decreased \(^{3}H\)Gua uptake into DNA by 20 ± 5% in HCT116 cells after 3 days and increased \(^{3}H\)Gua in DNA by 27 ± 7% in HCT116 3-6 cells after 3 days. Purified nucleic acid from \(^{3}H\)FdUrd-exposed cells was treated twice with 500 \(\mu\)g/ml DNase-free RNase A, and residual nucleotides were removed using Sephadex G-25 QuickSpin columns (Roche Applied Science) following the manufacturer’s instructions. Genomic DNA (0.10–0.25 \(\mu\)g) was treated with proteinase K, 0.5 \(\mu\)g of MBD4, pH 7.0, 1 mM dithiothreitol, 5 mM MgCl\(_2\), and 400 \(\mu\)g/ml bovine serum albumin at 37 °C for 16 h. MBD4 releases Ura or FU only if hydrogen-bonded to Gua (37). Reaction products (Ura or/\(^{32}P\)ATP and then annealed. DNA from unexposed cells was treated in prokaryotic form and incubated with \(^{3}H\)FdUrd-exposed cells was treated with 500 \(\mu\)g/ml DNase-
mediated [\(^3\)H]FU release. The percentage of [\(^3\)H]FU released due to MBD4 treatment was calculated as the amount of product divided by the total radioactivity (i.e. the amount of substrate and product), then multiplied by 100%. This percentage was then normalized for the amount of DNA replication using relative [\(^3\)H]dGua incorporation to yield the percentage of FU incorporated as FU:Gua in DNA, normalized for DNA replication.

RESULTS

MSH2-deficient Cells Were Resistant to FdUrd, but Not Tomudex—Clonogenic survival assays were performed to determine the effect of loss of MMR on response to 6-TG, FdUrd, or Tomudex treatments (Fig. 1). A human colon cell line deficient in MLH1, as well as both human and mouse cell lines deficient in MSH2, were simultaneously examined. As previously shown (9, 41), MLH1-deficient HCT116 cells were more resistant to 6-TG and FdUrd than their corrected, MMR-proficient HCT116 counterparts (Fig. 1, A and B). We then examined survival responses after treatment of these cells with the TS inhibitor, Tomudex. Whereas FdUrd has two major DNA-directed mechanisms of cell killing (i.e. DNA incorporation and inhibition of TS), Tomudex specifically inhibits TS; thus, treatment with Tomudex allows one to discriminate the relative contributions of DNA incorporation versus TS inhibition in MMR-dependent,
FdUrd-mediated cell killing. We noted very little difference in the survival of MMR-proficient versus MMR-deficient cells following Tomudex treatment, regardless of MLH1 status (Fig. 1C). TS activity in HCT116 cells was 0.208 ± 0.003 pmol/10^6 cells/min, compared with 0.304 ± 0.005 for HCT116 3-6 cells. Thus, differential TS activity appeared to explain the minor differences in cytotoxicity we found between these cells; increased resistance to TS inhibition has been correlated with increased TS levels (42, 43). If corrected for differential TS activities, near identical dose-response survival curves for HCT116 versus HCT116 3-6 cells were noted in response to Tomudex, suggesting that incorporation of FdUrd into DNA accounted for the difference in survival between these cells.

Survival was then assessed in HEC59 human endometrial carcinoma cells, which are deficient in MSH2, compared with their corrected HEC59 2-4 counterparts (Fig. 1, D–F). MMR-deficient HEC59 cells were more resistant to 6-TG and FdUrd. As with MMR-proficient HCT116 3-6 cells, MMR− HEC59 2-4 cells survived better than isogenic MMR− HEC59 cells following Tomudex treatments. The same trend after treatment with these three agents was also found when comparing Msh2−/− murine ES cells (Fig. 1, G–I). In all three cell systems, the MMR-deficient cells consistently displayed a substantial resistance to FdUrd (Fig. 1, B, E, and H) and a much smaller sensitivity to Tomudex (Fig. 1, C, F, and I). Thus, sensitivity to FdUrd was not merely specific to the expression of MLH1 and appeared not to simply reflect differences in TS inhibition.

