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p53 Modulates the Effect of Loss of DNA Mismatch Repair on the Sensitivity of Human Colon Cancer Cells to the Cytotoxic and Mutagenic Effects of Cisplatin[1]

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ABSTRACT

This study examined how the DNA mismatch repair (MMR) system and p53 interact to maintain genomic integrity in the presence of the mutagenic stress induced by cisplatin (DDP). Sensitivity to the cytotoxic and mutagenic effect of DDP was assessed using a panel of sublines of the MMR-deficient HCT116 colon carcinoma cells in which MMR function had been restored by transfer of a copy of MLH1 on chromosome 3 or in which p53 function had been disabled by expression of HPV-16 E6. Loss of p53 function by expression of E6 in MMR-proficient HCT116+ch3 cells conferred only 1.1–2.0-fold resistance to a panel of commonly used chemotherapeutic agents, whereas disruption of p53 in MMR-deficient HCT116 cells resulted in substantial levels of resistance to some agents (paclitaxel, 1.9-fold; gemcitabine, 2.7-fold; 6-thioguanine, 3.3-fold; and etoposide, 4.4-fold) but sensitization to other agents (topotecan, 2.5-fold; and DDP, 3.3-fold). Loss of MMR or p53 alone had only a minor effect on sensitivity to the mutagenic effect of DDP as measured by the appearance of variants resistant to 6-thioguanine, etoposide, topotecan, gemcitabine, and paclitaxel in the population 10 days later (1.0–2.4-fold), whereas loss of both p53 and MMR had a more profound effect (1.7–6.5-fold). Loss of both p53 and MMR increased the basal frequency insertion/deletion mutations detected by a shuttle vector-based assay to a greater extent than loss of either alone. In association with DDP-induced injury, loss of p53 or MMR alone resulted in 1.2- and 1.7-fold more mutations, whereas loss of both resulted in a 5.1-fold increase in mutant frequency. Examination of the impact of loss of p53 and/or MMR on the DDP-induced cell cycle checkpoint activation, p53 induction, ability of the cell to tolerate adducts in its DNA, and the rate of disappearance of platinum from genomic DNA indicated the effects of the loss of p53 and/or MMR on all of these parameters, suggesting a multifactorial etiology for the changes in sensitivity to the cytotoxic and mutagenic effects of DDP. These results indicate that p53 and MMR can cooperate to control sensitivity to the cytotoxic effect of DDP and to limit its mutagenic potential in the colon cancer cells.

INTRODUCTION

There is now a large body of evidence indicating that resistance to chemotherapeutic agents can arise through a process in which somatic mutation creates resistant variants in the tumor cell population that is then enriched for these cells by subsequent exposure to the therapeutic agent (1–3). A basic tenet of this model is that the rate of generation of drug-resistant variants in a population is a function of the mutation rate and that changes in genomic stability that increase the mutation rate will increase the probability that drug resistance will emerge during a course of treatment. There are substantial data indicating that the genomic instability that accompanies failure of DNA repair mechanisms and cell-cycle control checkpoints does in fact foster the generation of drug-resistant variants during tumor growth (4–9).

Among the types of defects that can cause genomic instability, the loss of MMR[3] is of particular interest with respect to resistance to chemotherapeutic agents. MMR is a postreplicative DNA repair process that corrects single-base mismatches and small mismatched loops in the daughter strand of newly replicated DNA (6). Failure to correct such mismatches results in an increased mutation rate, most readily apparent as instability in the length of microsatellite sequences. Loss of MMR because of mutation of MSH2 or MLH1 underlies the majority of cases of hereditary nonpolyposis colon cancer (8, 9) and is also common in a variety of sporadic cancers including endometrial, ovarian, breast, prostate, lung, and pancreatic cancer (10). In addition to increasing the rate of mutation throughout the genome, loss of MMR produces drug resistance directly by impairing the ability of the cell to detect adducts in its DNA that mimic base mismatches. Loss of MMR results in high-level resistance to the antimetabolite 6-thioguanine (11), moderate levels of resistance to the methylating agents such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (7) and temozolomide (12), and low-level resistance to DDP and carboplatin (13) in cell lines cultured *in vitro*. At concentrations attainable in patients, these agents cause cell death by apoptosis. Triggering apoptosis requires the cell to be able to recognize the presence of the damage in DNA, and the current hypothesis is that loss of MMR results in impaired adduct detection or lack of attempted repair and, thus, a diminished pro-apoptotic signal (7).

p53 is another protein critical to the maintenance of genomic integrity, particularly after genotoxic stress. Increases in p53 after cellular injury mediate G_1 checkpoint activation and enhanced DNA repair. Under circumstances where damage is extensive, p53 plays a direct role in triggering apoptosis (14). Cells lacking p53 function are genetically unstable and are predisposed to gross genomic alterations such as gene amplification, aneuploidy, translocation, and deletions. p53 dysfunction is associated with increased tumorigenesis in p53 knockout mice (15), and p53 is mutated in a very large fraction of human cancers (16).

DDP is widely used for the treatment of a variety of solid tumors. The most abundant lesions produced in DNA are intrastrand cross-links, which are believed to account for both the cytotoxicity and mutagenicity of the drug. Its mutagenic potential has been well documented in both bacterial (17, 18) and mammalian cells (19, 20). We have established that loss of MMR in the HCT116 cells causes low-level resistance to the cytotoxic effects of DDP (13) and also renders these cells hypersensitive to its mutagenic effects (21, 22). Because loss of p53 in human tumors is even more common than loss of MMR, it is of interest to determine how loss of these two genomic guardians interact. The aim of the current study was to determine whether loss of MMR function alters the effect of loss of p53 on parameters likely to be important to the development of drug resistance in the HCT116 model system.

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[3] The abbreviations used are: MMR, DNA mismatch repair; DDP, cisplatin.

MATERIALS AND METHODS

Cell Lines. The four sublines of the human colorectal adenocarcinoma cell line HCT116 used in this study were HCT116, HCT116+ch3, HCT116/E6, and HCT116+ch3/E6, representing $MMR^-/p53^+$, $MMR^+/p53^+$, $MMR^-/p53^-$, and $MMR^+/p53^-$ phenotypes, respectively. The hMLH1-deficient HCT116 was obtained from the American Type Culture Collection (ATCC CCL 247); HCT116 contains a hemizygous mutation in hMLH1 resulting in a truncated, nonfunctional protein (23). A subline complemented with chromosome 3 (HCT116+ch3) was obtained from Drs. C. R. Boland and M. Koi. The chromosome 3-complemented cells were competent in DNA mismatch repair (24). HCT116 and HCT116+ch3 sublines expressing papillomavirus E6 (identified here as HCT116/E6 and HCT116+ch3/E6) were obtained from Drs. D. A. Boothman and M. Meyers. In these cell lines, constitutive high-level expression of the human papillomavirus type-16 *E6* gene, which stimulates the degradation of p53 through a ubiquitin pathway, disrupted p53 function (25). All of the four cell lines were maintained in Iscove's modified Dulbecco's medium (Irvine Scientific, Irvine, CA) supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum. The chromosome-complemented lines were maintained in medium containing 400 μg/ml geneticin (Life Technologies, Inc., Grand Island, NY), and the cell lines expressing papillomavirus E6 were cultured in medium supplemented with 80 μg/ml hygromycin B (Boehringer Mannheim, Indianapolis, IN). HCT116 cells in which one or both *p53* alleles were deleted by targeted homologous recombination (26), designated as HCT116/$p53^{+/-}$ and HCT116/$p53^{-/-}$, were obtained from Dr. Bert Vogelstein. These were maintained in McCoy's 5A media (Irvine Scientific, Santa Ana, CA) supplemented with 10% FCS and penicillin/streptomycin.

