

Base Excision Repair as a Therapeutic Target in Colon Cancer¹

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ABSTRACT

Base excision repair (BER) is a fundamental cellular process used to reduce the cytotoxicity of alkylating agent chemotherapy. Heretofore, no therapeutic agents have targeted this DNA repair pathway. Methoxyamine (MX), which binds abasic sites, acting as an inhibitor of BER, was evaluated in combination with the methylating agent temozolomide (TMZ). Three human colon cancer cell lines were used, SW480 cells, which are wild-type for mismatch repair genes and have mutated p53, HCT116 cells, which are mutant in hMLH1 and wild-type for p53, and HCT15 cells, which are mutant in hMSH6 and mutant in p53 as well. Nude mice carrying these tumors received TMZ alone or in combination with MX or *O*⁶-benzylguanine (BG), an inhibitor of *O*⁶-alkylguanine DNA-alkyltransferase, daily i.p. for 5 consecutive days. At the highest tolerable dose of TMZ (120 mg/kg), a tumor growth delay of approximately 9.3 ± 1.2 days was noted in SW480. Addition of BG resulted in a tumor growth delay of 25 ± 2.4 days accompanied by significant weight loss (23%) and severe myelosuppression. In contrast, SW480 tumor-bearing mice treated with MX + TMZ had cessation of tumor growth for 50 ± 13 days and very slow regrowth, yielding tumor growth delays of >70 ± 14 days (*P* < 0.002) without additive systemic toxicity. HCT116 and HCT15 xenografts were completely resistant to treatment with TMZ alone or in combination with BG. However, treatment with MX + TMZ induced significant tumor growth delays (20 ± 1.4 days in HCT116 and 14 ± 3.1 days in HCT15 xenografts, *P* < 0.05). These studies demonstrate that a significant enhancement of the antitumor effect of TMZ by MX was observed in human colon cancer xenografts with mismatch repair proficiency and deficiency. DNA BER may be a useful pharmacological target through which tumor cells can be sensitized to alkylating therapeutic agents.

INTRODUCTION

Alkylating agents are commonly used in the treatment of brain tumors, ovarian cancer, malignant melanomas, and various hematological tumors (1, 2). These agents have either one or two reactive groups that interact covalently with nucleophilic centers in DNA. Such reactive sites are present in all four bases, and they are attacked with different affinities and specificities. Most reactive sites are the ring nitrogen atoms, in particular N7 of guanine and N3 of adenine, but alkylation also occurs at less nucleophilic oxygens, such as the *O*⁶ position of guanine. TMZ,³ a recently developed methylating drug, is approved for treatment of anaplastic astrocytomas and is in clinical trials for treatment of other solid tumors (3, 4). Under physiological conditions, TMZ is rapidly converted to a methylating species that reacts with DNA bases to N7mG, N3mA, and *O*⁶mG. Under normal circumstances, even though N7mG and N3mA are abundant, they are efficiently repaired by BER and are minimally cytotoxic. Likewise, *O*⁶mG is repaired by AGT. This DNA repair protein is often highly expressed in tumors; thus, it becomes an important drug resistance factor to diminish the efficacy of methylating and chloroethylating therapeutic agents (5). To block AGT repair, the novel AGT inhibitor BG was developed and is now in clinical trials (6, 7). The mechanism of *O*⁶mG-induced cytotoxicity appears to be activation of the MMR pathway through recognition and repetitive processing of the mispaired bases *O*⁶mG:T or *O*⁶mG:C, leading to DNA strand breaks and apoptotic death (8). Therefore, the absence of AGT activity and the presence of MMR function potentially contribute to the killing effect of methylating agent, such as TMZ. In contrast, sensitization to TMZ cytotoxicity by BG-induced depletion of AGT was not observed in MMR-deficient cell lines (9) because mispairs formed by *O*⁶mG fail to invoke a MMR response. Thus, even though *O*⁶mG lesions exist, they are not cytotoxic in the absence of MMR function.