**Fig. 2. Representative cell cycle arrest responses of MMR-deficient and MMR-proficient cells.** Representative histograms are shown for synchronized HCT116 and HCT116 3-6 cells as well as asynchronous Msh2−/− and Msh2+/− ES cells. HCT116 and HCT116 3-6 cells were synchronized in G1 by confluence and low serum treatment, then dissociated with trypsin and replated at low density in medium containing 10% FBS. 16 h later (a time just prior to entry into S phase), drug (6-TG, FdUrd, or Tomudex) was added; this time is now considered to be t = 0 h. Msh2−/− and Msh2+/− ES cells were not synchronized; they were treated in log-phase growth. Shown are HCT116 and HCT116 3-6 cells that are untreated (at 72 h) or treated with 6-TG (2.5 μM, 96 h of continuous treatment), FdUrd (0.25 μM, 48 h of continuous treatment), or Tomudex (0.1 μM, 24 h of continuous treatment), as well as Msh2−/− and Msh2+/− ES cells that are untreated (at 72 h) or treated with 6-TG (0.1 μM, 72 h of continuous treatment), FdUrd (0.0015 μM, 24 h of continuous treatment), or Tomudex (0.001 μM, 12 h of continuous treatment). The number in the upper right corner of each histogram represents the percentage of cells in G2.

FdUrd-mediated cell killing. We noted very little difference in the survival of MMR-proficient versus MMR-deficient cells following Tomudex treatment, regardless of MLH1 status (Fig. 1C). TS activity in HCT116 cells was 0.208 ± 0.003 pmol/10^6 cells/min, compared with 0.304 ± 0.005 for HCT116 3-6 cells. Thus, differential TS activity appeared to explain the minor differences in cytotoxicity we found between these cells; increased resistance to TS inhibition has been correlated with increased TS levels (42, 43). If corrected for differential TS activities, near identical dose-response survival curves for HCT116 versus HCT116 3-6 cells were noted in response to Tomudex, suggesting that incorporation of FdUrd into DNA accounted for the difference in survival between these cells.

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significant loss of survival as measured by clonogenic survival assay. As with MLH1-deficient cells, G2 arrest responses were abrogated in Msh2-deficient cells. Thus, G2 arrest in response to FdUrd exposure also relied on an intact MMR system and was not merely dependent on MLH1 expression. There were no differences in G2 arrest responses after Tomudex exposures in isogenic cell lines proficient or deficient in MLH1 or Msh2 (Figs. 2 and 3, C and F). In fact, there were very few cells in G2 at higher doses of Tomudex. Instead, Tomudex caused an increase of 12–23% in S phase cells in the HCT116 cell system and 24% in the ES Msh2 cell system, presumably due to dThd depletion in both cell systems as a consequence of the inhibition of TS activity, as previously reported (44, 45).

MMR-competent Cells Responded to FdUrd Treatment with a G2 Arrest within the First Cell Division—In its role in post-replicative DNA repair, MMR detects DNA mispairs/lesions in the context of a newly synthesized DNA strand. It is able to identify the incorrect base in a mispair (placed there by DNA polymerase error) due to its presence in the daughter strand (4). FdUrd relies on DNA replication for its incorporation into DNA, whereby this pyrimidine analog is incorporated (as the base FU) across from Ade or Gua. Additionally, FdUrd-mediated inhibition of TS causes an accumulation of dUMP in cells and results in the misincorporation of deoxyuridine (as Ura) into DNA (25). MMR might then direct the removal of FdUrd and deoxyuridine from DNA. Responses within the first cell division, therefore, would suggest that MMR could directly detect FdUrd and deoxyuridine from DNA. Responses within the first cell division, therefore, would suggest that MMR could directly detect FdUrd and deoxyuridine from DNA. Therefore, we examined the cell cycle arrest responses of HCT116 and HCT116 3-6 cells within the first cell division after treatment (Fig. 4). HCT116 and HCT116 3-6 cells were synchronized, then exposed to 0.25 μM FdUrd immediately prior to S phase as described previously (9) to allow
incorporation into DNA while avoiding p53-mediated, G1-S checkpoint arrest responses. The cell doubling times of HCT116 and HCT116 3-6 cells were 18/11006 and 20/11006 h, respectively (41). Interestingly, both cell lines responded with a strong G2 arrest by 20 h after FdUrd addition. However, the G2 arrest persisted only in the HCT116 3-6 cells (at least 96 h; data not shown), indicating that MMR is required to sustain G2 arrest. Similar G2 responses were noted in the first cell division in Msh2/11002/11002 versus Msh2/11001/11001 cells (data not shown).