Reagents. Cisplatin and paclitaxel were gifts from Bristol-Myers Squibb (Princeton, NJ). A stock solution of 1 mM cisplatin in 0.9% NaCl was stored in the dark at room temperature. Paclitaxel was dissolved in DMSO, diluted with saline to form a stock solution of 5 μM, and stored at −20°C. Topotecan was purchased from Smith Kline Beecham Pharmaceuticals (King of Prussia, PA), dissolved in deionized water, and stored as a 10 μM stock solution at −20°C. The clinical formulation of gemcitabine was purchased from Eli Lilly and Co. (Indianapolis, IN) and was diluted directly in tissue culture medium. The clinical formulation of etoposide was obtained from Bristol-Myers Laboratories (Syracuse, NY) and was diluted directly in tissue culture medium. 6-Thioguanine was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in 0.2 N sodium hydroxide to form a 20 mM stock solution and stored at −20°C.

Clonogenic Assay. Clonogenic assays were performed by seeding 250 cells into 60-mm plastic dishes in 5 ml of complete media. After 24 h, appropriate amounts of the drugs were added to the dishes, and the cells were exposed for 24 h (6-thioguanine, etoposide, and paclitaxel) or 1 h (all of the other drugs). Thereafter, the cells were washed, and fresh drug-free medium was added. Colonies of at least 50 cells were scored visually after 8–10 days. Each experiment was performed a minimum of three times using triplicate cultures for each drug concentration. IC_{50} values were determined using log-linear interpolation.

Mutant Frequency Assay. All of the HCT116 sublines were grown in hypoxanthine-aminopterin-thymidine medium containing 0.4 μM aminopterin, 16 μM thymidine, and 100 μM hypoxanthine for a minimum of 14 days and then were exposed for 1 h to increasing concentrations of DDP. Thereafter, the cells were washed twice and recultured in regular medium for 8 days during which the cultures were split 2:1 as needed to keep them from becoming confluent. All of the cells were then trypsinized and seeded into each of 10 100-mm tissue culture dishes at 100,000 cells/dish in the presence of 20 μM 6-thioguanine. At the same time, aliquots of 250 cells were seeded into each of three 60-mm dishes in drug-free medium for determination of cloning efficiency. After 14 days, colonies were counted after staining with 0.1% crystal violet. The procedure for determination of the frequency of mutation to the other drugs used in this experiment was the same except that the cells were not grown in hypoxanthine-aminopterin-thymidine medium before the start of the experiments. A concentration of drug that resulted in a cloning efficiency of approximately 0.0002% was added to identify the number of resistant clones. These concentrations were 20 μM 6-thioguanine, 10 μM etoposide, 200 nM topotecan, 100 nM gemcitabine, and 20 nM paclitaxel. The frequency of resistant variants was calculated as follows: variant frequency = $a/(b \times 10^6)$, where a is the number of colonies present in the 10 drug-treated dishes and b is the cloning efficiency. Each experiment was performed a minimum of three times, and the data are presented as mean ± SD.

Shuttle Vector Mutation Assay. The pZCA29 vector (27) was obtained from Dr. T. M. Runger (University of Gottingen, Gottingen, Germany). Four million cells were transfected with 2 μg of pZCA29 by electroporation on day 1. Replicated pZCA29 was recovered from aliquots of the transfected cells on days 3, 5, 7, 9, and 11 by a rapid alkaline lysis procedure. For the DDP treatment experiments, the cells were treated with 25 μM DDP for 1 h 2 days after transfection, and the vector was harvested on days 3, 5, 7, 9, and 11. Any pZCA29 that failed to replicate in the mammalian cells is characterized by the bacterial pattern of methylation. Such unreplicated plasmid DNA was removed by digestion with *Dpn*I, which cleaves the methylated DNA. *Escherichia coli* XL1-Blue MRF' (Stratagene) was transformed with the recovered pZCA29 and then selected on LB agar plates containing 5-bromo-4-chloro-3-indolyl-β-galactosidase, isopropyl-β-D-thiogalactoside, and ampicillin. Bacterial transformations were performed in triplicate for each of two to three independent samples of pZCA29 recovered at each time point. The mutant frequency was calculated as the mean of the total number of blue colonies divided by the mean of the total number of colonies.

Flow Cytometry. Subconfluent cultures growing in 10-cm tissue culture dishes were exposed to 25 μM DDP for 1 h. At 1, 2, 3, 4, 5, 6, and 7 days after DDP treatment, cells were harvested by trypsinization, washed twice with ice-cold PBS, fixed in ice-cold 70% ethanol, treated with RNase (Sigma) at 37°C for 30 min, and stained with 50 μg/ml propidium iodide (Sigma). After a 30-min incubation on ice, the cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using the FlowJo cell cycle analysis software (Tree Star, Inc., San Carlos, CA) and the "Watson Pragmatic" model.

Western Blotting. Cells were collected at times from 1 to 7 days after a 1-h treatment with 25 μM DDP and lysed in 100 μl of lysis buffer [10 mM Tris-HCl (pH7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 5 mM dithiothreitol, 1 mM sodium vanadate, 0.1 mM phenylmethylsulfonyl fluoride, and 5 mM aminocaproic acid] for 30 min on ice. The insoluble material was removed by centrifugation at 14,000 × g for 20 min at 4°C. Protein (10 μg) from each sample was electrophoresed through 10–20% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA). The membranes were blotted with monoclonal antibodies specific for p53 (Santa Cruz Biotechnology, Santa Cruz, CA). After application of a horseradish peroxidase-coupled secondary antibody, reactive proteins were visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Platinum-DNA Adduct Formation and Repair. The binding of platinum to DNA was determined in whole cells exposed to DDP. For dose-response studies, cells at 80% confluence in T-150 flasks were incubated in fresh medium containing 0–200 μM drug for 1 h. The cells were then trypsinized, washed three times with PBS, and incubated overnight at room temperature in a lysis buffer containing 0.67% Triton X-100, 2.6 M NaCl, 133 mM EDTA, and 2.6 M Tris-HCl (pH 8.0). DNA was isolated by phenol-chloroform extraction and dissolved in TE buffer (pH 8.0). Aliquots of the DNA were digested in 1 M HCl at 75°C for 2 h, and the hydrolysate was used for the quantitation of platinum by flameless atomic absorption spectrophotometry (Perkin-Elmer Model 2380). These experiments verified that platinum-DNA adduct levels were a linear function of DDP concentration in all of the four HCT116 sublines.

