Mitochondrial Targeting of Human O^6-Methylguanine DNA Methyltransferase Protects against Cell Killing by Chemotherapeutic Alkylating Agents

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Abstract

DNA repair capacity of eukaryotic cells has been studied extensively in recent years. Mammalian cells have been engineered to overexpress recombinant nuclear DNA repair proteins from ectopic genes to assess the impact of increased DNA repair capacity on genome stability. This approach has been used in this study to specifically target O^6-methylguanine DNA methyltransferase (MGMT) to the mitochondria and examine its impact on cell survival after exposure to DNA alkylating agents. Survival of human hematopoietic cell lines and primary hematopoietic $CD34^+$ committed progenitor cells was monitored because the baseline repair capacity for alkylation-induced DNA damage is typically low due to insufficient expression of MGMT. Increased DNA repair capacity was observed when K562 cells were transfected with nuclear-targeted MGMT (nucl-MGMT) or mitochondrial-targeted MGMT (mito-MGMT). Furthermore, overexpression of mito-MGMT provided greater resistance to cell killing by 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) than overexpression of nucl-MGMT. Simultaneous overexpression of mito-MGMT and nucl-MGMT did not enhance the resistance provided by mito-MGMT alone. Overexpression of either mito-MGMT or nucl-MGMT also conferred a similar level of resistance to methyl methanesulfonate (MMS) and temozolomide (TMZ) but simultaneous overexpression in both cellular compartments was neither additive nor synergistic. When human $CD34^+$ cells were infected with oncoretroviral vectors that targeted O^6-benzylguanine (6BG)–resistant MGMT ($MGMT^{P140K}$) to the nucleus or the mitochondria, committed progenitors derived from infected cells were resistant to 6BG/BCNU or 6BG/TMZ. These studies indicate that mitochondrial or nuclear targeting of MGMT protects hematopoietic cells against cell killing by BCNU, TMZ, and MMS, which is consistent with the possibility that mitochondrial DNA damage and nuclear DNA damage contribute equally to alkylating agent–induced cell killing during chemotherapy. (Cancer Res 2005; 65(8): 3319-27)

Introduction

DNA alkylating agents are common chemotherapeutic drugs used to treat pediatric and adult cancer (1). These agents are cytotoxic to cancer and normal cells, which leads to dose-limiting toxic effects including myelosuppresion. Dose escalation studies are often used to optimize dose regimens and to improve patient survival (2). Growth factors and stem cell support are also used to minimize myelosuppresion, but dose-limiting toxicity continues to be a major concern for patients treated with chemotherapeutic alkylating agents.

The cytotoxicity of alkylating agents may be directly linked to their ability to generate DNA adducts (2, 3). Different alkylating agents generate different adducts, and cellular DNA repair pathways have evolved with different specificities for specific alkylation products and/or other DNA lesions. For example, 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) generates several DNA adducts including O^6-methylguanine, which preferentially pairs with thymine and leads to GC to AT transitions (3, 4). BCNU also generates chloroethyl modifications at O^6, which can rearrange and lead to an interstrand cross-link (5). Interstrand DNA cross-links are particularly cytotoxic because they block DNA replication (6). O^6-methylguanine is repaired primarily by direct reversal-mediated O^6-methylguanine methyltransferase (MGMT; ref. 7). Temozolomide (TMZ) is a recently developed chemotherapeutic alternative to BCNU for patients with astrocytoma, glioblastoma, metastasic brain tumors, and malignant melanoma (8), TMZ methylates DNA primarily at the N^7 and O^6 positions of guanine and the N^3 of adenine (70%, 5%, and 9%, respectively; ref. 9) and these lesions are repaired by both the MGMT and base excision repair pathways (10).

If it is correct to assume that unrepaired DNA alkylation damage is directly linked to cellular cytotoxicity of DNA alkylating agents, then up-regulation of DNA repair proteins in bone marrow cells might prevent myelosuppression during alkylation chemotherapy. Several studies have been initiated to test this hypothesis by overexpressing enzymes involved in direct lesion reversal or DNA base excision repair in bone marrow cells during dose-intensified chemotherapy (11, 12). In addition, preclinical and clinical trials are under way to test the efficacy of this approach in the clinical setting (4, 12). Recent studies have also explored the relative importance for cytotoxicity of DNA damage/repair in the nuclear and mitochondrial genomes (13–16). The goal of this study was to determine whether targeted overexpression of MGMT in mitochondria protects against alkylation agent–induced cell killing. The results show that mitochondrial-targeted MGMT (mito-MGMT) protects K562 cells against methyl methanesulfonate (MMS), BCNU, and TMZ. In addition, it was unexpected that mitochondrial targeting of MGMT provided a greater level of resistance to alkylation-induced cell killing than nuclear targeting of MGMT for some drug treatments. Furthermore, mitochondrial targeting of O^6-benzylguanine (6BG)–resistant MGMT ($MGMT^{P140K}$) protects

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$CD34^+$ human cells against 6BG/BCNU and 6BG/TMZ. These studies suggest that mitochondrial DNA (mtDNA) damage/repair may play an equally important role in cell survival as nuclear DNA damage/repair.

Materials and Methods

Molecular biology and biochemistry techniques. DNA and RNA isolation, Northern and Western blot analysis, and SDS-PAGE were done as previously described (11, 17). Western blot analysis was done with the mouse anti-MGMT monoclonal antibody (mAb, MT 23.2; Novus Biologicals, Littleton, CO) and the goat anti-mouse IgG antibody (Chemicon International, Inc., Temecula, CA; ref. 17). MGMT activity assays were done using the HEX-labeled 18-mer oligonucleotide assay as previously described (17, 18).

