Blockage of abasic site repair enhances antitumor efficacy of 1,3-bis-(2-chloroethyl)-1-nitrosourea in colon tumor xenografts

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Abstract

Methoxyamine (MX) has been shown to potentiate the antitumor effect of temozolomide (TMZ) in human tumor xenograft models. This potentiation is due to the reactivity of MX with apurinic/pyrimidinic (AP) sites in DNA, which are formed following DNA glycosylase removal of TMZ-induced methyl-purine adducts. MX-bound AP sites cannot be further processed by base excision repair (BER), resulting in cell death. On the basis of this finding, we investigated *in vivo* whether MX enhanced therapeutic efficacy of other agents, such as 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) that generates AP sites during DNA repair. Nude mice carrying human colon tumor xenografts, HCT116 and HCT116-Ch3, were treated by a single injection (i.p) of BCNU alone (30 mg/kg) or MX (2 mg/kg) combined with BCNU. The effect on tumor growth of BCNU alone was very moderate. Combined administration of MX and BCNU produced significant inhibition of tumor growth. Tumor growth delays were 14 ± 3 days in HCT116 and 16 ± 2 days in HCT116-Ch3 tumors, respectively ($P < 0.05$ *versus* control or BCNU alone groups). Similar results were also observed in SW480 and DLD1 tumors. Importantly, no systemic toxicity was noted with BCNU and MX. In contrast, BCNU (at dose of 25 mg/kg) combined with O^6-benzylguanine (BG), an inhibitor of O^6-alkylguanine-DNA alkyltransferase (AGT) being tested in clinical trials, caused toxic death in all treated mice. However, a lower dose BCNU (10 mg/kg) combined with BG and MX had significant antitumor effect without toxic death. Thus, targeting BER with MX is a promising strategy to improve the antitumor activity of BCNU and perhaps other DNA-damaging agents. (Mol Cancer Ther. 2003;2:1061 – 1066)

Introduction

Alkylating agents are a major class of chemotherapeutic drugs. However, intrinsic or acquired drug resistances in human tumors to these agents are the common cause of treatment failure. An effort to overcome drug resistance by targeting a specific DNA repair pathway has yielded promising results. For instance, inactivation of O^6-alkylguanine-DNA alkyltransferase (AGT) with O^6-benzylguanine (BG) potentiates alkylating agents, such as temozolomide (TMZ) and 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU). Unfortunately, this combined treatment is limited by a narrow therapeutic index due to severe myelosuppression. As with other targeted therapeutics, agents that block a single pathway are not completely effective because tumors maintain a complex machinery to repair DNA lesions induced by therapeutic agents. Thus, to develop new targets to improve therapeutic efficacy is particularly urgent.

We have previously shown that methoxyamine (MX) potentiates the cytotoxic effect of methylating agent, TMZ, both *in vitro* and in human colon tumor xenograft models (1, 2). We proposed that this potentiation was mediated through the known ability of MX to bind apurinic/pyrimidinic (AP) sites in DNA generated by methyl-purine glycosylase, the first step to remove abnormal bases induced by alkylating agents in base excision repair (BER). MX-bound AP sites are very poor substrates for AP endonuclease (APE), so that BER pathway is interrupted and cell death ensues (1, 2). Because AP sites are the common intermediates produced during repair of a number of DNA adducts, we hypothesized that MX-initiated interruption of BER pathway would potentiate the cytotoxic effects of another alkylating agent, BCNU.

MX was first introduced as a tool to study BER pathway in 1985 (3). The specific action of MX in disruption of BER has been clearly demonstrated: (*a*) MX reacts with the tautomeric open-ring form of deoxyribose generated from the removal of an abnormal base by any one of the DNA glycosylases (4). (*b*) The reaction of MX with AP sites is fast, even faster than APE (4). (*c*) MX blocks repair of AP site due to the chemical modification of AP sites, rather than poisoning of the enzyme, APE (3). (*d*) MX-bound AP sites are resistant to cleavage by dRP lyase activity of DNA polymerase β by a factor of 300-fold (5). Although MX has been a known inhibitor of BER for over 20 years, we were the first to explore MX as a modulator of alkylating agents in anticancer treatment.