For measurement of the disappearance of platinum from DNA, subconfluent cells in T-150 flasks were treated for 1 h with DDP concentrations that yielded the same initial adduct level, and the pg platinum/μg DNA was measured at 0, 3, 6, 12, and 24 h after the drug treatment.

Statistics. All of the data were analyzed by use of a two-sided paired Student's t test with the assumption of unequal variance.

RESULTS

Effect of Loss of p53 or MMR Function on Sensitivity to the Cytotoxic Effect of DDP and Drugs Often Used in Combination with DDP. These studies were conducted using a panel of four human colon carcinoma HCT116 sublines molecularly engineered to have the following combinations of proficient and deficient phenotypes: $p53^+/MMR^+$, $p53^-/MMR^+$, $p53^+/MMR^-$, and $p53^-/MMR^-$

Table 1. *IC_{50} values for HCT116 sublines*[a]

Cell line (phenotype)	Cisplatin (μM)	6-Thioguanine (μM)	Etoposide (μM)	Topotecan (nM)	Gemcitabine (nM)	Paclitaxel (nM)
HCT116+ch3 ($p53^+/MMR^+$)	12.5 ± 2.5	2.1 ± 0.5	0.39 ± 0.1	52.8 ± 10.3	89 ± 17.4	2.6 ± 0.5
HCT116 ($p53^+/MMR^-$)	23 ± 3.4	10.7 ± 1.4	0.6 ± 0.2	123 ± 14.6	124.3 ± 20.5	2.1 ± 0.7
HCT116+ch3/E6 ($p53^-/MMR^+$)	14.8 ± 2.7	2.9 ± 1.1	0.76 ± 0.2	82.3 ± 11.8	114.1 ± 10.2	2.8 ± 1.2
HCT116/E6 ($p53^-/MMR^-$)	7.5 ± 1.8	34.9 ± 6.8	2.5 ± 0.3	55.2 ± 9.6	334.4 ± 15.7	3.8 ± 1.1

[a] Each value represents mean ± SD of three to five independent experiments each performed with triplicate cultures for each drug concentration.

corresponding to the HCT116+ch3, HCT116+ch3/E6, HCT116, and HCT116/E6 sublines, respectively. Clonogenic assays were used to determine the effect of loss of p53, MMR, or both on the sensitivity to the cytotoxic effects of DDP and to a panel of chemotherapeutic agents often used in combination with DDP. The IC_{50} values are presented in Table 1, and the ratios of IC_{50} values are summarized in Table 2. Loss of p53 function in MMR-proficient HCT116+ch3 cells conferred statistically significant resistance only for topotecan. However, disruption of p53 in MMR-deficient HCT116 cells produced significant degrees of resistance to gemcitabine, 6-thioguanine, and etoposide by a factor of 2.7-, 3.3- and 4.4-fold, respectively, but paradoxically rendered the cells hypersensitive to topotecan and DDP by factors of 2.5- and 3.3-fold, respectively. Thus, the effect of disabling p53, MMR function, or both together was unique to each drug, consistent with the fact that each has a different mechanism of action. This suggests that the interaction between p53 and MMR with respect to cytotoxicity varies depending on the type of injury inflicted.

Loss of p53 function has been reported to produce resistance to DDP (28–30), whereas in these colon cancer cells, loss of p53 function because of expression of E6 produced sensitization. E6 can affect the function of other proteins in addition to that of p53. To determine whether loss of p53 function alone was sufficient to produce this change in sensitivity, a truly isogenic set of HCT116 cell lines in which either one or both *p53* alleles had been somatically deleted using a knockout vector (26) was tested for sensitivity to DDP. As shown in Fig. 1, the parental cells and the heterozygote with one deleted *p53* allele demonstrated similar sensitivity with IC_{50} values of 24.4 μM and 18.4 μM, respectively. However, the homozygote in which both *p53* alleles were disrupted was markedly more sensitive with an IC_{50} of 5.7 μM, thus confirming the results obtained with the E6-expressing cells. This finding is in good agreement with prior observations showing that knockout of p53 makes these human colorectal cancer cells more sensitive to apoptosis induced by other agents capable of inducing DNA damage such as doxorubicin (26) and γ-radiation (31).

Effect of Loss of p53 or MMR Function on the Ability of DDP to Generate Resistant Variants. Cells were exposed for 1 h to increasing concentrations of DDP, and then 8 days later, the number of clonogenic cells demonstrating high level resistance to 6-thioguanine, etoposide, topotecan, gemcitabine, and paclitaxel was determined. We have demonstrated previously (21) that, over the concentration range of 0–75 μM, DDP produces a linear increase in the number of such resistant clones and that the effect is well-described by the slope of the number of resistant clones plotted as a function of the concentration of DDP used to mutagenize the cells. Table 3 presents these slopes for 6-thioguanine, etoposide, topotecan, gemcitabine, and paclitaxel for the four HCT116 sublines. On the basis of the ratio of the slopes, as shown in Table 4, loss of p53 in the MMR-proficient HCT116+ch3 cells had only a minor effect on the ability of DDP to generate variants resistant to 6-thioguanine, etoposide, topotecan, gemcitabine, or paclitaxel. In contrast, loss of p53 in the MMR-deficient HCT116 cells resulted in larger increases in slope for all of these drugs. Stated the other way around, loss of MMR in the p53-proficient HCT116+ch3 cells had a modest impact, producing increases of 1.1–2.4-fold, whereas loss of MMR in the p53-deficient HCT116+ch3/E6 cells had more profound effect and increased the generation of resistant variants by 2.3–6.5-fold. These results indicate that p53 and MMR both operate to prevent the emergence of resistant clones after exposure to DDP and that they do so by at least partially independent mechanisms because loss of both functions produced a greater effect than loss of either alone.

To confirm this finding, the isogenic sets of $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ HCT116 cell lines were tested for generation of 6-thioguanine-resistant variants. Fig. 2 shows that when both *p53* alleles were deleted from the parental cells, DDP was 3.9-fold ($P < 0.01$) more effective at generating 6-thioguanine-resistant variants. Interestingly, when one *p53* allele was deleted there was little change in sensitivity at low DDP concentrations; however, at higher DDP concentrations there was nearly the same increase in resistant variants generated as was observed when both alleles were deleted. This gene dosage effect is consistent with the hypothesis that at low adduct burdens the

Table 2. *Magnitude of change in drug sensitivity as a result of the loss of p53, MMR, or both functions expressed as IC_{50} ratios*[a]

	Effect of loss of p53		Effect of loss of MMR	
Drug	in MMR^+ HCT116+ch3 cells	in MMR^- HCT116 cells	in $p53^+$ HCT116+ch3 cells	in $p53^-$ HCT116+ch3/E6 cells
DDP	1.2 ± 0.1	0.3 ± 0.1[b]	1.9 ± 0.3[c]	0.5 ± 0.0[d]
6-Thioguanine	1.4 ± 0.6	3.3 ± 0.6[b]	5.1 ± 0.8[c]	12.0 ± 7.4[d]
Etoposide	2.0 ± 0.5	4.4 ± 1.1[b]	1.6 ± 0.6	3.4 ± 0.7[d]
Topotecan	1.6 ± 0.3[e]	0.4 ± 0.1[b]	2.4 ± 0.6[c]	0.7 ± 0.2[d]
Gemcitabine	1.3 ± 0.1	2.7 ± 0.3[b]	1.4 ± 0.2	2.9 ± 0.2[d]
Paclitaxel	1.1 ± 0.3	1.9 ± 0.6	0.8 ± 0.2	1.4 ± 0.5