N7mG and N3mA DNA adducts are processed through BER. BER is a multiprotein pathway initiated by diverse damage-specific glycosylases. These glycosylases process damaged bases with high specificity, and despite having very little sequence homology, they have similar functions. For instance, methylpurine glycosylase recognizes methylated bases, whereas the T:G mismatch, uridine, and 8-oxo-7,8-dihydroguanine are recognized by their respective glycosylases. However, the action of these glycosylases generates a common intermediate, AP sites, through a similar chemistry reaction (10). After enzymatic hydrolysis of the N-glycosidic bond and release of the abnormal

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³ The abbreviations used are: TMZ, temozolomide; BER, base excision repair; MX, methoxyamine; MMR, mismatch repair; BG, *O*⁶-benzylguanine; AGT, *O*⁶-alkylguanine DNA-alkyltransferase; N7mG, N7-methylguanine; N3mA, N3-methyladenine; *O*⁶mG, *O*⁶-methylguanine; AP, apurinic/aprimidinic; APE, AP endonuclease; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; SCE, sister chromatid exchange; ARP, aldehyde reactive probe.

base, APE then exaggerates the kink and hydrolyzes the phosphodiester backbone 5' to the lesion (11). DNA polymerase β catalyzes release of the phosphodeoxyribose moiety 5' to the AP site (deoxyribosephosphodiesterase activity) and filling of the single-nucleotide gap; the nick is sealed by DNA ligase I or III (10). MX specifically binds to AP sites (12, 13) and reduces the APE cleavage of the backbone by more than 300-fold compared with the cleavage of normal AP sites (14). We previously demonstrated that when using MX to interrupt repair of DNA adducts by the BER process, MX synergistically increased TMZ-induced cytotoxicity *in vitro* of both MMR-proficient (SW480) and MMR-deficient (HCT116) colon cancer cells (15, 16). Whereas the exact mechanism of cell death and the fate of the MX-adducted AP site remain to be identified, these data indicated that BER had a clear protective effect in tumor cells treated with TMZ and that MX could target TMZ-induced AP sites, disrupting BER and causing cell death. Thus, we proceeded to further test this hypothesis *in vivo* using human colon cancer xenografts. We demonstrate here that nontoxic doses of MX markedly potentiate the TMZ-induced antitumor effect in MMR-proficient and -deficient tumors.

MATERIALS AND METHODS

Tumors in Nude Mice. Tumor cells (5×10^6) were injected into the bilateral flanks of female athymic nude mice (6–8 weeks of age). The tumors were measured with calipers using the formula: $V = L \text{ (mm)} \times I^2 \text{ (mm)} / 2$, where V is the volume, L is the largest diameter, and I is the perpendicular diameter of the tumor. When the volume of tumor nodules reached 100–150 mm³, mice were randomly assigned to control or treatment groups (6–9 mice/group). Mice were housed in the Case Western Reserve University Cancer Center athymic animal facility.

Antitumor Effects of TMZ and MX + TMZ. Athymic mice carrying tumors were treated i.p. with TMZ alone, MX alone, MX + TMZ, BG + TMZ, or the combination of all three agents daily for 5 consecutive days. Doses used per group were as follows: (a) TMZ alone (120 or 40 mg/kg); (b) MX alone (2 mg/kg); (c) BG (30 mg/kg) + TMZ (120 or 40 mg/kg); and (d) MX (2 mg/kg) + TMZ (120 or 40 mg/kg). In the present studies, we used BG treatment as a control: (a) these three tumor cell lines have high levels of AGT activities (Ref. 9; 6.7 fmol/ μ g DNA in SW480 cells; 7.0 fmol/ μ g DNA in HCT116 cells, and 22.5 fmol/ μ g DNA in HCT15 cells); (b) we have shown that *in vitro*, BG failed to potentiate TMZ cytotoxicity in MMR-deficient cell lines; therefore, we predict that the failure of BG potentiation in MMR-deficient tumors will also be observed *in vivo*; and (c) we attempt to determine whether three combinations of BG, MX, and TMZ (at lower doses) significantly increase the antitumor effect in MMR-proficient tumor due to blockage of the AGT and BER DNA repair pathways.

Experimental End Point. Tumor measurements were taken every 3 days. Tumor responses were quantified by tumor regrowth delay. Tumor growth delays were calculated according to the following formula: tumor growth delay = $T2x - C2x$, where $T2x$ and $C2x$ represent the number of days that treated and control tumors take to double in size from the day of treatment, respectively.