Construction of nuclear and mitochondrial wild-type O^6-methylguanine DNA methyltransferase constructs. The human MGMT or MGMT with the 72 bp mitochondrial targeting sequence from the human manganese superoxide dismutase enzyme (*MnSOD* gene) was cloned into pcDNA3.1 (19–21). Purified plasmid DNA (Qiagen, Chatsworth, CA) was used to transfect K562 cells using the Lipofectin transfection reagent (Life Technologies, Rockville, MD) as per manufacturer's protocol. Individual clones were selected using 0.75 mg/mL G418 (Life Technologies) and expression of the nuclear-targeted MGMT (nucl-MGMT) or mito-MGMT was characterized using Northern and Western blot analysis. Cells were maintained at 37°C, 5% CO_2 in RPMI medium supplemented with 5% fetal bovine serum (FBS), 100 units/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate (Life Technologies). Individual clones were selected and further characterized for expression of nucl-MGMT or mito-MGMT.

Additionally, the mito-MGMT was cloned into the pSF91.1 retroviral vector that is based on the murine stem cell virus (MSCV) and was a gift of Dr. C. Baum (University of Hamburg, Hamburg, Germany; ref. 22). This vector is a bicistronic retrovirus with an internal ribosome entry site (IRES) immediately preceding the enhanced green fluorescence protein (EGFP) and has been previously used by us in other studies (22, 23). Nucl-MGMT cells were infected with the mito-MGMT-EGFP-pSF91.1 construct and 2 days later were sorted using FACStar^{Plus} (Becton Dickinson, San Jose, CA) as EGFP-expressing cells. EGFP expression levels in transduced cell populations were determined using FACSCalibur (Becton Dickinson) by measuring fluorescence intensities of cells. Flow cytometry data were analyzed by CellQuest 3.3 software (Becton Dickinson). EGFP expression levels were also visualized by fluorescent microscopy as we have previously described (22, 23). Individual colonies were then chosen and mito-MGMT and nucl-MGMT expression confirmed.

***O^6*-methylguanine DNA methyltransferase P140K retroviral vector construction.** The bicistronic retroviral vector, SF1-nuclP140K-IRES-EGFP (SF1-nuclP140K), that coexpresses nuclear-localized $MGMT^{P140K}$ (P140K) and the enhanced green fluorescent protein (EGFP) has been previously described (24). P140K is a mutant of MGMT that is resistant to the effects of 6BG, which is used to deplete cells of MGMT activity (7, 24). The SF91-EGFP vector that expresses EGFP was used as a control. For targeting $MGMT^{P140K}$ to the mitochondria, the $MGMT^{P140K}$ cDNA was fused by PCR to the mitochondrial targeting sequence derived from the human MnSOD sequence (21) as described above for the wild-type MGMT constructs. The SF91-WTmitoMGMT vector was digested with *Eco*RI and *Sal*I (both from Roche Diagnostic Corporation, Indianapolis, IN) to release the wild-type mito-MGMT and the vector-purified fragments. A 5′ *Eco*RI-mitoMGMT^{P140K}-3′ *Sal*I fragment generated by PCR was then subcloned into the digested SF91 vector upstream of the IRES element. In contrast to the wild-type MGMT, expression of mitoP140K from the SF91 vector was low to nondetectable in human $CD34^+$ cells (data not shown). In an attempt to increase expression of the transgene, the mitoP140K cassette was subcloned into the MIEG3 and SF1 retroviral vectors. The MIEG3 vector was used because it uses a different promoter/enhancer for transgene expression than the SF vectors. The MIEG3 vector utilizes the PCMV promoter enhancer (25, 26) and the SF1 and SF91 vectors use the spleen focus-forming virus promoter enhancer for transgene expression. The SF1 and SF91 vectors differ in the leader sequence 5′ to the mitoP140K cDNA. The SF1 vector contains seven pre-AUG initiation codons, whereas the SF91 vector does not contain pre-AUG codons (27). Depending on the transgene used, differences in the leader sequence can influence efficiency of protein translation from vector-derived RNA transcripts.[3] A 5′ *Eco*RI-mitoP140K-*Sal*I 3′ fragment was subcloned into the MIEG3 vector upstream of the IRES element by ligation into MIEG3 digested with *Eco*RI and *Xho*I. The mitoP140K cassette was subcloned into the SF1 retroviral vector by linearizing the MIEG3-mitoP140K vector with *Eco*RI, filling in, and releasing the mitoP140K-IRES-EGFP fragment with *Sal*I. The 5′ blunt-ended mitoP140K-IRES-EGFP-*Sal*I-3′ was then subcloned into the SF1 vector blunted at the 5′ end and cut at the 3′ end with *Sal*I. Both MSCV-mitoP140K and SF1-mitoP140K constructs expressed mitoP140K in hematopoietic $CD34^+$ cells and expression of mitoP140K using the SF1 vector was slightly higher (data not shown). Therefore, in studies using human $CD34^+$ cells, we used the SF1-mitoP140K vector.

Pseudotyping of retroviral vectors. Retroviral vectors were pseudotyped with the gibbon ape leukemia virus envelope using the PG13 packaging cell line (American Type Culture Collection, Manassas, VA; ref. 28) as previously described (24).