The impact of BER on BCNU cytotoxicity has not been fully evaluated. A recent study has shown that cells from mice deficient in 3-methyladenine DNA glycosylase have increased sensitivity to BCNU (6). Reduced DNA damage and cytotoxicity induced by BCNU was observed in human lung cells transfected with genes of *Escherichia coli* formamidopyrimidine-DNA glycosylase or human 8-oxoguanine-DNA glycosylase (7). Overexpression of polymerase β, the major polymerase involved in short patch BER, increased

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cell resistance to both mono- and bifunctional-alkylating agents (8). It has also been suggested that BCNU resistance is associated with enhanced capacity for repairing AP sites, because the predominant DNA adducts involve alkylation at N7 position of guanine, and these lesions appear to be substrates for BER (9–11).

In this study, we used colon tumor xenograft models to investigate the potential of MX-mediated BER blockage to enhance antitumor effect of BCNU. We chose colon tumor cells because these cells are well characterized for genetic defects/status in DNA repair, such as mismatch repair (MMR), p53, and AGT, each of which may confer selective resistance to different DNA damages induced by BCNU. Thus, they may serve to link specific genetic defect with drug target. We initially failed to observe significant potentiation of BCNU by MX *in vitro* (1), presumably due to different microenvironments *in vitro versus in vivo* that influence drug sensitivities. For instance, *in vivo*, angiogenesis affects drug pharmacokinetics that may affect the duration of interaction between drug and molecular targets; or signal transduction cascades and cell to cell interaction differ from *in vitro* may affect sensitivity of cells to the apoptotic signaling. In this report, we provide evidence that MX potentiates BCNU antitumor effect without additive systemic toxicity in *in vivo* studies. When BG, BCNU, and MX were used together, lower doses of BCNU are comparable or more effective than higher doses of BCNU and either agent alone. Thus, blocking both repair pathways, BER and AGT with MX and BG, improved BCNU therapeutic efficacy.

Materials and Methods

Tumors in Nude Mice

Tumor cells (5×10^6) were injected into the bilateral flanks of female athymic nude mice, at 6–8 weeks of age. The tumors were measured with calipers using the formula: $V = L$ (mm) $\times W^2$ (mm)/2, where L is the largest diameter and W is the perpendicular diameter of the tumor. When the volume of tumor nodules achieved 100 mm^3, mice were assigned randomly to control or treatment groups (10–15 mice with 20–30 tumors/group).

Experimental End Point

Tumor measurements were taken every 3 days. Experiments were terminated when tumor volume reached greater than 1000 mm^3. The relative tumor volume (V/V_0) was calculated by dividing the measured tumor volume (V) by the initial tumor volume (V_0) at day 0. Tumor responses were quantified by tumor growth delays. Tumor growth delays were calculated according to: tumor growth delays = $T_{2\times} - C_{2\times}$, where $T_{2\times}$ and $C_{2\times}$ represent the number of days treated and control tumors taking to double in size from the day of treatment, respectively (2).

AP Sites Measured by Aldehyde Reactive Probe-Slot-Blot

We used aldehyde reactive probe (ARP) reagent to measure AP sites formed by BCNU and blocked by MX. ARP and MX have a similar reactivity with AP sites. They react specifically with an aldehyde group, an open-ring form of the AP sites. The AP site assay was essentially performed as described by Nakamura *et al.* (12). Briefly, DNA (15 μg) extracted from cells with different treatments was incubated with 1 mM ARP (Dojindo Molecular Technologies Inc., Gaithersburg, MD) at 37°C for 10 min. After precipitation and wash with ethanol, DNA was resuspended in TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 7.2)]. DNA was heat-denatured at 100°C for 5 min, quickly chilled on ice, and mixed with an equal amount of ammonium acetate (2 M). The single-stranded DNA was then immobilized on a BAS-85 NC membrane (Schleicher and Schuell, Keene, NH), using a vacuum filter device (Schleicher and Schuell). The NC membrane was incubated with streptavidin-conjugated horseradish peroxidase (BioGenex, San Ramon, CA) at room temperature for 30 min. After NC membrane was rinsed with washing buffer containing NaCl (0.26 M), EDTA (1 mM), Tris-HCl (20 mM), and Tween 20 (1%), ARP-AP sites were visualized with ECL reagents (Amersham Corp., Piscataway, NJ).