[a] Ratio of the IC_{50} value in the functionally deficient to proficient cells determined by clonogenic assay. Each value is mean ± SD.
[b] $P < 0.05$, comparison between the $MMR^-/p53^-$ and the $MMR^-/p53^+$ cells.
[c] $P < 0.05$, comparison between the $MMR^-/p53^+$ and the $MMR^+/p53^+$ cells.
[d] $P < 0.05$, comparison between the $MMR^-/p53^-$ and the $MMR^+/p53^-$ cells.
[e] $P < 0.05$, comparison between the $MMR^+/p53^-$ and the $MMR^+/p53^+$ cells.

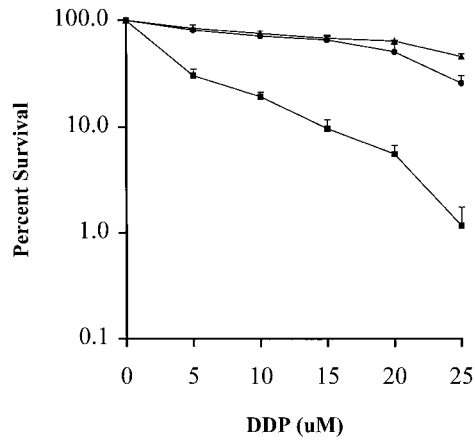

Fig. 1. DDP concentration-survival curves for the $p53^{+/+}$ (▲), $p53^{+/-}$ (●), and $p53^{-/-}$ (■) HCT116 cells. Each point represents the mean of three experiments with triplicate cultures. *Bars*, SD.

Table 3. *Slope of a plot of the number of resistant colonies as a function of DDP concentration for HCT116 sublines*[a]

Cell line (phenotype)	6-Thioguanine (uM)	Etoposide (uM)	Topotecan (nM)	Gemcitabine (nM)	Paclitaxel (nM)
HCT116+ch3 ($p53^+$/MMR^+)	2.1 ± 0.8	0.4 ± 0.1	1.2 ± 0.1	0.8 ± 0.2	0.4 ± 0.1
HCT116 ($p53^+$/MMR^-)	4.8 ± 1.5	0.6 ± 0.1	1.9 ± 0.6	1.0 ± 0.2	0.4 ± 0.1
HCT116+ch3/E6 ($p53^-$/MMR^+)	4.1 ± 1.6	0.4 ± 0.0	1.3 ± 0.3	0.8 ± 0.3	0.6 ± 0.1
HCT116/E6 ($p53^-$/MMR^-)	21.3 ± 2.5	2.8 ± 0.6	3.1 ± 0.7	2.7 ± 0.6	1.3 ± 0.2

[a] Each value represents mean ± SD of three independent experiments.

amount of p53 generated by a single allele is sufficient to guard against mutation at most sites, but at high adduct burdens it is inadequate.

Induction of Mutations by DDP. Another way to assess the effect of loss of p53 or MMR function on sensitivity to the mutagenic potential of DDP is to measure its ability to produce insertion/deletion mutations in a defined DNA sequence. The stability of repetitive sequences replicated episomally was compared between the four sublines using the shuttle vector pZCA29. This β-galactosidase expression vector contains a 94-bp insertion that includes within it a 28-bp CA repeat tract and a 30 bp GT repeat tract arranged palindromically such that the β-galactosidase sequence is out of frame (27). The vector also contains SV40 T-antigen, origin, and enhancer to allow episomal replication in the human cells. In the absence of a frameshift mutation generated during replication of the vector in the tumor cell, β-galactosidase is not expressed when the plasmid is rescued from the mammalian cell and electroporated into appropriate bacteria. However, +2 or +4 insertions or −2 or −4 deletions in the repeat tract introduced during replication in the tumor cells restore the reading frame and permit expression of β-galactosidase when the vector is transferred to the bacteria. The fraction of the plasmids containing such a mutation recovered after passage through the tumor cells can be quantified by determining the fraction of blue *versus* white bacterial colonies after addition of isopropyl-β-D-thiogalactoside and 5-bromo-4-chloro-3-indolyl-β-galactosidase exposure.

Fig. 3*A* shows the spontaneous mutant frequencies observed after replication of the pZCA29 vector in the HCT116+ch3 (MMR^+/$p53^+$), HCT116+ch3/E6 (MMR^+/$p53^-$), HCT116 (MMR^-/$p53^+$), and HCT116/E6 (MMR^-/$p53^-$) cell lines for various periods of time. These frequencies reflect the ability of the cell to faithfully replicate the out-of-frame vector under basal conditions in the absence of any exogenous insult. For each cell line, the number of mutations increased as a function of the time during which the vector was allowed to replicate in the tumor cell. Differences between the cell lines were already apparent after just 3 days of vector replication. The mutant frequency was lowest for the MMR and p53-proficient HCT116+ch3 cell line. Loss of either p53 function or MMR function alone resulted in a small increase in the number of mutants; the increases were 1.2- and 1.7-fold, respectively ($P > 0.05$ for all of the time points relative to the MMR^+/$p53^+$ cells). However, the greatest increase was observed in the cells that had lost both p53 and MMR function (5.1-fold). This increase was statistically significant ($P < 0.05$) for comparison with all of the other three sublines at all of the time points measured. Thus, the pZCA29 vector detected the type of genomic instability produced by loss of either p53 or MMR, and this type of instability was augmented in cells burdened by loss of both functions.

Fig. 3*B* shows that treatment of cells with 25 μM DDP for 1 h starting 48 h after introduction of the vector into the tumor cells increased the number of pZCA29 mutants in all of the four cell lines. On the basis of the slopes of the curves, loss of p53 permitted DDP exposure to generate an average of 1.2-fold more mutants ($P = 0.67$), whereas loss of MMR function permitted it to generate an average of 1.7-fold more mutants ($P = 0.13$). Loss of both p53 and MMR function in the HCT116/E6 cells resulted in 5.1-fold more mutants ($P = 0.03$). Loss of p53 function had a larger effect in MMR-deficient cells (2.9-fold; $P = 0.05$) than in MMR-proficient cells (1.2-fold; $P = 0.67$). Likewise, loss of MMR had a larger effect in p53-deficient cells (4.3-fold; $P = 0.04$) than in p53-proficient cells (1.7-fold; $P = 0.13$). Thus, DDP was much more effective at producing substitution and/or deletion mutations when both p53 and MMR function were lost.