Isolation of granulocyte-mobilized peripheral blood $CD34^+$ cells. The protocol for the collection and processing of granulocyte colony-stimulating factor (G-CSF)–mobilized peripheral blood were approved by the Institutional Review Board at the Indiana University School of Medicine. For isolation of mobilized peripheral blood $CD34^+$ cells, healthy adult volunteers were treated s.c. for 4 days with 10 μg/kg/d of human G-CSF (Filgrastim, Neupogen; Amgen, Thousand Oaks, CA). WBC were collected by apheresis and $CD34^+$ cells were isolated by immunomagnetic methods using the Isolex 300i cell selection device according to the manufacturer's instructions (Baxter Immunotherapy, Irvine, CA). The range in purity of $CD34^+$ cells was 80% to 96%.

Transduction of mobilized peripheral blood $CD34^+$ cells. Mobilized peripheral blood $CD34^+$ cells were transduced as previously described (24). $CD34^+$ cells were prestimulated at a cell density of 5×10^5 cells/mL in Iscove's modified Dulbecco's medium (IMDM, Life Technologies, Grand Island, NY) containing FBS (Hyclone, Logan, UT), 20 μg/mL gentamicin (Sigma, St. Louis, MO), and 2 mmol/L L-glutamine (Life Technologies). The medium was supplemented with G-CSF, stem cell factor, and megakaryocyte growth and development factor (a generous gift from Amgen). Each cytokine was used at 100 ng/mL for prestimulation. Nontissue culture plates (Falcon, Franklin Lakes, NJ) were coated with 2 $\mu g/cm^2$ Retronectin (Takara Shuzo, Otsu, Japan) for 2 hours at room temperature or overnight at 4°C. Cells were plated at a concentration equal to 1×10^5 $cells/cm^2$ for transduction. Cells were infected with a 1:1 ratio of retrovirus supernatant/complete media with cytokines for 4 hours on 2 consecutive days, with a change to complete medium containing cytokines for overnight incubation. After the second round of infection, cells were allowed to remain on Retronectin-coated plates overnight in the presence of cytokines. The following day, the cytokine-containing medium was removed and replaced with new medium containing 100 ng/mL stem cell factor and expanded for 2 days.

K562 cell survival and drug treatments. Following DNA-damaging agent exposure, K562 cells expressing vector, nucl-MGMT, or mito-MGMT constructs were measured 48 hours after treatment using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide survival assay (19, 29). Vector-infected cells were used as the control in these experiments, and survival data was normalized to the survival of untreated vector control cells. Experiments were done in triplicate and repeated thrice. Statistical analysis was done using SigmaStat (Jandel Scientific, San Rafael, CA) software package (*t* test and ANOVA).

DNA alkylating agents used in these experiments include TMZ (a kind gift from Dr. Robert Bishop, Schering-Plough Corp., Kenilworth, NJ), BCNU, and MMS from Sigma-Aldrich (St. Louis, MO). 6BG (Sigma) were prepared as previously described (26).

[3] S. Cai and K.E. Pollok, unpublished observations.

Colony-forming-unit assays. Transduced CD34$^+$ cells were exposed to 20 μmol/L 6BG in serum-free IMDM containing 100 ng/mL G-CSF for 1 hour followed 1 hour later with increasing concentrations of BCNU (20-80 μmol/L) or TMZ (100-400 μmol/L). For colony-forming-unit assays, transduced cells were plated at 2,000 cells per plate in complete methlycellulose (Methocult H4434, Stem Cell Technologies, Vancouver, BC, Canada) in the presence of 10 μmol/L 6BG to maintain depletion of wild-type MGMT activity for a longer period of time. Colonies were scored 14 days later by microscopy.

Confocal microscopy. K562 or human CD34$^+$ cells transduced with the SF91 control, nuclP140K, or mitoP140K vectors were analyzed for expression and localization by confocal microscopy. The cells were incubated with 0.125 μmol/L MitoTracker Red (CMXRos, Molecular Probes, Eugene, OR) for 1 hour at 37°C and washed with PBS thrice. The cells were then fixed with 4% paraformaldehyde (Sigma) for 1 hour at 4°C and washed with PBS thrice. The cells were then permeabilized with 0.2% Triton X-100 (Sigma) for 5 minutes and washed with PBS thrice. Then, 1: 200 dilution of anti-MGMT (mAb clone MT3.1, 200 μg/mL, Kamiya Biomedical Company, Seattle, WA) was added to cells and incubated at 4°C overnight. After washing with PBS thrice, 1:200 dilution of Alexa-Fluor 647 goat anti-mouse IgG (H + L, 2 mg/mL, Molecular Probes) was added and incubated at room temperature for 2 hours and then washed with PBS thrice. An aliquot of cells resuspended in mounting media (70% glycerol/30% PBS/1.5 μg/mL 4′,6-diamidino-2-phenylindole; Sigma) was placed on glass slides. The confocal analysis were done on a Zeiss LSM-510 confocal microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a UV argon laser (351 and 364 nm excitation), a visible argon laser (458, 488, and 514 nm excitation), and two helium-neon lasers (543 and 633 nm excitation).

Results

Nuclear and mitochondrial targeting of O^6-methylguanine DNA methyltransferase in K562 cells. K562 cells, which are deficient in endogenous MGMT (2, 11, 12, 17), were stably transfected with pcDNA3.1 engineered to overexpress MGMT in nuclei or mitochondria. Positive clones were selected and characterized by Northern blot analysis (data not shown) and Western blot analysis. Total protein was collected from G418-resistant clones or vector-transfected control cells and these samples were tested for expression of nucl-MGMT or mito-MGMT in the pooled clones (data not shown). Individual clones were analyzed by Western blot using anti-MGMT antibody (Fig. 1*A*). A band comigrating with human MGMT (21 kDa) was detected in mito-MGMT clones 1, 2, 3, 5, and 6 and nucl-MGMT clones 1, 2, and 3. No MGMT was detected on Western blots with extracts from vector transfected K562 cells (Fig. 1*A*). Subsequent analyses were done using mito-MGMT clone 1 and nucl-MGMT clone 3, which express MGMT at a similar level.