Comet Assay

The principle of Comet assay is based on the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of an electric field. Undamaged DNA migrates slower and remains within the confines of the nuclei when a current is applied. DNA damage was assessed in a cell based on evaluation of the DNA ''comet'' tail shape and migration distance (13). Cells were harvested and washed with PBS after exposure to BCNU (25 μM) or MX (25 mM) plus BCNU (25 μM) for 2 h. Cell suspension (1×10^5/ml cold PBS) was mixed with 1% low gelling temperature agarose at 42°C at a ratio of 1:10 (v/v) and immediately pipetted 75 μl onto CometSlide (Trevigen, Inc., Gaithersburg, MD). When the low gelling temperature agarose had set, the slides were submerged in prechilled lysis buffer [2.5 M NaCl, 100 mM EDTA, and 10 mM Tris-HCl (pH 10.5–11.5), containing 1% Triton X-100, added just before use] at 4°C for 1 h. After lysis, the slides were washed with distilled water. Slides were arranged lengthwise in an electrophoresis tank and submerged in alkali buffer [50 mM NaOH and 1 mM EDTA (pH 12–12.5)] for 30 min. Slides were then electrophoresed in both alkali (pH > 13, 300 mM NaOH, 1 mM EDTA) and neutral solution (1 × TBE) for 25 min at 18 V (0.6 V/cm), 250 mA. Alkaline electrophoresis detects both single- and double-stranded DNA breaks, resulting from AP sites as well as other alkali labile DNA adducts (*e.g.*, phosphoglycols, phosphotriesters), whereas neutral electrophoresis detects predominantly double-stranded DNA breaks.

The slides were removed and washed with neutralization buffer [0.5 M Tris-HCl (pH 7.5)] for 10 min, followed by a PBS wash. The slides were then left to air dry overnight at room temperature.

The DNA was stained using Silver Staining Kit (Trevigen) following manufacturer's procedures. The comets were visualized using an Olympus microscope. Images were captured using a digital camera and analyzed using NIH image software. The tail moment for each image was

defined as the product of the percentage in the comet tail, and the distance between the means of the head and tail distributions (13).

$$\text{Tail moment} = \%\ \text{of DNA in the tail} \times \text{Tail distance}$$

where % of DNA in the tail = tail area (TA) × tail average intensity (TAI) × 100/(TA × TAI) + [head area (HA) × head area intensity (HAI)].

Statistical Method

Tumor growth delay means ± SD (20–30 tumors/group) were tested by *t* test for statistical significance. *P* values are two sides. nQuery Advisor program was used to test power between control and treated groups.