To confirm the results obtained when expression of E6 was used to disable p53 function, the pZCA29 vector was used to measure the basal and DDP-induced frequency of mutations in the HCT116 sublines in which one or both *p53* alleles had been deleted. The basal mutant frequencies after passage of the pZCA29 vector through the HCT116 $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ cell lines are shown in Fig. 4*A*. The $p53^{+/-}$ cells replicated the vector as faithfully as wild-type cells over the period tested. However, loss of both *p53* alleles rendered the vector replication error-prone by a factor of 1.9-fold ($P = 0.12$). Fig. 4*B* shows that when cells were treated with 25 μM DDP for 1 h starting 48 h after vector introduction, the number of mutants produced was increased in all of the three cell lines. On the basis of the

Table 4. *Effect of the loss of p53, MMR, or both on the ability of DDP exposure to generate drug-resistant variants*

Drug	Effect of loss of p53[a]		Effect of loss of MMR[a]	
	in MMR^+ HCT116+ch3 cells	in MMR^- HCT116 cells	in $p53^+$ HCT116+ch3 cells	in $p53^-$ HCT116+ch3/E6 cells
6-Thioguanine	2.0 ± 0.4	4.7 ± 1.0[b]	2.4 ± 0.4	5.6 ± 1.7[c]
Etoposide	1.2 ± 0.1	5.0 ± 0.9[b]	1.6 ± 0.1[d]	6.5 ± 1.3[c]
Topotecan	1.1 ± 0.2	1.7 ± 0.4	1.6 ± 0.4	2.4 ± 0.3[c]
Gemcitabine	1.0 ± 0.5	2.7 ± 0.5[b]	1.3 ± 0.1	3.3 ± 0.8[c]
Paclitaxel	1.6 ± 0.5[e]	3.0 ± 0.4[b]	1.1 ± 0.1	2.3 ± 0.4[c]

[a] Ratio of the slope of the number of resistant colonies/10^6 clonogenic cells as a function of DDP concentration. Each value is mean ± SD; $n = 3$ for all of the drugs.
[b] $P < 0.05$: comparison between the MMR^-/$p53^-$ and the MMR^-/$p53^+$ cells.
[c] $P < 0.05$: comparison between the MMR^-/$p53^-$ and the MMR^+/$p53^-$ cells.
[d] $P < 0.05$: comparison between the MMR^-/$p53^+$ and the MMR^+/$p53^+$ cells.
[e] $P < 0.05$: comparison between the MMR^+/$p53^-$ and the MMR^+/$p53^+$ cells.

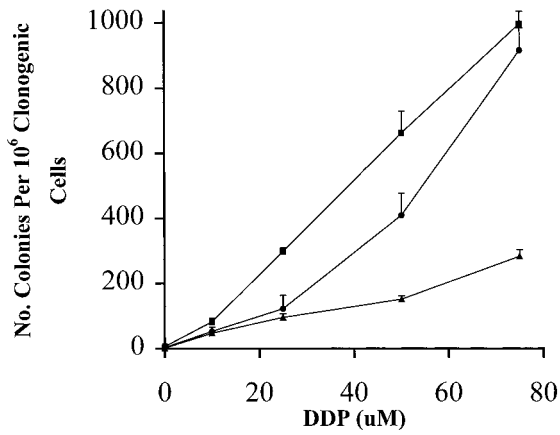

Fig. 2. Number of 6-thioguanine-resistant colonies/10^6 clonogenic cells as a function of DDP concentration for the $p53^{+/+}$ (▲), $p53^{+/-}$ (●), and $p53^{-/-}$ (■) HCT116 cells. Each point is the mean (± SD) of three experiments for each concentration of DDP.

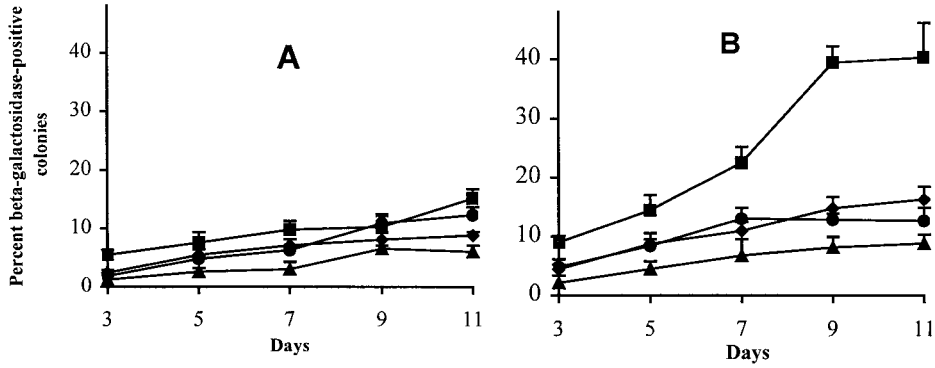

Fig. 3. Frequency of pZCA29 mutation in cells without (*Panel A*) and with (*Panel B*) DDP treatment. The graph shows the frequency of blue bacterial colonies obtained after passage of pZCA29 through the HCT116+ch3 (▲), HCT116 (◆), HCT116+ch3/E6 (●), and HCT116/E6 (■) cells. Plasmid DNA was isolated at the indicated times after introduction into the tumor cells and subsequently transduced into permissive bacteria, *E. coli* XL1-Blue MRF. One h of 25 μM DDP treatment was initiated at 24 h after pZCA29 transfection. Each data point represents the mean (± SD) of three experiments.

ratio of the slopes, DDP generated 1.5-fold ($P = 0.19$) and 2.9-fold ($P = 0.04$) more mutants, respectively, in the $p53^{+/-}$ and $p53^{-/-}$ cells compared with the parental $p53^{+/+}$ HCT116 cells. Thus, the simple repetitive sequences in pZCA29 were hypermutable after DDP exposure in the cells in which p53 function was inactivated.

Cell Cycle Checkpoint Activation by DDP. The ability of DDP to activate the G_1 and G_2-M cell cycle control checkpoints was examined in cells that had lost p53 alone, MMR alone, or both. As shown in Fig. 5, in the p53- and MMR-proficient HCT116+ch3 cells, DDP induced a marked G_2-M phase arrest that peaked at 24 h at which point 65.1% of the DDP-treated cells were in G_2-M compared with 29.5% of the untreated control population. The G_2-M arrest gradually resolved over the ensuing days such that the cell cycle phase distribution had returned to normal by 7 days after DDP exposure. Loss of p53 alone resulted in a greater depletion of cells from G_1 at early time points such that at 24 h after DDP exposure only 6.6% of the cells were in G_1 compared with 23.4% of cells with intact p53 function. The peak accumulation of cells in the G_2-M phase was also increased (to 63.8%) and delayed to 48 h compared with the p53-proficient cells. Loss of MMR-function alone also resulted in a greater loss of cells from G_1 over the first 24 h. However, the largest effect was a more rapid resolution of the G_2-M phase accumulation consistent with failure of the cells to remain arrested in G_2-M. When both MMR and p53 functions were disabled, the perturbations produced by loss of p53 predominated over those produced by loss of MMR. There was excessive depletion of cells from G_1 at all of the time points examined and a substantial delay in the resolution of the G_2-M phase arrest. Thus, loss of either p53 or MMR function alone produced unique patterns of checkpoint failure, and the greatest difference from the pattern observed in the fully proficient cells occurred when both functions were lost.