Previous studies showed that a MnSOD mitochondrial targeting sequence efficiently targets proteins to which it is linked to mitochondria (13, 19, 30–32). Mitochondrial expression of MGMT was confirmed by subcellular fractionation followed by Western blot analysis. In mito-MGMT clones, MGMT was detected in the mitochondrial fraction but not in the nuclear fraction (Fig. 1*B*). Western blots were probed with cytochrome *c* as a positive control.

K562 cells were also engineered to simultaneously overexpress nucl-MGMT and mito-MGMT by infecting nucl-MGMT cells with a retroviral bicistronic construct for mito-MGMT and EGFP. Infected cells were analyzed by flow cytometry and sorted according to presence or absence of green fluorescence (i.e., EGFP positive or EGFP negative). EGFP-positive cells were plated and individual clones were analyzed by Northern blot and Western blot for MGMT expression (Fig. 2). Figure 2*B* shows the results with clonal cell lines expressing nucl-MGMT, mito-MGMT, or both. As expected, cells with nucl-MGMT and mito-MGMT seem to express approximately twice as much MGMT protein as cells expressing MGMT only in the nuclear or mitochondrial compartment. MGMT activity assays were in agreement with the Western blot analysis and confirmed equal amounts of activity in the nucl-MGMT or mito-MGMT cell lines and twice as much in nucl-MGMT plus mito-MGMT cells (data not shown). Previous studies have shown a strong correlation between protein level and activity. This is consistent with the fact that MGMT acts in a stoichiometric ratio with its substrate and must be regenerated after each catalytic cycle (33).

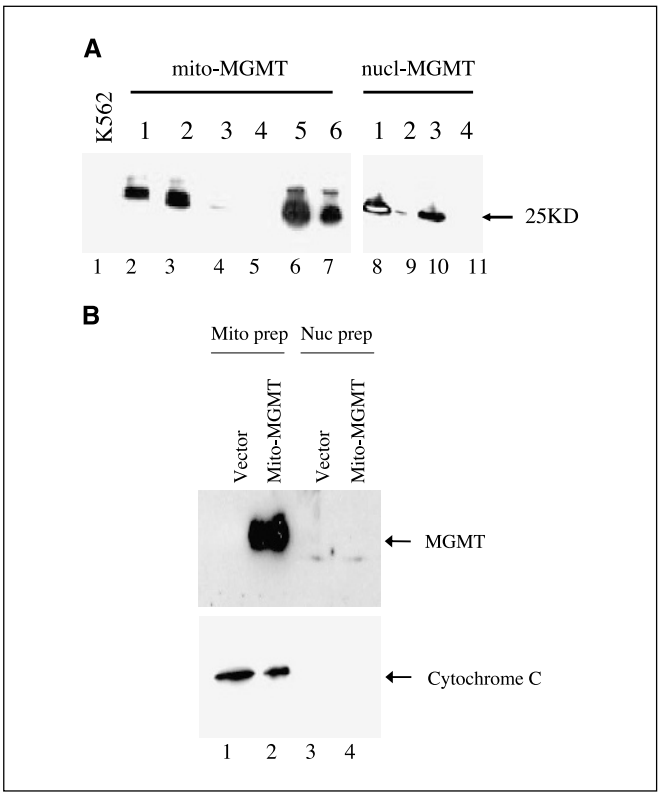

Figure 1. Characterization of K562 cell lines overexpressing either nucl-MGMT or mito-MGMT and specificity of the MnSOD mitochondrial targeting sequence. *A*, Western blot analysis of six clones positive for mito-MGMT (*lanes 2-7*; *numbers 1-6*) and four clones positive for nucl-MGMT (*lanes 8-11*; *numbers 1-4*). K562 control cell extract was run in the first lane (*lane 1*) and is negative for MGMT. Blot was probed with a monoclonal antibody to human MGMT as described. *B*, subcellular fractionation on mito-MGMT clone 1. Clone 1 of the mito-MGMT (*A*) was used for mitochondria and nuclear extracts and analyzed using Western blot analysis with the MGMT monoclonal antibody as in (*A*). K562 cells with vector alone were negative controls (*lanes 1* and *3*). *Lanes 1 and 2,* extracts from the mitochondrial preparation; *lanes 3 and 4,* extracts from the nuclei. As shown, mito-MGMT only was present in the mitochondrial fraction and not in the nuclei. The blot was stripped and reprobed with antibody to cytochrome *c* to show the purity and specificity of the mitochondrial preparations versus the nuclear preparations. The MnSOD targeting sequence is highly effective in targeting proteins, even those with nuclear targeting sequences preferentially into the mitochondria as we have previously shown (19).