Results

BCNU plus MX Enhanced Antitumor Effect

Four human colon cancer cell lines expressing high levels of AGT but having different MMR activity and p53 status were used in these studies: HCT116 (hMLH1 mut, p53 wt); HCT116-Ch3 (having restored hMLH1 activity); DLD1 (hMSH6 mut and p53 mut); and SW480 (MMR wt and p53 mut). These tumor cells were inoculated into the bilateral flanks of nude mice at 6–8 weeks of age. When the tumor volume reached 100 mm^3, mice received a single injection (i.p) of MX (2 mg/kg) or BCNU (30 mg/kg) alone or MX (2 mg/kg) combined with BCNU. MX alone has no observed cytotoxicity in both tumor and bone marrow tissues. BCNU alone had a very mild effect on tumor growth. Combined administration of MX and BCNU produced significant tumor growth inhibition in all tested xenograft tumors (Fig. 1). Tumor growth delays in mice receiving MX and BCNU *versus* control were 11 ± 3 days in DLD1 (10 mice/group), 14 ± 3 days in HCT116 (15 mice/group), 16 ± 2 days in HCT116-Ch3 (10 mice/group), and 18 ± 4 days in SW480 tumors (15 mice/group), respectively ($P < 0.05$ *versus* either groups of control, in which $C_{2\times} = 3 \pm 0.7$ days; or groups of BCNU alone, in which $T_{2\times} - C_{2\times} = 0.9 \pm 0.5$ days). Considering the fact that antitumor effect was not observed in mice treated with MX alone, enhanced inhibition of tumor growth resulted from a synergistic interaction that MX amplified BCNU cytotoxicity. Because there was no significant difference in therapeutic efficacy among these four tumors while they have different patterns of p53 and MMR functional mutations, we conclude that antitumor effect exerted by the treatment with BCNU and MX is independent on p53 and MMR status.

We were surprised to find no systemic toxicity in mice treated with BCNU and MX. In contrast, BCNU (even at a lower dose of 25 mg/kg) combined with BG (30 mg/kg), an AGT inhibitor, caused toxic deaths in all treated mice. To determine whether antitumor efficacy could be achieved by combined treatment with three drugs, we treated mice bearing tumors with BG (30 mg/kg, i.p.) 1 h before MX (2 mg/kg) and a low dose of BCNU (10 mg/kg). As shown in Fig. 1, E and F, BCNU alone at this low dose had no effect on tumor growth delay in these colon tumor xenograft models. However, the combined treatment of BG, MX, and low-dose BCNU induced tumor growth delays of 12 ± 2.0 days *versus* 0.7 ± 0.5 days in mice treated with BG (30 mg/kg) + BCNU (10 mg/kg), $P < 0.05$, in HCT116 (15 mice/group) and 21 ± 3.3 days in SW480 ($P < 0.05$, compared to 0.5 ± 0.4 days in the BG + BCNU group, 15 mice/group). These results suggest that inhibition of two DNA repair pathways—AGT and BER, two DNA repair mechanisms mainly responsible for repair of DNA lesions induced by BCNU, significantly improves therapeutic efficacy of BCNU.

MX Bound to AP Sites Generated by BCNU

BCNU produces a wide spectrum of modified bases that could be converted to AP sites by initial reaction with DNA *N*-glycosylases (7, 9–11). We evaluated the formation of AP sites with ARP reagent in cells after exposure to