Effect of Loss of MMR on p53 Response to DDP Injury. The effect of loss of MMR on the ability of DDP-induced DNA damage to signal to ATM and CHK2 to stabilize p53 was measured by quantifying p53 levels by Western blot for up to 7 days after exposure to 25 μM DDP for 1 h. As shown in Fig. 6, in the $p53^{+}$/MMR^{+} cells, p53 levels were increased by 1 day after exposure, and high levels persisted for up to 6 days. This response was blunted in cells in which MMR had been disabled, with both the magnitude and duration of the change in p53 levels being reduced. As expected based on the ability of E6 to mediate the degradation of p53, although p53 was detectable in the E6-expressing HCT116+ch3/E6 and HCT116/E6 cells, the levels did not change significantly after DDP exposure, and they were consistently below those found in the non-E6-expressing cells (data not shown). Thus, loss of MMR had a substantial effect on the extent to which DDP-DNA adducts generated the signals responsible for the stabilization of p53.

Effect of Loss of p53 and MMR on the Disappearance of Platinum from DNA. The rate of disappearance of platinum from the DNA accurately mirrors the rate of removal of the most common DDP adducts (32, 33). The rate of disappearance of platinum from total cellular DNA was measured in each subline. Because removal may be a saturable phenomenon, the DDP concentrations were separately adjusted for each subline so that during the 1-h exposure nearly the same amount of platinum was loaded onto the DNA. The starting loads were 282.4, 277.5, 270.5, and 279.8 pg platinum/μg DNA, respectively, for the HCT116+ch3, HCT116, HCT116+ch3/E6, and HCT116/E6 cells. Fig. 7 shows that there were substantial differences in the kinetics of platinum disappearance from DNA among the four sublines. By 24 h after the start of the 1-h drug exposure, only 5% of the platinum remained in the DNA of the HCT116+ch3 cells. Loss of either p53 or MMR function alone reduced the disappearance rate such that by 24 h 20% and 17%, respectively, of the platinum remained in the DNA. Loss of both p53 and MMR resulted in further slowing of platinum disappearance with persistence of 39% of the initial load in the DNA at 24 h. To better quantify the platinum clearance from DNA among the four sublines, the area under the curve for the percentage of platinum-DNA adducts remaining over

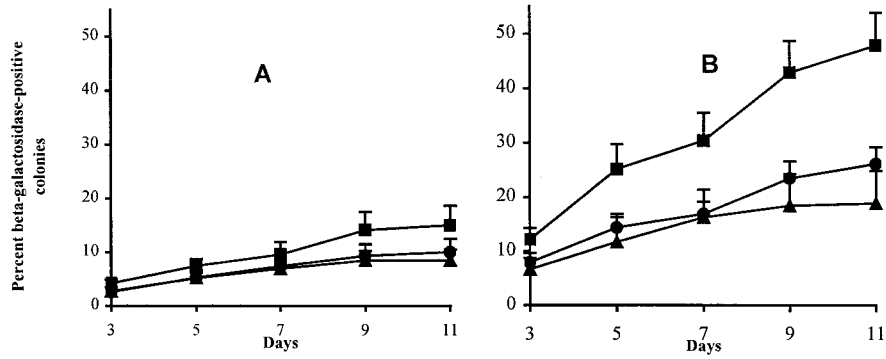

Fig. 4. Frequency of pZCA29 mutation in cells in the absence (*Panel A*) and presence (*Panel B*) of DDP exposure. The graph represents the frequency of blue bacterial colonies obtained after passage of pZCA29 through the HCT116 $p53^{+/+}$ (▲), $p53^{+/-}$ (●), and $p53^{-/-}$(■) cells. Harvest procedures and treatment conditions were identical to those in Fig. 3.

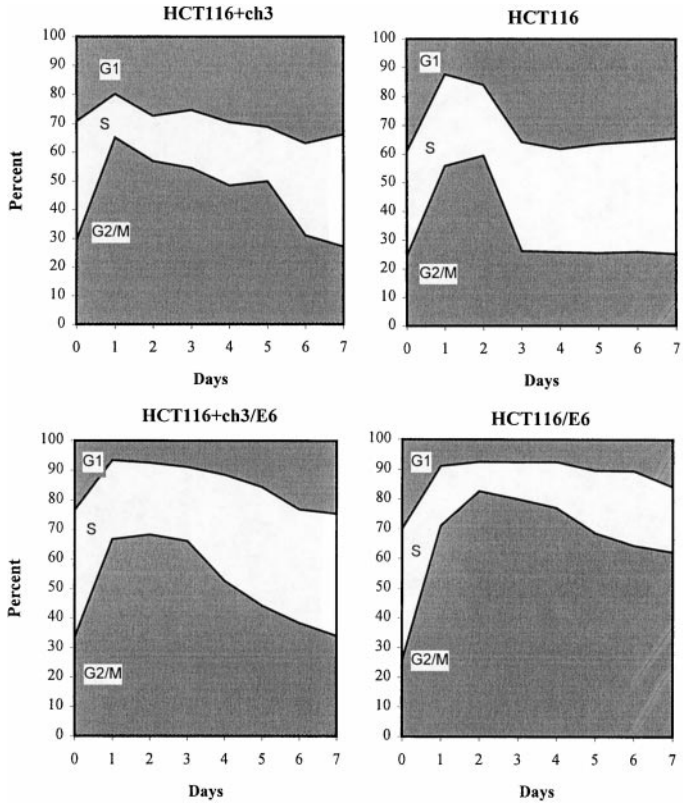

Fig. 5. Effect of DDP on cell cycle phase distribution. Cells were exposed to 25 μM DDP for 1 h and harvested at the indicated times. The cell cycle phase distribution was determined by flow cytometry.

time (percentage × h) was calculated using trapezoidal rule. The area under the curve for HCT116+ch3, HCT116+ch3/E6, HCT116, and HCT116/E6 cells was 619.5, 1042.5, 1141.5, and 1537.5 (percentage of platinum remaining × h), respectively. This indicates that loss of p53 alone resulted in a 1.7-fold increase in total adduct exposure, whereas loss of MMR alone produced a 1.8-fold increase, and loss of both caused a 2.5-fold increase.

Effect of Loss of p53 and MMR on Tolerance to DDP Adducts. One way in which loss of either MMR or p53 may influence the cytotoxicity and mutagenicity of DDP is by altering the ability of the cell to tolerate DDP adducts in its DNA without induction of apoptosis. DDP adduct tolerance can be quantified by measuring the extent of cell kill as a function of the total amount of platinum in the DNA, which is a measure of the cytotoxic potency of the adducts. Because of limitations on the ability to measure the small amounts of platinum present in the DNA after exposure to low DDP concentrations, the total platinum/μg DNA was measured over a range of higher DDP concentrations that were then transformed to multiples of the IC_{50} concentration for each subline. This approach is validated by the log-linear nature of the clonogenic survival curve for DDP at concentrations above the IC_{50}.