Effect of nuclear or mitochondrial targeting of O^6-methylguanine DNA methyltransferase on sensitivity to alkylating agents. Cells expressing nucl-MGMT or mito-MGMT were exposed to three alkylating agents, each of which has a different mode of action. MMS is a monofunctional methylating agent that reacts with cellular DNA via a second-order S_N2-type reaction. MMS primarily methylates DNA at N^7-guanine and N^3-adenine nucleophilic centers. MMS-induced DNA lesions include 82% N^7-methylguanine, 10.8% N^3-methyladenine, and 0.6% N^3-methylguanine. These lesions are repaired by the DNA base excision repair pathway. Only a small

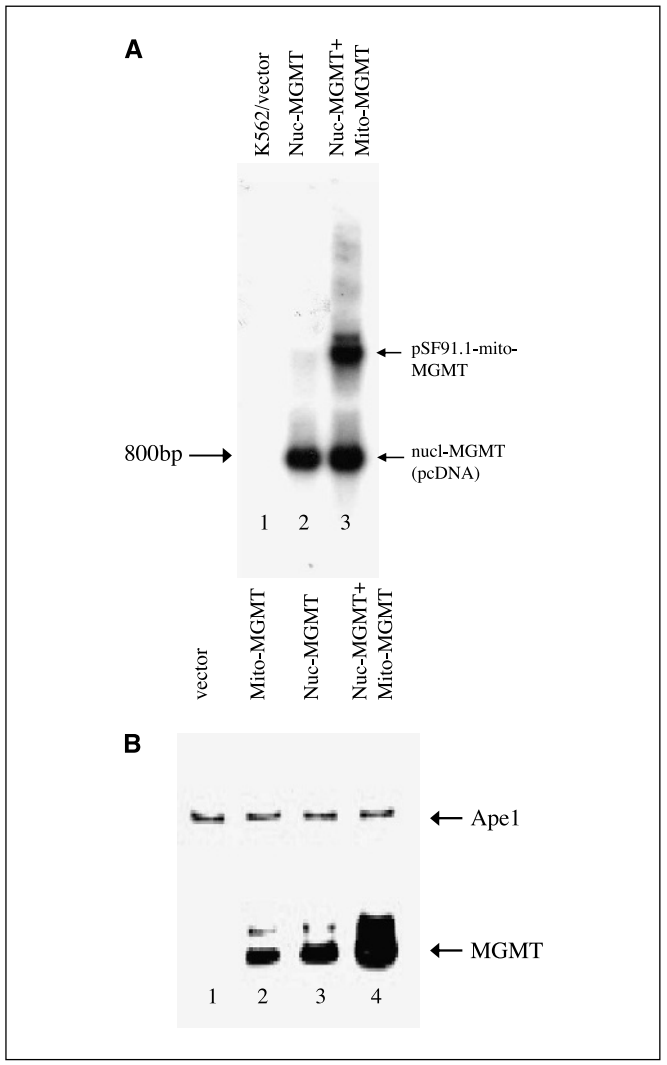

Figure 2. Isolation of cells with both nucl-MGMT and mito-MGMT. Nucl-MGMT clone 3 (see text and Fig. 1*A*) was infected with a retroviral construct containing mito-MGMT and EGFP. Following sorting, EGFP cells were picked and isolated. *A*, Northern blot analysis of a representative clone containing both the plasmid-encoded nucl-MGMT and the retroviral mito-MGMT. Blot was probed with a MGMT cDNA. *Lane 1,* vector control negative for MGMT; *lane 2,* nucl-MGMT clone 3; *lane 3,* nucl-MGMT with retroviral mito-MGMT. *Upper band,* retroviral transcript containing the mito-MGMT; *lower band,* nucl-MGMT transcript. *B*, Western blot analysis of the clones shown in (*A*) using the human MGMT antibody. *Lane 1,* vector control negative for MGMT; *lane 2,* mito-MGMT clone; *lane 3,* nucl-MGMT; *lane 4,* mito-MGMT and nucl-MGMT clone. The blot was reprobed with the human Ape1 antibody to normalize for loading (29, 30).

fraction of MMS-induced adducts (0.3%) are O^6-methylguanine (34, 35); thus, a small fraction of MMS-induced DNA damage is repaired by MGMT. In K562 cells, which are differentially susceptible to deleterious effects of O^6-methylguanine, MGMT overexpression in nuclei, mitochondria, or both decreased susceptibility to cell killing by MMS by ~2-fold ($P < 0.01$) at all doses (Fig. 3*A*). The ability of nucl-MGMT to confer such protection was anticipated, but the ability of mito-MGMT to confer equally strong protection was not anticipated. It is also interesting to note that simultaneous overexpression of MGMT in nuclei and mitochondria did not confer greater protection than expression in nuclei or mitochondria alone.

BCNU and TMZ are DNA-alkylating agents that are more clinically relevant than MMS. BCNU forms a chloroethyl adduct at O^6-guanine, which can rearrange into an interstrand ethyl bridge between N^1-guanine and N^3-cytosine (5). Interstrand DNA cross-links are particularly cytotoxic because they block DNA replication (6). Figure 3*B* shows that overexpression of MGMT in