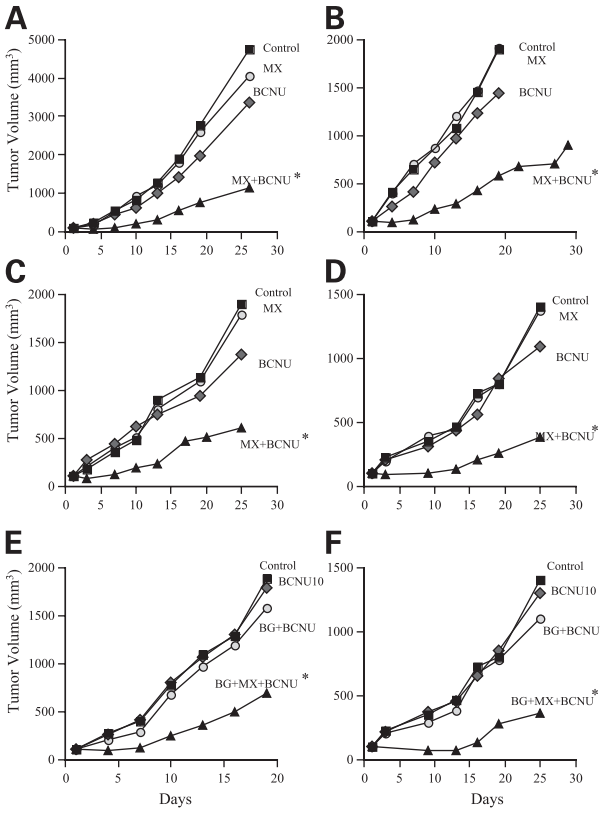

Figure 1. Effect of BCNU or MX and BCNU (single i.p. injection) on colon tumor growth *in vivo*. **A,** DLD1 tumor volume (10 mice with 20 tumors/group). **B,** HCT116-Ch3 tumor volume (10 mice with 20 tumors/group). **C,** HCT116 tumor volume (15 mice with 30 tumors/group). **D,** SW480 tumor volume (15 mice with 30 tumors/group). Athymic mice carrying tumors were treated with drugs. Doses used per group were as follows: BCNU alone (30 mg/kg); MX (2 mg/kg) + BCNU (30 mg/kg). **E** and **F** show the effect on tumor growth by the treatments with three drugs, in which mice carrying HCT116 tumor (**E**: 15 mice with 30 tumors) or SW480 tumor (**F**: 15 mice with 30 tumors) were treated with BG (30 mg/kg, injected 1 h before other drugs) + MX (2 mg/kg) + BCNU (10 mg/kg). *, $P < 0.05$ *versus* control, BCNU alone or BG and BCNU groups. Power test (using nQuery Advisor program) shows greater than 90% power between control and treated groups.

BCNU and the ability of MX to bind these AP sites that would competitively reduce ARP reactivity with AP sites (12) in cells treated with MX and BCNU. As shown in the images of ARP-AP sites (Fig. 2A), in HCT116 cells, AP sites in DNA increased proportionally (r = 0.995) with the concentrations of BCNU (Fig. 2B). Co-treatment with MX (25 mM) reduced detectable AP sites to control levels (Fig. 2C). This is due, not to the absence of AP sites, but to the occupancy of AP sites by MX, making them unavailable for ARP. We then determined the time course of BCNU-produced AP sites. Cells were treated with BCNU (50 μM) alone or BCNU plus MX (25 mM) for 2 h and then prolonged exposure to MX with lower concentrations (2.5 mM) for 72 h. With BCNU alone (Fig. 2D), the levels of AP sites peaked at 4 h and slowly decreased. At 72 h, 50% of AP sites still remained over this period and MX apparently blocked AP site to react with ARP at 2 h after treatment; however, MX-blockage was observed over 72 h even maintained at low doses.

BCNU plus MX Increased in DNA Strand Breaks

We have previously reported that treatment with TMZ plus MX increases cell death mediated by apoptosis and DNA strand breaks (2, 14). Here, we used the Comet assay to measure DNA strand breaks in individual cells 2 h after exposure to BCNU alone or BCNU plus MX. Comet images are shown in Fig. 3A. After treatment with BCNU plus MX, distinct comets were observed, tail length was 4 times greater compared with cells treated with BCNU alone, in which much smaller comets were observed (Fig. 3B). In cells treated with BCNU plus MX, tail moment, a measurement of the amount of genetic material within a tail together with the distance of migration of the presumably damaged DNA, was 3-fold higher than that in cells treated with BCNU alone (Fig. 3C).

Discussion

Targeting BER Is a Rationale Approach to Enhance Cytotoxicity of BCNU

Although 90% of total DNA adducts generated by BCNU are substrates for BER (15–19), BER has been known to contribute little to BCNU resistance, particularly compared to AGT, which has been recognized as a crucial BCNU-resistant factor due to its ability to repair cytotoxic O^6mG DNA adduct, a precursor of lethal DNA cross-linking lesions (20, 21). Thus, in this study, we investigated whether BER pathway might be also an important mechanism that confers resistance to BCNU by developing a therapeutic strategy that targets BER. We demonstrated that MX potentiated BCNU antitumor effect in four colon cancer xenografts that differ in p53 and MMR status. That enhanced therapeutic efficacy of BCNU was induced by MX at nontoxic doses strongly suggested a synergistic interaction between BCNU and MX. MX efficiently magnifies BCNU cytotoxicity through interruption of BER, which makes most of adducts (90%) formed by BCNU known as less or non-toxic lesions lethal.