The hMSH2-hMSH6 Heterodimer Recognized FU:Gua Base Pairs in DNA—We tested the ability of purified hMSH2-hMSH6 or hMSH2-hMSH3 heterodimers to recognize FP lesions (specifically FU base-paired with Ade or Gua) in 41-mer oligonucleotide substrates and thereby activate their inherent ATPase activities. Mismatch-provoked ATP hydrolysis is a better indication of MMR activity than electrophoretic mobility shift assays, because MMR binding does not necessarily denote MMR activation (33, 46). We examined oligonucleotides that contained a central FU:Ade base pair (we believed Ade would be the predominant base pairing partner employed by the Thy analog FU), as well as Ura:Ade (to eliminate the influence of the presence of fluorine-5 in found in FU but not in Ura) and Thy:Ade (a negative control for MMR recognition). We also employed oligonucleotides containing a central FU:Gua base pair (to present FU in the context both of a base analog and a mispair), as well as Ura:Gua (Ura is a natural base analog, present here as a mispair) and Thy:Gua (a natural mispair that would serve as a positive control for MMR recognition). The duplex containing a Thy:Gua base pair significantly stimulated the hMSH2-hMSH6 ATPase (i.e. the ATPase velocity) compared with the duplex containing a Thy:Ade base pair, as expected (Fig. 5A). Interestingly, FU:Gua and Ura:Gua base pairs, but not FU:Ade or Ura:Ade ones, were able to significantly activate MMR activity. Thus, MMR was not capable of recognizing the dThd analogs, Ura or FU, directly when base-paired with Ade, but only when mispaired with Gua.
in DNA. The data represent the mean hMSH3 heterodimer, which chiefly recognizes insertion/deletion loops Thy:Ade control and the hMSH2-hMSH6 (A) and hMSH2-hMSH3 (B) heterodimers. We also examined the ability of hMSH2-hMSH3 complexes to activate the ATPase activities of the hMSH2-hMSH6 heterodimer, which predominately recognizes simple mispairs in DNA. (Cyt-Ade) loop) than the negative control (Thy:Ade). As expected, neither FU:Ade nor FU:Gua were substrates for MMR and, therefore, did not significantly activate the ATPase of the hMSH2-hMSH3 complex above the activity observed with the negative control. Results with both MMR complexes are summarized in Table I. MMR-deficient Cells Incorporated Higher Levels of FdUrd in Their DNA than MMR-proficient Cells—One of the three major mechanisms of FP-mediated cytotoxicity is the incorporation of antimetabolites into DNA (25). To determine if MMR status influenced the overall amount of radiolabeled FP incorporated into DNA, MMR-deficient and MMR-proficient cells of both MLH1 (HCT116) and Msh2 (ES) systems were treated with various doses of FdUrd spiked with 20–50 μCi of [3H]FdUrd for 3 days, and genomic DNA purified and assayed for antimetabolite-related, incorporated radioactivity (Fig. 6). DNA of MLH1-deficient HCT116 and Msh22−/− ES cells treated with 2.5 μM FdUrd contained 2.7- and 2.3-fold greater incorporated radioactive FdUrd, respectively, than their MMR-proficient counterparts. The incorporation levels of FdUrd in the DNA of both HCT116 and HCT116 3-6 cells were lower and nearly equivalent after shorter exposures (data not shown); this is consistent with our observation that shorter exposures of FPs did not result in survival differences in MMR− compared with MMR− cells. Addition of excess dThd, but not Urd, prevented incorporation of FdUrd into DNA (data not shown), consistent with the ability of dThd to rescue FdUrd-induced toxicity in these cells, as previously reported (9).