Toxicity Evaluation. Toxicity after treatment was evaluated by body weight measurements and peripheral WBC counting. Body weight was measured three times weekly from the first treatment until 2 weeks after the last treatment. The weight loss was expressed as a percentage of the initial weight (initial weight – lowest weight/initial weight \times 100%). Peripheral WBCs were monitored 5 and 10 days after the end of treatment and compared with WBCs of control mice.

AP Site Assay. The AP site assay was essentially performed as described by Nakamura *et al.* (17) and Atamna *et al.* (18). Briefly, DNA from either cells or tumor tissue was isolated by using the DNeasy Tissue Kit (Qiagen, Valencia, CA). Ten μ g of DNA in 10 mM Tris (pH 8)-1 mM EDTA were incubated with 50 μ l of 10 mM ARP (Dojindo Molecular Technologies Inc., Gaithersburg, MD) for 1 h at 37°C in 150- μ l reaction volume. ARP reaction mixture was transferred to a filtration tube, and the tube was spun to isolate ARP-labeled DNA. The DNA was quantitated, and 1 μ g of DNA was incubated with avidin-horseradish peroxidase at 37°C for 1 h and then washed with PBS/0.05% Tween 20. Substrate solution was added, and absorbance was read at 650 nm. A standard curve for AP sites was generated using ARP-labeled standard DNA solutions (Dojindo Molecular Technologies Inc.). The detection limit of this assay estimated by the company is 1 AP site/10⁵ bp.

TUNEL Assay. The TUNEL assay was performed in paraffin-embedded sections to facilitate the identification of apoptotic cells or DNA strand breaks. The 3'-OH end labeling of apoptotic cell DNA was performed by using an ApopTaq Plus *in situ* peroxidase detection kit (Oncor). After deparaffinization and rehydration in graded alcohols, tissue sections were incubated with proteinase K. Endogenous peroxidase was inhibited with 3% hydrogen peroxide. Terminal deoxynucleotidyl transferase enzyme was then applied to catalyze the addition of digoxigenin-labeled nucleotides to the 3'-OH ends of the fragmented DNA (37°C, 1 h, in a humidified chamber). Subsequently, the slides were incubated with a peroxidase-conjugated antidigoxigenin antibody. Staining was developed in 3,3'-diaminobenzidine, and sections were counterstained with methyl green. Negative controls were run in which terminal deoxynucleotidyl transferase was omitted.

SCE and Chromosomal Aberrations. Tumor cell suspensions were isolated from fresh tumor tissue by mincing and digestion with medium containing collagenase (0.5 mg/ml), 0.1% protease, and 0.25% trypsin. Cells (1×10^6) were plated overnight and treated with 10 μ M bromodeoxyuridine for two rounds of DNA replication (approximately 48 h). Two h after the addition of 0.2 μ M Colcemid, mitotic cells were collected by shaking, treated in hypotonic solution (0.2% KCl, 0.2% sodium citrate, and 10% fetal bovine serum) at 37°C for 15 min, and fixed in Carnoy's solution. Slides were stained with Hoechst 33258 (5.0 μ g/ml) for 20 min, mounted in 0.067 M Sorensen's buffer [$\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (pH 6.8)] with a coverslip, exposed to a General Electric 15-W black light at 55°C for 20 min, heated at 65°C in 20 \times SSC for 20 min, rinsed, and stained in a 5% Giemsa solution to produce "harlequin" chromosomes.

Statistical Method. Tumor growth delay means \pm SD (12–18 tumors/treatment group) were tested by *t* test for statistical significance. *P*s are two-sided.