MX-Modified AP Sites Are Lethal

Alkylating agents do not directly cause DNA strand breaks. They attack bases at several positions on DNA.

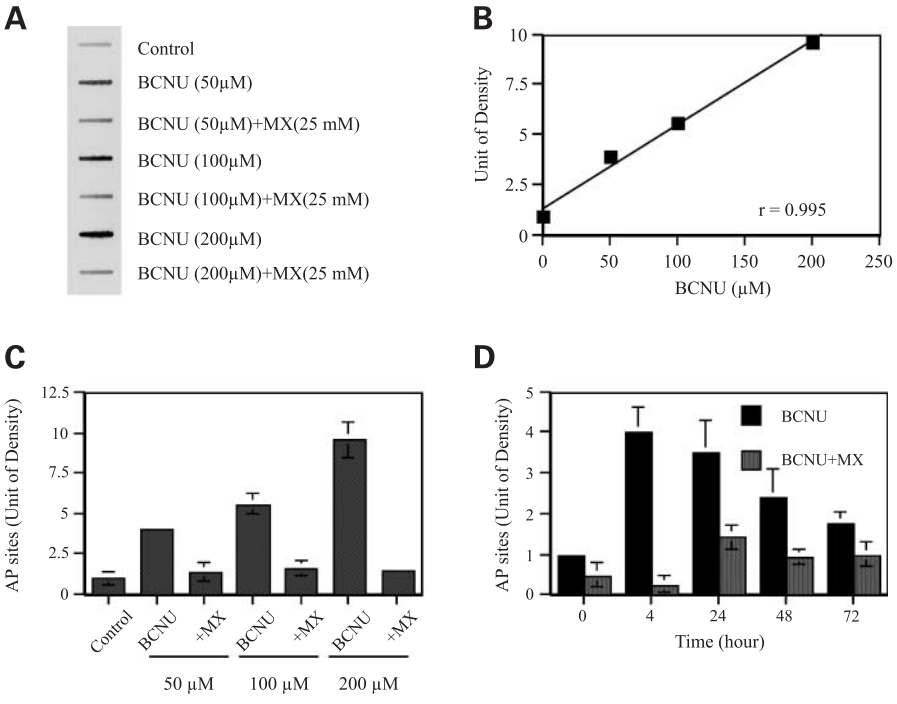

Figure 2. AP sites detected in HCT116 cells treated with either BCNU alone or BCNU plus MX. **A,** image of AP sites in cells treated with BCNU alone *versus* BCNU plus MX showed the reduction of AP sites by MX. **B,** the levels of AP sites increased in a dose-dependent fashion (r = 0.995). **C,** AP sites induced by different doses of BCNU (0 – 200 μM) and reduction of AP sites by MX. **D,** AP sites induced by BCNU alone (50 μM for 2 h treatment) decreased in a time-dependent fashion. When low doses of MX were present, reduction of AP sites was observed over 72 h. Cells were treated with BCNU (50 μM) + MX (25 mM) for 2 h and changed in drug-free medium and low doses of MX (2.5 mM) were added in. Samples for slot blot assay were blotted in duplicate and SD values represent three independent experiments.