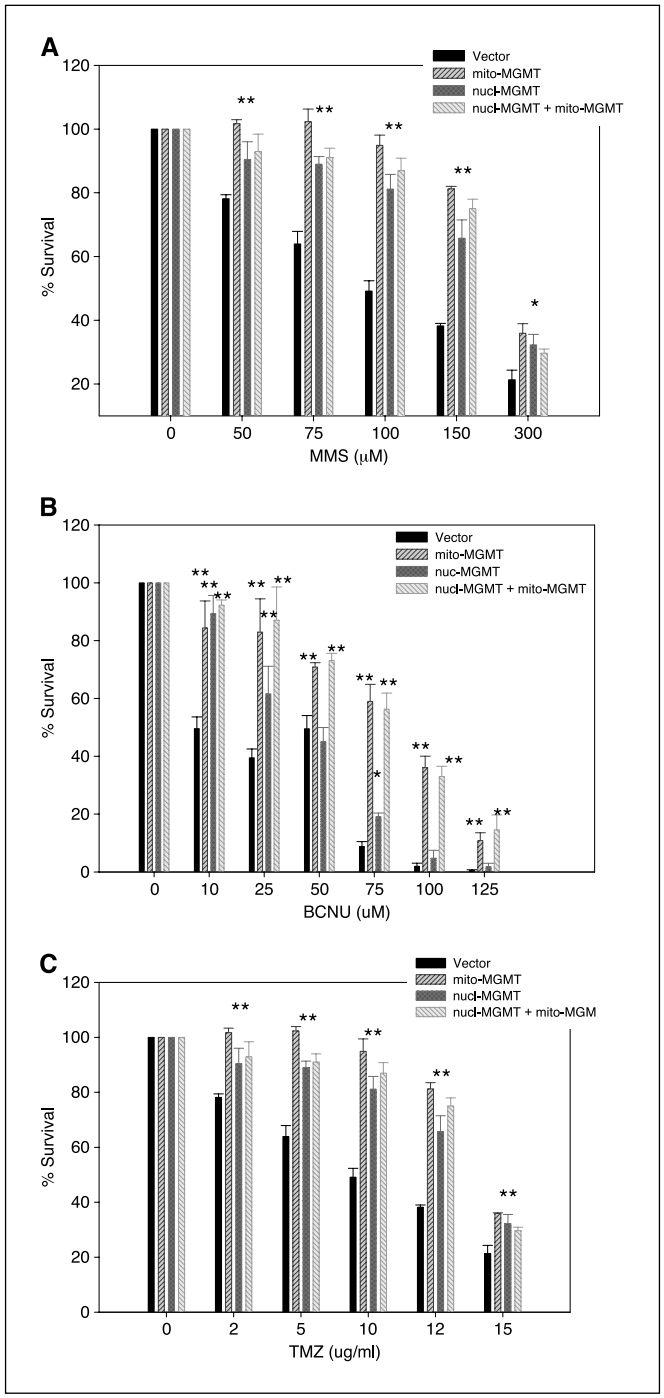

Figure 3. Protection of K562 cells with nucl-MGMT, mito-MGMT, or both. Cell survival assays were done as discussed in Materials and Methods and in previous publications (17, 19, 29, 30). Cells were treated with MMS (*A*), BCNU (*B*), or TMZ (*C*). In (*A*) and (*C*), all groups were statistically different from the vector control at the $*P < 0.05$ or $**P < 0.01$ levels. In (*B*), the statistical level is indicated above each bar and represents comparison to the vector control at the 10 and 25 μmol/L levels, while it also indicates significance in a comparison between the mito-MGMT and nucl-MGMT + mito-MGMT compared with the nucl-MGMT for the 25, 50, 75, 100, and 125 μmol/L levels. The significance is indicated as $*P < 0.05$ or $**P < 0.01$.

nuclei, mitochondria, or both protected against cell killing by BCNU. In addition, overexpression of MGMT in mitochondria conferred greater protection against BCNU at 10 μmol/L or higher than overexpression in nuclei ($P < 0.01$), and an equivalent level of protection as overexpression in nucl-MGMT and mito-MGMT (Fig. 3*B*). These results suggest that mitochondrial expression of MGMT may play as or more important a role in preventing cell killing by BCNU than nuclear expression of MGMT.

TMZ is another DNA-alkylating agent that is increasingly used instead of BCNU in the clinic. TMZ is nonenzymatically hydrolyzed in solution to the active compound 3-methyl-(triazen-1-yl)imidazole-4-carboxamide, which is also the biologically relevant metabolite of dacarbazine. TMZ reacts with the same functional sites in DNA as MMS, but the relative reactivity of these groups is different than for MMS. In particular, ~5% of all TMZ DNA adducts are O^6-methylguanine; the rest of the adducts are ~70% N^7-guanine adducts and 9% N^3-adenine adducts (9). TMZ does not produce cross-links. Therefore, TMZ is considered to be a clinically relevant analogue of MMS. This view is consistent with the fact the effect of overexpression of MGMT on cell survival is similar for K562 cells treated with MMS or TMZ (i.e., expression of MGMT in nuclei, mitochondria, or both conferred a similar level of protection against cell-killing; $P < 0.01$; Fig. 3*C*).

Similar results were observed with other cell lines, indicating that these observations are unlikely to be an artifact specific to one particular cellular genotype or phenotype (data not shown).

Overexpression of O^6-methylguanine DNA methyltransferase protects O^6-methylguanine DNA methyltransferase–proficient cells against alkylation-induced cell killing. The results presented above were done in MGMT-deficient K562 cells. If similar results were observed in MGMT-proficient cells, it would strengthen the evidence that mitochondrial MGMT plays a role in protecting against alkylation-induced cell killing, and confirm that this role is important in the presence of endogenous nuclear MGMT. This experiment was done in A204 rhabdomyosarcoma cells, which express a high level of nuclear MGMT (Fig. 4*A* and *B*). A204 cells were engineered to overexpress nuclear or mitochondrial MGMT, as confirmed by coexpression of EGFP and Western blotting for MGMT (Fig. 4*C*). Figure 4*C* confirms expression of a slow-migrating protein band corresponding to MGMT plus its mitochondrial-targeting motif, which is observed in whole cell extracts but not in mitochondrial extracts. Figure 4*D* shows that expression of ectopic nuclear or mitochondrial MGMT increases cell survival at all doses of BCNU, but control cells carrying vector only do not show increased survival. In addition, in A204 cells as in K562 cells, overexpression of mitochondrial MGMT reduces BCNU-induced cell killing more than overexpression of nuclear MGMT.