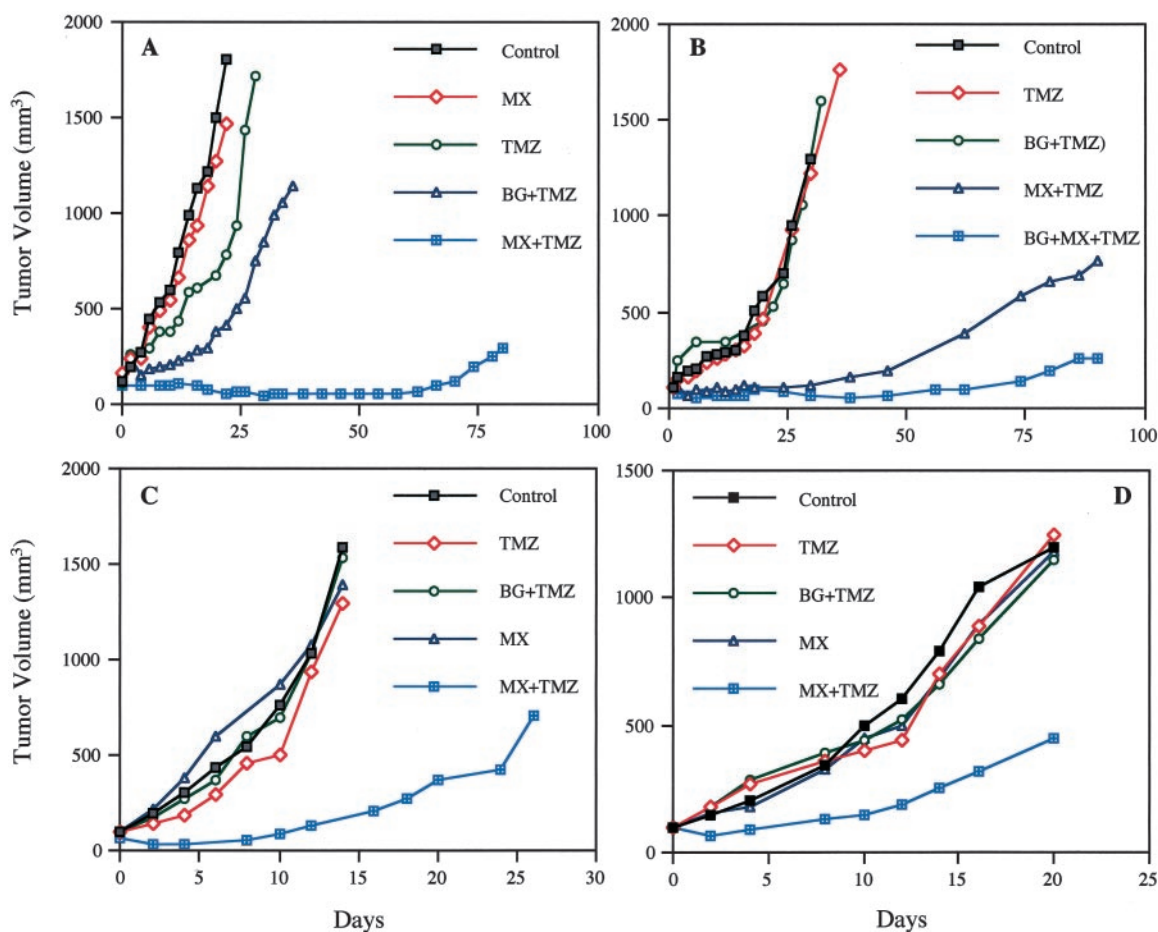


Fig. 1 *In vivo* efficacy of i.p. administered MX, TMZ, BG + TMZ, or MX + TMZ (once daily for 5 consecutive days) against human colon cancer xenografts in nude mice. **A**, SW480 tumor volume in mice (9 mice with 18 tumors/group); **B**, SW480 tumor volume in mice with the same treatments as in **A**, except that lower doses of TMZ were used (40 mg/kg); **C**, HCT116 tumor volume in mice (9 mice with 18 tumors/group); and **D**, HCT15 tumor volume in mice (6 mice with 12 tumors/group). The treatments in **C** and **D** were the same as those in **A**.

RESULTS

Acute Toxicity of MX. MX at doses of 2, 10, 20, 40, 60, 100, 150 and 200 mg/kg was injected i.p. into 3 mice/group for the test of acute toxicity. MX was lethal at 200 mg/kg, causing death within 10 min. At 150 mg/kg, mice had transient decreased mobility for approximately 1 h. Lower doses were well tolerated. MX at 2 mg/kg was used in the xenograft experiments.