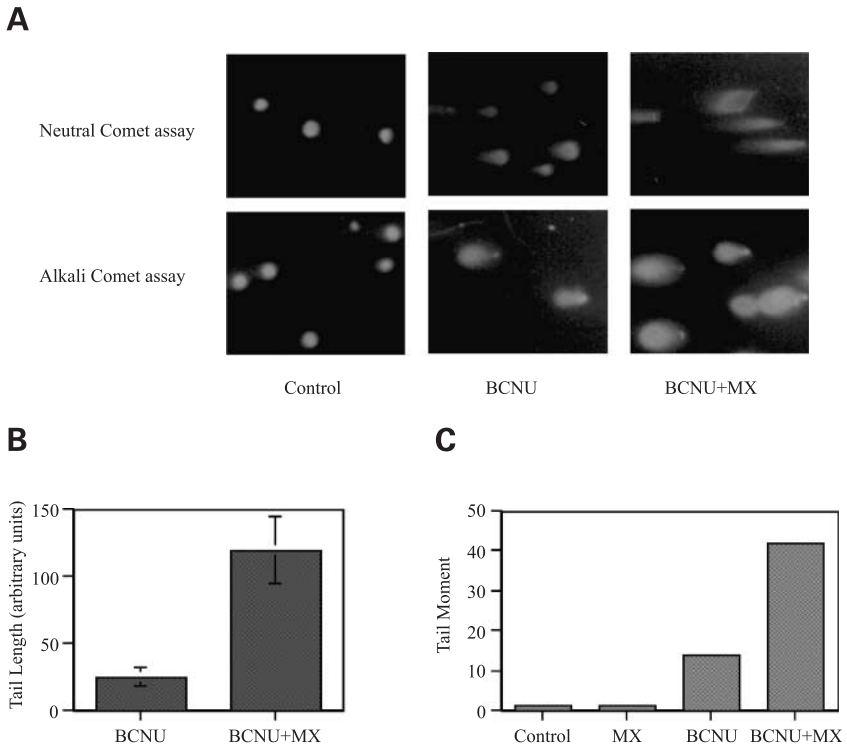

Figure 3. Effect of MX on BCNU on DNA strand breaks assayed by Comet assay. **A,** comet images. **B,** comparison of comet tail length between cells treated with BCNU alone and BCNU plus MX. **C,** comet tail moment: Tail moment = % of DNA in the tail × Tail distance; where % of DNA in the tail = tail area (TA) × tail average intensity (TAI) × 100/(TA × TAI) + [head area (HA) × head area intensity (HAI)]. The displayed data are the mean value of the measurement of 50 randomly selected comets from two slides per experimental point.

These alkylated bases lead to the formation of AP sites either spontaneously or enzymatically. The removal of AP sites is accomplished by APEs, which cleave DNA adjacent to AP sites. In general, AP sites are rapidly repaired in mammalian cells (22). It has been estimated that the BER pathway can repair more than 10,000 AP sites spontaneously formed per day per cell (22). Therefore, AP sites would not cause lethal cytotoxicity in a normal condition even though they are abundant. To explain the results of increased DNA strand breaks and cell death after treatment with BCNU and MX, we propose that the repair of AP sites generated by BCNU is inhibited due to the structural modification by MX, thus, accumulation of MX-AP sites become toxic during DNA replication (23, 24). Moreover, when MX-AP sites are located within topoisomerase II (topo II) cleavage sites, they may act as poisons to produce a ''cleavable complex'' resulting in protein-associated DNA double-stranded breaks (25–28). As we have noted with MX and TMZ (2), DNA double-stranded breaks occurring in tumors lead to chromosomal aberrations and the apoptotic death. Thus, a broader implication of these results is that MX-modified AP sites induced by whatever mechanism are lethal.

Broad Therapeutic Index of MX and BCNU

An unexpected finding was less evidence of systematic toxicity of BCNU and MX compared to BCNU plus BG. This is reminiscent of the decreased myelosuppression and less body weight loss observed with TMZ and MX compared to TMZ plus BG, although the former had greater antitumor efficacy (2). This suggests that the combination of MX with BCNU has a wider therapeutic index compared to BG and BCNU. In our preliminary studies, we have found that MX-bound AP sites are a substrate for topo II and increased cell death was associated with the induction of topo II in colon tumor cell lines. In contrast, much lower levels of topo II were present in normal bone marrow cells compared to tumors cells (unpublished data). Thus, we propose that the wider therapeutic index of BCNU and MX compared to BCNU plus BG is due to the differences in topo II levels between normal bone marrow and tumors that may determine cytotoxic effect selectively occurring in highly proliferative tumor cells.

In summary, to explore the use of MX in chemotherapy is important because MX-bound AP site in DNA is a new therapeutic target that appears to potentiate the cytotoxicity of a number of alkylating therapeutic agents. This study represents a new opportunity in anticancer drug development.

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