Protection of human CD34$^+$ cells from 6BG/BCNU or 6BG/TMZ by overexpression of nuclear or mitochondrial O^6-methylguanine DNA methyltransferase P140K. Experiments similar to those described above were also done in mobilized peripheral blood CD34$^+$ human progenitor cells expressing nuclear MFMT, mitochondrial MGMT, or vector control. When CD34$^+$

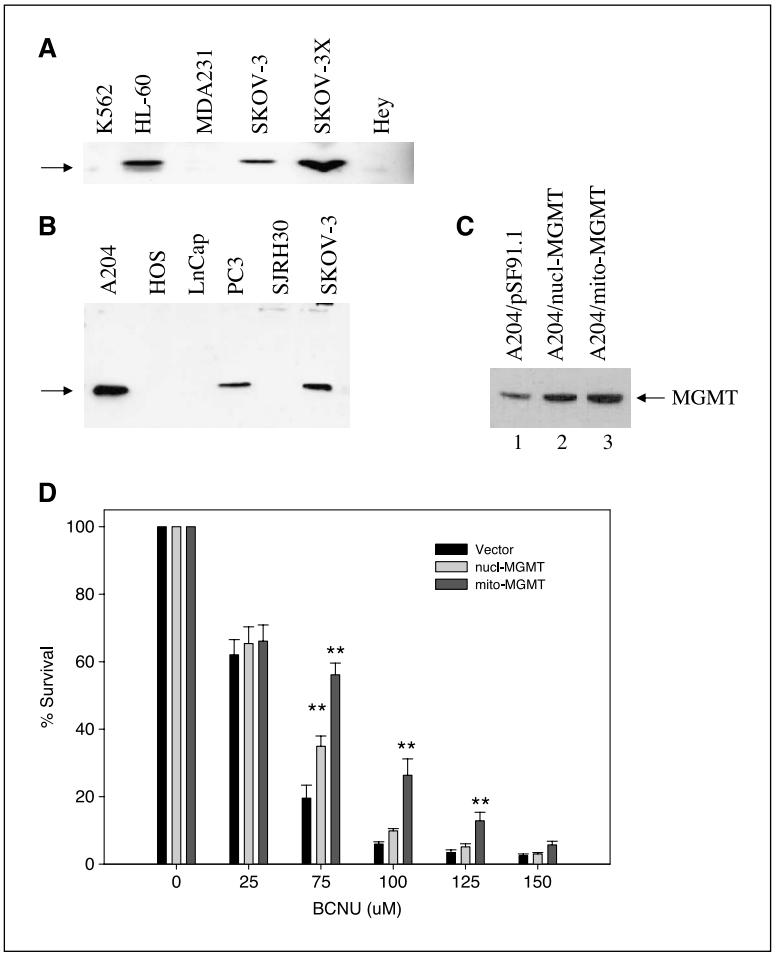

Figure 4. Protection of MGMT-proficient cells with nucl-MGMT or mito-MGMT. *A and B,* Western blots of various cancer cell lines to determine endogenous MGMT levels. Blots were probed with the human monoclonal MGMT antibody as previously discussed. K562 (*A*) is a negative control, whereas the A204 cell line in (*B*) is one of the highest MGMT cell lines tested. *C*, Western blot of total extracts from A204 cells with vector alone (*lane 1*), nucl-MGMT (*lane 2*), or mito-MGMT (*lane 3*) inserted into the cells. The upper band in lane 3 is the mito-MGMT, which sometimes migrates at a slightly higher molecular weight in crude extracts presumably because of the uncleaved MnSOD targeting sequence. *D*, cell survival assay of A204 cells with either vector, nucl-MGMT, or mito-MGMT constructs following BCNU treatment. Comparisons between the nucl-MGMT or mito-MGMT and vector were determined and the statistical significance was determined at the $^{**}P < 0.01$ level where indicated.

human progenitor cells were exposed to BCNU, ectopic nuclear or mitochondrial MGMT increased survival to a higher level than the vector control. However, cell colonies derived from the $CD34^+$ progenitor cells were less susceptible to 80 μmol/L BCNU when nuclear MGMT, but not when mitochondrial MGMT, was overexpressed (data not shown). These data suggest that mitochondrial MGMT is not sufficient to protect from BCNU-induced DNA damage in all cell types and that expression of nuclear MGMT also plays a critical role.

Previous studies showed an effective *in vivo* method for differential selection of specifically engineered hematopoietic stem cells or their progeny (18, 26, 36). Here, this approach was applied to murine stem cells or K562 cells, which were transduced with an expression construct for 6BG-resistant MGMT mutant $MGMT^{P140K}$; recipient cells were then exposed to 6BG/BCNU or 6BG/TMZ. A similar approach was previously used to select human progenitor cells in nonobese diabetic/severe combined immunodeficient mice (24, 37, 38).