Antitumor Effect of TMZ, TMZ + BG, TMZ + MX, or TMZ + BG + MX in a MMR-proficient Tumor Xenograft. The response of SW480 (MMR wild-type) xenografts to TMZ alone and in combination with BG, MX, or both is shown in Fig. 1, **A** and **B**. At the highest tolerable dose of TMZ (120 mg/kg), a tumor growth delay of approximately 9.3 ± 1.2 days was noted, indicating that MMR-proficient SW480 tumor was sensitive to TMZ (Fig. 1A). Treatment with BG + TMZ resulted in a tumor growth delay of 25 ± 2.4 days accompanied by significant weight loss (maximum body weight loss from 26 to 20 g; 23%), and very low leukocyte counts (90% decreased) 5 days after the last treatment. In contrast, mice treated with MX + TMZ had cessation of tumor growth lasting 50 ± 13 days and

very slow regrowth, yielding tumor growth delays of $>70 \pm 14$ days ($P < 0.002$). Compared with BG + TMZ, treatment with MX + TMZ (120 mg/kg) showed less body weight loss and less myelosuppression. In mice carrying SW480 tumors treated with lower-dose TMZ (40 mg/kg), BG had no potentiating effect, whereas MX enhanced the TMZ antitumor effect significantly. At this dose of TMZ, the addition of BG and MX induced a remarkable response, even though TMZ alone had no effect (Fig. 1B). There was no evidence of toxicity in mice treated with this combination.

Antitumor Effect of TMZ, BG, and MX Combinations in MMR-deficient Tumor Xenografts. In contrast to SW480, HCT116 (hMLH1 mutant) and HCT15 (hMSH6 mutant) xenografts were resistant to treatment with either TMZ alone or the combination of BG + TMZ (Fig. 1, **C** and **D**). However, MX + TMZ induced significant tumor growth delays (20 ± 1.4 days in HCT116 and 14 ± 3.1 days in HCT15) compared with control tumors ($P < 0.05$), indicating that MX also enhanced the antitumor effect of TMZ in human colon cancer xenografts with MMR deficiency.

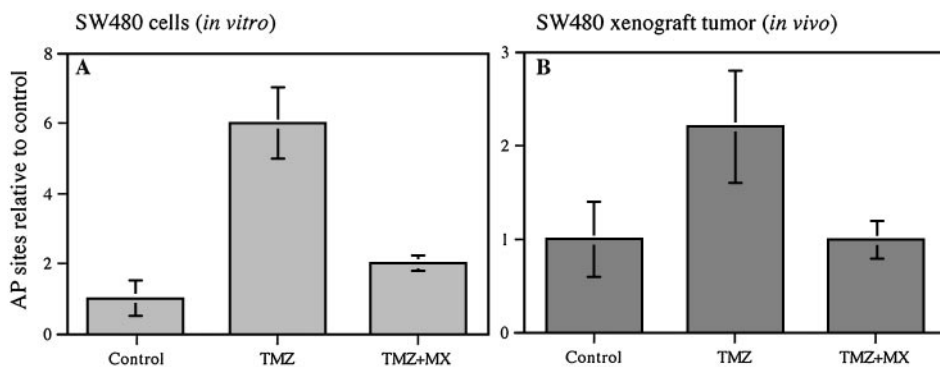


Fig. 2 AP site formation after exposure to TMZ alone or MX + TMZ. A, SW480 cells were treated with 375 μ M TMZ (equivalent to IC_{50}) or 6 mM MX + 375 μ M TMZ for 2 h. B, nude mice carrying SW480 tumors were treated (a single dose) with TMZ (120 mg/kg) or MX (2 mg/kg) + TMZ (120 mg/kg), and tumors were harvested 2 h after the treatment.