FdUrd-treated MMR-deficient Cells Selectively Incorporated Significant Levels of FU:Gua in Their DNA—Because FU stimulated the hMSH2-hMSH6 ATPase only when it was mispaired with Gua, we examined the frequency of FU:Gua base pairs in the DNA of FdUrd-treated HCT116 and HCT116 3-6 cells relative to total incorporated FU. To distinguish FU:Ade from FU:Gua radiolabeled lesions, MBD4 was used. MBD4 (also known as methyl-CpG-binding endonuclease 1) recognizes only Ura:Gua or FU:Gua, but not Ura:Ade or FU:Ade, in DNA (37). In contrast, UDG removes Ura and FU from DNA regardless of their base pairing partners; it can recognize Ura:Ade, FU:Ade, Ura:Gua, and FU:Gua, as well as other base pairings (38). UDG (as a positive control for this assay) recognized both Ura:Ade and Ura:Gua, as indicated by the generation of a cleavage product following hot alkali treatment (Fig. 7A). MBD4 only recognized DNA substrates containing FU:Gua lesions. In fact, MBD4 recognized FU:Gua regardless of Cyt methylation status (methylation in human DNA occurs on Cyt in a CpG context) (35, 48).

Using MBD4, MMR-deficient and -proficient cells were analyzed for the incorporation of FU:Gua into DNA. HCT116 and HCT116 3-6 cells were incubated with 2.5 μM [3H]FdUrd for 3 or 10 days and genomic DNA was isolated (Fig. 7B). DNA was then treated with MBD4 to determine the amount of FU incorporated into FU:Gua lesions. Whereas the level of FU incorporated across from Gua (determined as described under “Materials and Methods”) was somewhat higher (1.7-fold) in MMR HCT116 cells compared with MMR− HCT116 3-6 cells after 3 days of treatment, there was 5.0-fold more FU:Gua in HCT116 DNA at day 10. This incorporation difference correlated well with lethality. We have found that the difference in survival between HCT116 and HCT116 3-6 cells becomes more pronounced after longer exposures to FdUrd; there was virtually no survival difference after ≈24 h of treatment, but HCT116 cells were 17-fold more resistant to a 10-day exposure to 7.5 μM FdUrd (9). Because MBD4 recognizes the FU:Gua lesion (37), it can interact with MLH1 (35), and HCT116 cells have a mutation in MBD4 (a frameshift in the exon 3 A10 tract of one of their alleles on chromosome 3) that is corrected in HCT116 3-6 cells (49, 50); we wanted to rule out the possibility that MBD4 levels might be different between MMR− and MMR+ cells. The levels of MBD4 protein were examined and found to be equivalent in HCT116 and HCT116 3-6 cells (Fig. 7C), as well as in the other cell systems used (HCT116 3-6 cells had 1.07 times as much MBD4 as HCT116 cells, HEC59 2-4 had 0.98 times as much as HEC59 cells, and Msh22−/− ES cells had 1.17 times as much as Msh2−/− ES cells).

**DISCUSSION**

MMR is involved in cell cycle and cell death responses to agents such as MNNG, 6-TG, cisplatin, FU, and IR, which...
produce a broad spectrum of DNA lesions (5–8, 41). Previously, we reported that human colon cancer cells deficient in MLH1 were more resistant to continuous FdUrd treatment than MLH1-proficient cells (9). Here, we demonstrated that increased resistance to FdUrd was not limited to a deficiency in MLH1, but rather was a general feature of the absence of MMR. Response to FdUrd treatment, as indicated by a pronounced G2 arrest, occurred quickly in MMR-proficient cells, perhaps indicating a direct signaling from MMR to the cell cycle machinery. Interestingly, hMSH2-hMSH6 only recognized FdUrd (as FU) in DNA when mispaired with Gua. We found a greater overall level of FdUrd in the DNA of MMR-deficient cells, and a large proportion of this FdUrd was mispaired with Gua. Thus, we demonstrated that MMR recognizes FU:Gua and is involved in FdUrd-induced cytotoxicity.