As shown in Table 5, total platinum/μg DNA at the end of a 1-h exposure increased linearly with DDP concentration for all of the four sublines, and the adduct potency was remarkably constant at different DDP concentrations within each subline. Loss of p53 function alone increased adduct tolerance by 2.0-fold ($P = 0.0001$), whereas loss of MMR alone increased tolerance by a factor of 2.7-fold ($P = 0.0083$). Thus, both functional deficits permitted cells to survive with a higher adduct load in their DNA. Interestingly, instead of generating an even higher level of tolerance, loss of both MMR and p53 function resulted in only a 1.8-fold increase in tolerance ($P = 0.0144$). This suggests that, with respect to adduct tolerance, both p53 and MMR may be operating through the same mechanism.

DISCUSSION

DDP is a widely used chemotherapeutic agent, but resistance emerges routinely during treatment. One paradigm that may explain this phenomenon is that the genomic instability inherent to tumors somatically generates mutants resistant to DDP before the time the tumor can be diagnosed and that subsequent treatment with DDP enriches for these clones (34). In principle, anything that further increases the mutation rate in the tumor has the potential of enhancing the rate at which drug resistance develops during treatment. Because DDP is itself a mutagen, this agent has the potential of generating variants resistant to both itself and other drugs with which it is often combined and, thus, positively augmenting the process by which resistance emerges.

Loss of MMR results in genomic instability characterized by small insertion and deletion mutations in repetitive sequences throughout the genome. Our prior work (21, 22) demonstrated that loss of MMR rendered cells hypersensitive to the ability of DDP to generate clones resistant not only to 6-thioguanine but also to etoposide, topotecan, gemcitabine, and paclitaxel. Loss of MMR is particularly important with respect to the emergence of drug resistance because MMR-deficient cells are not only hypersensitive to exogenous and endogenous mutagens (35, 36) but also resistant to DDP because of an apparent reduced ability of the cell to sense the presence of adducts in DNA (37, 38). It has been well documented, using both *in vitro* and

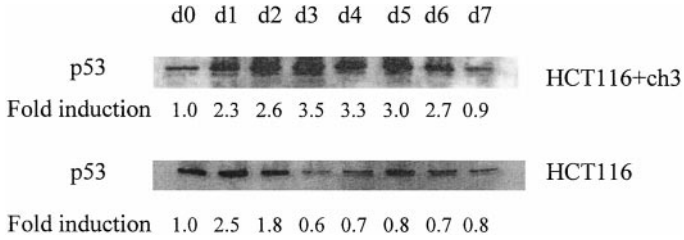

Fig. 6. Change in p53 level in response to DDP. Cells were exposed to 25 μM DDP for 1 h and tested at the time points indicated from the beginning of exposure. Equal amounts of protein were separated by electrophoresis and subjected to Western blot analysis with p53-specific monoclonal antibodies.

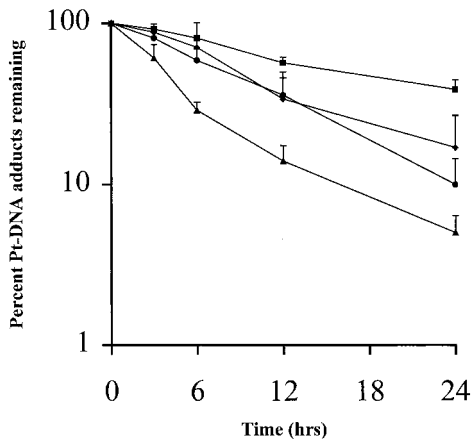

Fig. 7. Disappearance of platinum from DNA. Cells were treated for 1 h with DDP at concentrations designed to give an initial adduct level of approximately 280 pg platinum/μg DNA. The concentrations used were: 200, 160, 120, and 100 μM for HCT116+ch3 (▲), HCT116 (◆), HCT116+ch3/E6 (●), and HCT116/E6 (■) cells, respectively. Platinated DNA was isolated at the indicated times after treatment and quantified by atomic absorption spectrophotometry. Each data point represents the mean (± SD) of two experiments.

Table 5. *Platinum DNA adduct levels after equitoxic DDP exposures*[a]

Cell line (phenotype)	IC_{50} (uM)	Exposure concentration (uM)	Multiple of IC_{50}	pg platinum/μg DNA	pg platinum/μg DNA/IC_{50} multiple	Mean ± SD pg platinum/μg DNA/IC_{50} multiple for all concentrations tested
HCT116+ch3 ($p53^+/MMR^+$)	12.5	100	8.0	136.4	17.1	
						16.7 ± 0.98
		120	9.6	159.6	16.6	
		160	12.8	196.3	15.3	
		200	16.0	282.4	17.6	
HCT116 ($p53^+/MMR^-$)	23.0	100	4.3	186.7	42.9	
						45.2 ±9.85[bc]
		120	5.2	200.2	38.4	
		160	7.0	277.5	39.9	
		200	8.7	519.4	59.7	
	14.8	100	6.8	234.6	34.7	
						33.1 ± 1.58[de]
HCT116+ch3/E6 ($p53^-/MMR^+$)						
		120	8.1	270.5	33.4	
		160	10.8	334.4	30.9	
		200	13.5	452.3	33.5	
HCT116/E6 ($p53^-/MMR^-$)	7.5	100	13.3	279.8	21.0	
						25.0 ± 3.16[f]
		120	16.0	391.6	24.5	
		160	21.3	556.5	26.1	
		200	26.7	760.3	28.5	

[a] Pt-DNA adduct levels were determined as a function of increasing DDP concentrations and relative potency of each adduct formed in the four HCT116 sublines with respect to cytotoxicity was expressed in terms of the amount of platinum in the DNA after exposure to different concentrations of DDP which were converted into the multiple fold of IC_{50}.
[b] $P = 0.0083$ compared with $p53^+/MMR^+$.
[c] $P = 0.0164$ compared with $p53^-/MMR^-$.
[d] $P = 0.0001$ compared with $p53^+/MMR^+$.
[e] $P = 0.0307$ compared with $p53^-/MMR^-$.
[f] $P = 0.0144$ compared with $p53^+/MMR^+$.

in vivo models, that the degree of resistance is great enough so that subsequent treatment with DDP enriches for these genomically unstable cells (39).

The effect of the loss of p53 and MMR function alone on chemotherapeutic agent cytotoxicity appears to be unique to the cell line under study, and both increased and decreased sensitivities have been observed in different model systems (13, 26, 40–43). The results reported here are in general agreement with prior studies in colon cancer cell lines (13, 26). In the colon carcinoma cells used in this study, the effect of loss of both p53 and MMR varied among the drugs tested. In the case of 6-thioguanine, etoposide, and gemcitabine, loss of both p53 and MMR incrementally increased the level of resistance over that observed with loss of either function alone. Thus, it would be reasonable to expect that treatment with any of these agents would enrich the tumor for doubly deficient cells.