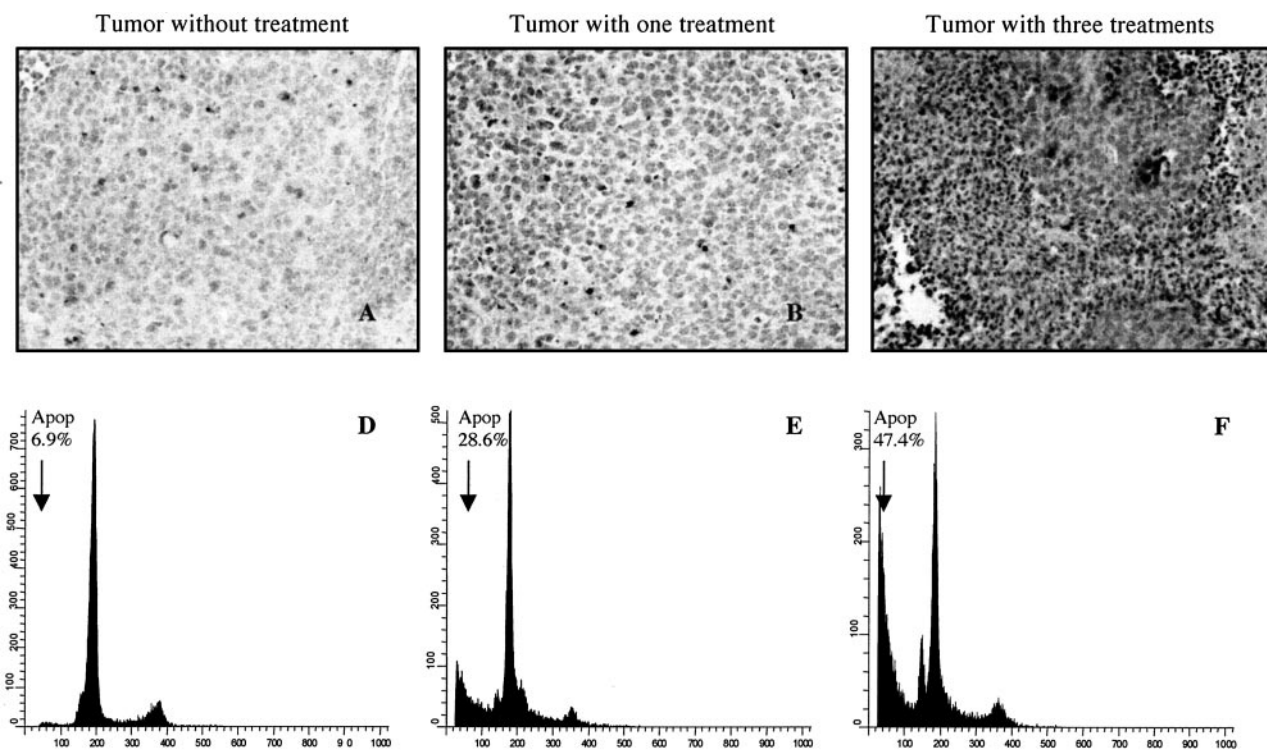


Fig. 3 Induction of apoptosis in SW480 tumors by MX + TMZ as determined by the TUNEL assay. Nude mice carrying SW480 tumors were treated with MX (2 mg/kg) + TMZ (120 mg/kg). A, untreated tumor; B, tumor harvested 24 h after one treatment; C, tumor harvested 24 h after three treatments (daily for 3 days). The percentage of apoptotic cells was analyzed by flow cytometric assay (D–F). Single cell suspensions were isolated from untreated xenograft tumors (D) or from xenograft tumors 24 h after one treatment (E) and three treatments (F). Cells were stained with propidium iodide and analyzed by flow cytometry.

Blockage of Repair of AP Sites Induced by MX. To test our hypothesis that potentiation of the TMZ cytotoxic effect by MX is the result of MX targeting AP sites and subsequently interrupting the BER pathway, we used the ARP reagent to measure AP sites formed by TMZ and blocked by MX because MX and ARP have similar reactivity toward the aldehyde group at AP sites. Thus, MX will block AP sites and reduce ARP binding. Fig. 2A shows that TMZ produces high levels of AP sites in SW480 cells and that MX decreases the number of ARP-reactive AP sites. *In vivo*, TMZ induced AP sites 2 h after a single i.p injection, and MX reduced these levels to back-

ground (Fig. 2B). Of note, the *in vivo* detection of both TMZ-induced AP sites and MX-blocked AP sites was much lower than that observed *in vitro*. However, it is important to remember that this low level of MX-blocked AP sites (about 50% of the AP sites generated by TMZ) is associated with significant tumor growth delays and cell death (see below).