The damage tolerance response to FPs appeared to be a general defect in MMR-deficient cells. We employed two systems (one human and one murine) involving altered functional systems (one human and one murine) involving altered functional expression of MSH2 to show that cell death (Fig. 1, B, E, and H) and G2 responses (Fig. 3, B and E) following continuous FdUrd exposure involved MMR as a whole and not merely MLH1. FPs exert their cytotoxic effects through various mechanisms (51). Because we demonstrated that FdUrd-mediated cytotoxicity was DNA-directed (9), we sought to elucidate which DNA-directed mechanism (direct incorporation into DNA or TS inhibition) was more important. To do this, we utilized Tomudex, an antifolate-based antimetabolite that very specifically inhibits its TS (52). Since Tomudex cannot be incorporated into DNA, it is a useful tool to determine the effect of TS inhibition directly, independently of DNA incorporation. In contrast to FdUrd treatment (Fig. 1, B, E, and H), the survival of MMR− versus MMR+ cells following Tomudex treatment was much less pronounced and, in fact, reversed (Fig. 1, C, F, and I). Furthermore, no significant differences in G2 cell cycle checkpoint responses following treatment with Tomudex (Fig. 3, C and F) were noted. Thus, DNA incorporation of FdUrd antimitabolites appeared to be the important factor in FdUrd-induced, MMR-mediated cytotoxicity.

Although it was not surprising that hMSH2-hMSH6 was able to detect FdUrd moieties in DNA, we found it interesting that FU:Ado was not detected. Apparently, the presence of the fluorine atom (with an atomic radius of 1.35 Å, compared with 1.2 Å for hydrogen) imparted too subtle a change to allow its direct detection by MMR. In light of the fact that other enzymes such as DNA polymerase, RNA polymerase, UDPG, MBDA4, dThd kinase, dThd phosphorylase, and ribonucleotide reductase can metabolize fluorinated analogs almost as readily as their normal substrates (53), this was not unexpected. MMR proteins can directly recognize DNA substrates containing bulkier lesions, such as O6-methylguanine paired with Cyt or Thy, without requiring the context of a mispair (30, 54). Alternatively,
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We have also shown that MMR-deficient cells incorporated elevated levels of the FdUrd antimetabolite in their DNA compared with isogenic wild-type cells. Historically, it had been difficult to demonstrate the presence of FP moieties in DNA until the availability of radiolabeled FU with a high specific activity and use of high performance liquid chromatography (55). The efficient actions of dUTP diphosphohydrolase (which keeps levels of dUTP, as well as FdUTP, low by converting these molecules to dUMP or FdUMP, respectively) and UDG limit the amount of FdUrd moieties in DNA to nearly undetectable levels (39). In addition to UDG, other DNA base excision repair (BER) enzymes such as G:T mismatch-specific thymine-DNA glycosylase and single strand-selective monofunctional uracil-DNA glycosylase also remove FU from DNA (25, 56). Thus, FPs may be incorporated only at low levels into DNA (due to low FdUtp levels), or they may be incorporated at higher levels but are readily removed through the actions of BER and MMR. Thus, we measured the steady-state levels of incorporated, radiolabeled FdUrd moieties in DNA directly. We found 2- to 3-fold more FdUrd in the DNA of MMR-deficient cells compared with their MMR-proficient counterparts (Fig. 6). These FU-Gua base pairs made up a significant portion of the DNA lesions created after FdUrd exposure (Fig. 7B). These data strongly suggest that the presence of FU-Gua is extremely lethal, sufficient to trigger MMR-mediated G2 cell cycle checkpoint arrest and cell death responses.