Specifically with respect to changes in sensitivity to the cytotoxic effect of DDP, some investigators have reported that loss of p53 function renders cells hypersensitive whereas others have found that it renders them resistant (summarized in Ref. 44) even in cells that are likely to have intact MMR (42, 45). In the model system used in the current study, loss of p53 had relatively little effect in MMR-proficient cells but conferred substantial hypersensitivity in MMR-deficient cells, a finding that is consistent with prior observations by Vikhanskaya *et al.* (43). Although a central role for p53 in mediating activation of the caspase cascade after DNA damage has been extensively reported, and DDP evokes a large increase in p53 level in the $p53^+/MMR^+$ subline, any proapoptotic effect of this change is apparently offset in these cells by other antiapoptotic roles played by this protein. In the absence of functional MMR, these cells appear to be more dependent on this protective effect of p53, as the impact of the loss of p53 was substantially larger in MMR-deficient than MMR-proficient cells. This finding is consistent with experiments in other cellular systems. Brown *et al.* (28) reported that transfection of a dominant negative mutant p53 in A2780 cells (MMR proficient) did not significantly change DDP-induced cytotoxicity, whereas the same vector transfected into an MMR-deficient subline of A2780 induced a significant increase in DDP sensitivity. In addition, by comparing E6-transfected clones obtained from HCT-116 or MCF cells, it was noted that disruption of p53 in MMR-deficient cells (HCT116) greatly enhanced sensitivity to DDP (43, 46), whereas in MCF-7 cells in which MMR function was normal, the effects were much diminished (41). Therefore, our findings extend observations made in other cell lines and further support the hypothesis that p53 cooperates with MMR in determining the cellular sensitivity to DDP.

A clear interaction between loss of p53 and loss of MMR was apparent also with respect to the mutagenic potential of DDP. Loss of both p53 and MMR function rendered the cells substantially more sensitive to the ability of DDP to generate variants resistant to a variety of other drugs than loss of either p53 or MMR alone. Although we did not document that the resistant clones were true mutants, the presence of such clones capable of withstanding very high level exposures to these drugs is likely to contribute to the emergence of drug resistance. This finding brings into sharp focus the concern that initial treatment with agents such as DDP, although it may help reduce tumor burden, also sows the seeds that will eventually cause treatment failure by generating clones within the tumor that are resistant to many other types of drugs. The specific mechanisms by which DDP generates these resistant variants are not yet understood. However, it has been demonstrated that single mutations in topoisomerase II can produce resistance to etoposide (47). In the case of topotecan and gemcitabine, it has already been established that resistance can result from mutations in the topoisomerase I (48) and deoxycytidine kinase (49) gene, respectively. As for paclitaxel, the target of which is β tubulin, the role of single mutations in mediating resistance has not been as clearly established. However, previous studies have documented that paclitaxel-resistant variants arise at a rate as high as that for etoposide (50). That p53 and MMR both function to modulate the ability of DDP to produce mutations was documented further by the experiments with the pZCA29 vector. An increased frequency of small insertion/deletion mutations is a feature of the genomic instability produced by the loss of either p53 or MMR (6, 27, 51), and the pZCA29 vector proved capable of detecting these changes in the basal

rate of mutation. It also effectively detected the mutagenic effect of DDP exposure in the $p53^+/MMR^+$ cells. Although loss of either p53 or MMR alone increased DDP-induced mutations, the observation that the loss of both functions produced a further increase argues that p53 and MMR operate in different pathways to offset the mutational risk posed by DDP adducts in DNA.

Previous studies (35) from this laboratory have demonstrated that p53 and MMR-deficient HCT116/E6 cells are resistant to exogenous H_2O_2 but hypersensitive to the ability of H_2O_2 to generate mutants resistant to 6-thioguanine and ouabain. The current study did not investigate the biochemical nature of the interaction between loss of p53 and MMR; *i.e.,* it cannot be determined from the studies reported here whether the interaction is truly synergistic, only additive, or even partially antagonistic. However, it is clear that DDP is more cytotoxic and mutagenic when both functions are disabled. Caution is needed in interpreting the resulting effects of E6 expression and chromosome 3 transfer as being entirely because of the loss of p53 and complementation of MMR, respectively, because E6 can affect other cellular proteins, and transfer of the chromosome 3 also introduces other genes into the cell.

What is the mechanism by which loss of either p53 or MMR causes DDP to be more mutagenic? The results presented here suggest multiple effects. DDP exposure induced an increase in p53 and activated the G_1 checkpoint in the $p53^+/MMR^+$ cells, and the differences observed in cell-cycle phase distribution when p53 function was lost are consistent with the premature release of cells into S phase. Mammalian DNA polymerases that can bypass adducts in DNA, but create mutations as they do so, have recently been identified (52, 53). Both p53 and MMR may play a role in preventing such mutagenic bypass replication (54, 55). Alternatively, if polymerase fidelity is influenced by MMR or p53, mutations may be introduced during the gap-filling step after processing of the adduct by one or another of the DNA repair mechanisms.

Among the known DNA repair mechanisms, nucleotide excision repair appears to be quantitatively the most important for removal of DDP adducts. Evidence for a direct role for p53 protein in this and other types of DNA repair is accumulating rapidly. Wang *et al.* (56) reported that p53 can bind to several proteins known to play central roles in nucleotide excision repair, including XPD (Rad3), XPB, and CSB, and Therrien *et al.* (57) reported direct involvement of p53 in both global and transcription-coupled nucleotide excision repair. Very recently, Tanaka *et al.* (58) reported that p53 regulates the transcription of a catalytic subunit of ribonucleotide reductase, an enzyme essential to the supply of deoxynucleotides for nucleotide excision repair. MMR also modulates at least one subtype of nucleotide excision repair, transcription-coupled repair. The observation that the kinetics of platinum disappearance from DNA are delayed is consistent with a cooperative interaction between p53 and MMR with respect to nucleotide excision repair. Although variable degrees of continued proliferation can contribute to differences in the disappearance of platinum from DNA, this is unlikely because of the high concentrations of DDP (100–200 μM) used in these kinetic studies.

One might have expected impairment of nucleotide excision repair because of loss of p53 or MMR function to result in hypersensitivity to the cytotoxic effect of DDP. Other genetic lesions that disable nucleotide excision repair clearly produce such an effect (59). Instead, loss of p53 or MMR diminished the cytotoxic potency of DDP adducts by a factor of 2.0- and 2.7-fold, respectively. Thus, the reduced disappearance of adducts from DNA was offset in the p53- and MMR-deficient cells by improved adduct tolerance. This is of particular concern with respect to the emergence of drug resistance because the persistence of such adducts in DNA is likely to contribute additional mutations if the cell can complete another round of DNA synthesis. Such adduct tolerance may be important for other DNA-interacting drugs, although changes in sensitivity to drugs that do not produce adducts must be explained by effects on signaling or apoptotic mechanisms.

Mutations that disable p53 are found very frequently in human cancers (16), often in association with tumor progression or high-grade malignancy (15). Loss of MMR function is a less common but well-described phenomenon, particularly in colon and endometrial cancer (8, 9). Thus, there is a reasonable likelihood that many tumors contain at least a few cells in which both functions have been disabled. The results presented in this study suggest that these cells are particularly dangerous as potential mediators of the continued accumulation of somatic mutations that provide the heterogeneity that favors emergence of drug resistance.

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