Tumor Apoptosis Induced by TMZ + MX. Tumor apoptosis occurred in a dose- and time-dependent manner after treatment with MX + TMZ (Fig. 3, A–C). Apoptotic cells showed a scattered distribution 24 h after a single treatment (Fig. 3B) in the SW480 tumor (4-fold above control levels, 28

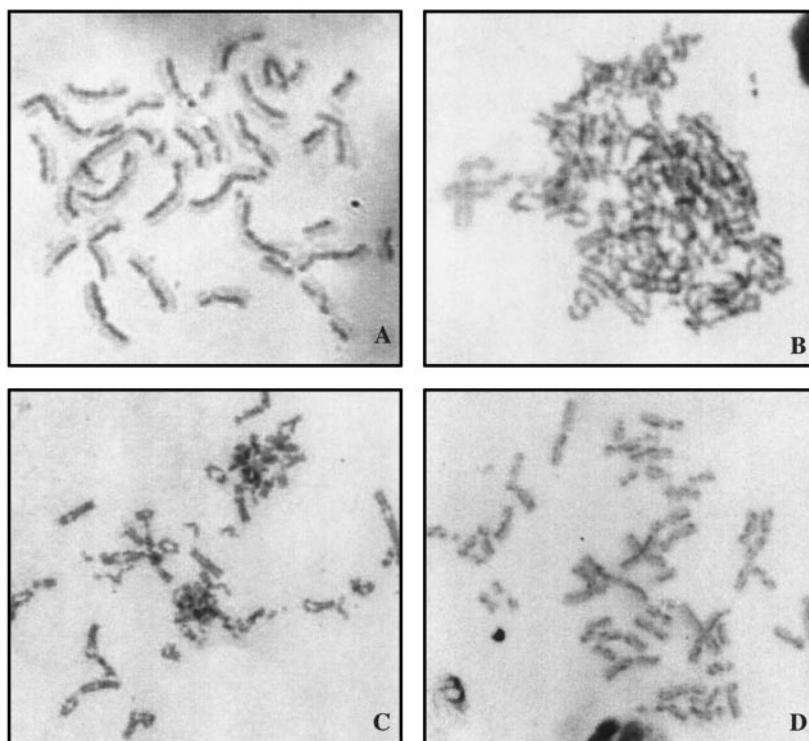


Fig. 4 Induction of chromosomal aberrations in SW480 tumors by MX + TMZ. Nude mice carrying SW480 tumors were treated with MX (2 mg/kg) + TMZ (120 mg/kg). *A*, chromosome isolated from untreated SW480 tumor. *B*, aneuploidy detected in SW480 tumor after three treatments. *C*, chromosomal breakage detected in SW480 tumor after three treatments. *D*, SCE detected in SW480 tumor after three treatments.

apoptotic-positive cells/slide *versus* 10^7 cells/slide), whereas after three treatments, the number of apoptotic cells was greatly increased. Results from flow cytometric assay (Fig 3, *D–F*) of single cell suspensions isolated from the xenograft tumors treated for 3 days (Fig. 3*F*) and stained with propidium iodide indicated that 46–50% of tumor cells underwent apoptotic death.

Induction of Chromosomal Aberration. MX and TMZ also induced extremely high frequencies of chromosomal aberration [present in >90% of chromosomes (SCE, 44%; DNA breakage, 32%; and aneuploidy, 20%)] in SW480 tumor after three daily treatments (Fig. 4). The single-stranded DNA and the double-strand DNA breaks (Fig. 4*B*) are visible at metaphase, indicating blocking replication forks and disrupting chromatin condensation caused by MX-AP sites. A high incidence of SCE (Fig. 4*C*) was also observed. Thus, it appears that aberrant processing of the MX-adducted AP site results in gross chromosomal disruption coupled with apoptotic signaling and cell death.

DISCUSSION

In this report, we provide experimental evidence that non-toxic doses of MX, a BER inhibitor, combined with TMZ result in a significant antitumor effect in MMR-proficient and -deficient tumors. The combination is associated with less evidence of systemic toxicity compared with the combination of BG + TMZ. Thus, BER may be a new target for agents to modulate chemotherapy resistance to alkylating agents in human tumors.