MMR-proficient cells responded to FdUrd exposures with a G2 arrest within the first cell division (Fig. 4). This is similar to MNNG treatment, whereby the Gua of a Gua:Cyt base pair is rapidly methylated at the O6-position, which then allows it to template Thy in the first round of replication. This O6-methyl-Gua:Thy base pair is a good substrate for hMSH2-hMSH6 (30, 54). In fact, FU-Gua is a better substrate than O6-methyl-Gua:Thy (30). However, the response to 6-TG treatment is greatly delayed compared with FdUrd; 6-TG must first be incorporated, then undergo a low frequency (0.016%) methylation to form S6-methyl-6-TG, which can then template Thy to form a substrate recognizable by MMR (57). It takes at least three replication cycles for a G2 arrest to develop following exposure to 6-TG (41). Significantly, recognition by MMR in the first cell division after FdUrd treatment would be expected to keep FdUrd from being mutagenic.

The fact that MMR-proficient cells responded to FdUrd exposures within the first cell division is consistent with FU-Gua serving as the primary signal for MMR in controlling G2 cell cycle checkpoint arrest responses and cell death. Other lesions, such as DNA double-strand breaks, typically appeared after several days of FdUrd exposure (9). MMR might interact with other proteins; MLH1 and/or MSH2/3/6 can associate with or influence the activities of molecules such as proliferating cell nuclear antigen (58–61), Bloom’s syndrome helicase (62), and in yeast) the homologs of the human DNA nucleotide excision repair factors XPF, XPG, XPD, ERCC1, XPA, and XPB (66). In addition, the mechanism of MMR is unique among DNA repair pathways in that a large tract (≥1 kb) of DNA is removed upon each repair event; this presents an opportunity to re-incorporate even more of the antimetabolites, resulting in futile cycles of repair (67). Alternatively, MMR might collide with BER enzymes as they simultaneously repair FU-Gua and Ura-Gua lesions. Additionally, in the case of cisplatin treatment, one group suggested that MMR proteins might form a complex with cisplatin adducts in DNA that make it refractory to nucleotide excision repair; slow repair would result in the selective retention of the signal that triggers cell death (68). A comparable phenomenon may occur as MMR interferes with BER processes.
(e.g. recognition of a glycosylase-generated abasic site) (69). A recent study found that a dominant missense mutation in 
Msh6 (Thr-1217 → Asp) in mice caused loss of DNA repair function while having no effect on induction of apoptosis, in response to exposure to MNNG, 6-TG, or cisplatin (70). This mutant could bind mispaired bases but was resistant to ATP-induced release, thereby interfering with repair (resulting in increased mutation rate, MSI, and cancer development). The authors concluded that the increased mutation frequency in these mice was sufficient to drive tumorogenesis, regardless of the status of their DNA damage response function (70). This supports the model that MMR serves as a damage sensor to signal apoptosis and/or cell death (either directly via a signal transduction cascade or indirectly by blocking DNA replication, transcription, and/or repair processes). This is in contrast to the futile repair cycles model, whereby excision by MMR leads to double-strand break creation; these double-strand breaks then signal apoptosis.

We likewise favor the hypothesis that MMR serves as a sensor of DNA damage. We theorize that it is likely that the formation of a FU:Gua lesion following FP treatment triggers G2 arrest and cell death responses. Additionally, this indicates that tumors with this type of MMR mutation (i.e. the previously described missense mutation in 
Msh6 with separation of repair and apoptotic functions) (70) may remain responsive to chemotherapeutic agents such as FPs. However, mutations in 
MSH6 are uncommon in human familial or sporadic colorectal cancers (71), and most mutations in MMR genes result in complete loss of function (72).

The overall response of MSI+ tumors to FP-based therapies remains controversial. Although tumors with MSI+ have an overall better prognosis (73), several studies have demonstrated that FP-based chemotherapy did not provide a significant survival advantage to patients with MSI+ tumors (27–29). Clinical studies using FP therapies are hard to interpret, because FPs are always given in combination with other agents, and other genetic determinants may influence the response (74). Studies are undergoing in our laboratory to elucidate MMR-mediated signal transduction responses that cause cell cycle arrest and cell death, and resolving these signaling pathways may shed light on determinants that more efficaciously affect overall responses of tumors to therapies.

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Mark Meyers, Mark W. Wagner, Anthony Mazurek, Christoph Schmutte, Richard Fishel
and David A. Boothman

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