BER is an important pathway by which tumor cells remove TMZ-induced methylated bases, such as N3mA and N7mG, or

potentially T mispaired with O⁶mG. The excision of these aberrant bases from DNA by glycosylases results in the formation of AP sites. MX has reactivity with the free aldehyde group at the abasic site to form a MX-AP complex (12, 13). The MX-adducted AP sites are refractory to cleavage of APE, perhaps by stabilizing the phosphodiester bonds adjacent to the AP site (12, 13). In the present studies, we demonstrate that MX induces the reduction of AP sites generated by TMZ in tumor cells and xenograft tumors compared with TMZ alone, suggesting that MX is able to bind to AP sites and block repair of AP sites. On the basis of our results showing that TMZ + MX causes high numbers of broken and recombined chromosomes and aneuploidy, it is reasonable to link these MX-adducted AP sites to the enhanced cytotoxicity by MX: (a) we propose that MX-adducted AP sites, compared with the regular AP sites, have similar abilities to disrupt DNA replication (19, 20); and (b) recent studies have also demonstrated that AP sites, when located within a topoisomerase I/II DNA cleavage site, act as topoisomerase poisons and stimulate enzyme-mediated DNA scission, leading to the formation of permanent DNA strand breaks (21, 22). These breaks may also induce recombination events that lead to chromosomal translocations or trigger the cell death pathway. Moreover, it has been suggested that topoisomerase II poisons are able to interfere with topoisomerase II-dependent decatenation at the start of anaphase, thereby preventing normal chromosome segregation during anaphase and leading to aneuploidy (23). Thus, our data suggest that MX-adducted AP sites serve as poisons for topoisomerase II, resulting in cell death.

p53 suppresses tumor growth by either inducing apoptosis

or halting the cell cycle. Consistent with this idea, loss of p53 function leads to a decrease in apoptosis induced by γ -radiation and an increase in resistance to many types of chemotherapeutic agents (24). However, our results provide strong evidence that p53 is not required for cell death induced by MX + TMZ. Profound inhibition of tumor growth effect coupled with remarkable apoptosis was observed in a SW480 tumor that is p53 mutant. It seems likely that unrepaired MX-AP site lesions are responsible for eliciting these responses in the p53 mutant tumors. Furthermore, it was also reported that cells exposed to topoisomerase II poisons might be more susceptible to apoptosis than to DNA-reactive agents because they trigger apoptotic cell death through p53-independent pathways (25). This reinforces the suggestion that MX-AP sites are topoisomerase poisons, which are effective in the absence of p53.

Of note, MX significantly enhanced the antitumor effect of TMZ in both MMR-proficient and -deficient colon tumor xenografts. Although the greatest tumor regrowth delays were observed in the SW480 MMR wild-type tumor, antitumor efficacy of MX and TMZ was also observed in MMR-deficient tumors (HCT116 and HCT15 xenografts), suggesting that MMR status *per se* does not appear to impact MX-associated synergy. Importantly, MX can overcome the profound degree of resistance to methylating agents that is characteristic of MMR-defective tumors. MMR deficiency can arise through mutations or promoter methylation and is selected for during drug treatment *in vitro* (26) and in the clinical setting (27). The efficacy of a number of chemotherapeutic agents may be diminished by emergence of MMR deficiency; thus, our studies may provide a means to bypass MMR deficiency-mediated clinical drug resistance.

MX-modified AP sites specifically block polymerase β -dependent single-nucleotide BER (14). MX-modified AP sites may be repaired by long patch BER, but this process is much slower, a rate that is only 0.3% of the repair rate of intact AP sites (14). Because it appears that the only remaining pathway of repair of MX-modified AP sites is long patch BER, the cellular capacity to initiate long patch BER may theoretically decrease the toxicity of TMZ + MX combinations. Therefore, additional studies will be pursued to determine whether differential sensitivity to MX + TMZ results from heterogeneous capacity for short BER compared with long patch BER in tumor cells.

Overall, these studies demonstrate that DNA BER pathway is a useful anticancer pharmacological target and that disruption of BER by MX-bound AP sites sensitizes MMR-proficient and -deficient tumors to methylating therapeutic agents. MX is the lead compound in what should become a large set of BER inhibitors to be used in combination with AP site-generating DNA-damaging agents. Our data suggest a new strategy for mechanism-based anticancer therapy.

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