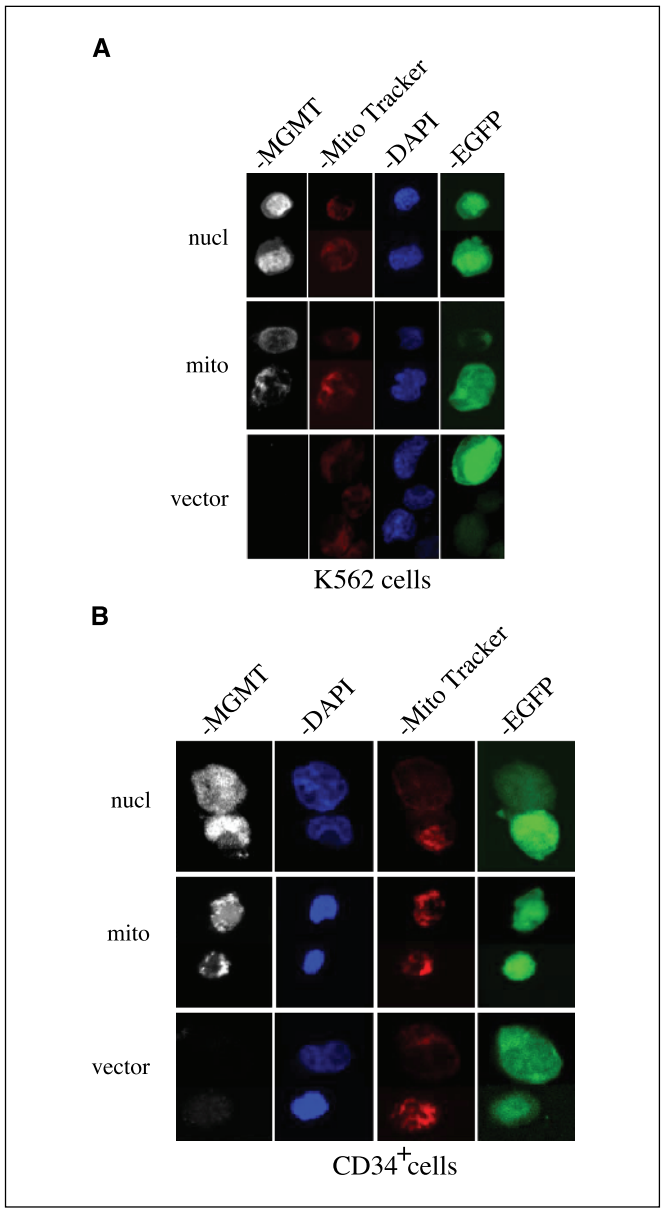

Figure 5. Confirmation of $MGMT^{P140K}$ localization to the mitochondria by confocal microscopy. K562 cells (*A*) and $CD34^+$ cells (*B*) were transduced with control, nuclP140K, or mitoP140K vectors. Cells were fixed, permeabilized, and stained with anti-MGMT antibody (clone MT3.1) and anti-mouse IgG-conjugated antibody. To detect the mitochondria, some samples were incubated with the MitoTracker Red dye before the fixing and staining procedure.

Expression and subcellular localization of mitochondrial and nuclear $MGMT^{P140K}$ in targeted K562 cells (Fig. 5*A*) and $CD34^+$ (Fig. 5*B*) cells was confirmed using a specific MitoTracker dye, antibody staining, and confocal microscopy (Fig. 5*A*). Specific colocalization of mitotracker dye with mitochondrial $MGMT^{P140K}$ or nuclear DNA with nuclear $MGMT^{P140K}$ was observed in appropriate cell lines. Relative transduction efficiency was determined to be similar (35-40% average for three independent experiments) for the control plasmid and for plasmids overexpressing nuclear or mitochondrial $MGMT^{P140K}$ (Fig. 6*A*) using EGFP fluorescence as a reference. The mean fluorescence intensity of the $EGFP^+$ cells varied slightly between the nuclear $MGMT^{P140K}$ (mean fluorescence intensity = 826) and the mitochondrial $MGMT^{P140K}$ (mean fluorescence intensity = 732; Fig. 6*A*). Analysis of MGMT expression by Western blots and enzyme activity showed that mitochondrial $MGMT^{P140K}$ was expressed at a lower level than nuclear $MGMT^{P140K}$ (~4-fold lower by enzyme assay; Fig. 6*B*).

When mobilized peripheral blood $CD34^+$ cells were exposed to 6BG and increasing doses of BCNU or TMZ, colony-forming unit assays indicated that committed progenitor cells overexpressing nuclear or mitochondrial 6BG-resistant $MGMT^{P140K}$ were indeed more resistant to alkylation-induced cell killing than control cells. However, overexpression of nuclear $MGMT^{P140K}$ conferred the greatest resistance to 6BG/BCNU. In contrast, nuclear and mitochondrial $MGMT^{P140K}$ provided a similar level of resistance to 6BG/TMZ up to 200 μmol/L TMZ and nuclear expression of $MGMT^{P140K}$ only provided greater resistance at 400 μmol/L TMZ.

These data were also analyzed to assess the relative ability of mitochondrial and nuclear $MGMT^{P140K}$ to protect progenitor $CD34^+$ cells from 6BG/BCNU or 6BG/TMZ (Table 1). In this analysis, we determined how many progenitor colonies would be theoretically protected if mitoP140K activity was equivalent to nuclP140K activity in the $CD34^+$ cells. Interestingly, for cells treated with 6BG/BCNU, increased numbers of progenitors were protected at 20 to 40 μmol/L BCNU. Because there were no mitoP140K-progenitor colonies protected at 80 μmol/L BCNU (Fig. 6*A*), normalization could not be determined at this concentration. For mitoP140K-transduced cells treated with 6BG/TMZ, increased numbers of progenitor colonies were protected at all doses tested compared with nuclear P140K–transduced cells. These data show that localization of $MGMT^{P140K}$ to the mitochondria increases the survival of committed human hematopoietic progenitor cells. In future studies, a bicistronic vector that coexpresses nuclP140K and mitoP140K will be used to test the hypothesis that increased protection of human progenitor cells from alkylator-mediated DNA damage can be obtained by coexpression of the mitoP140K and the nuclP140K DNA repair proteins.

Discussion

This report shows that overexpression of MGMT enhances the survival of hematopoietic cells challenged with MMS, BCNU, or TMZ and that targeted overexpression of MGMT in mitochondria enhances survival as well as or better than targeted nuclear overexpression. Whereas protection of cells by nuclear-localized MGMT has been documented in previous reports (7, 24), the

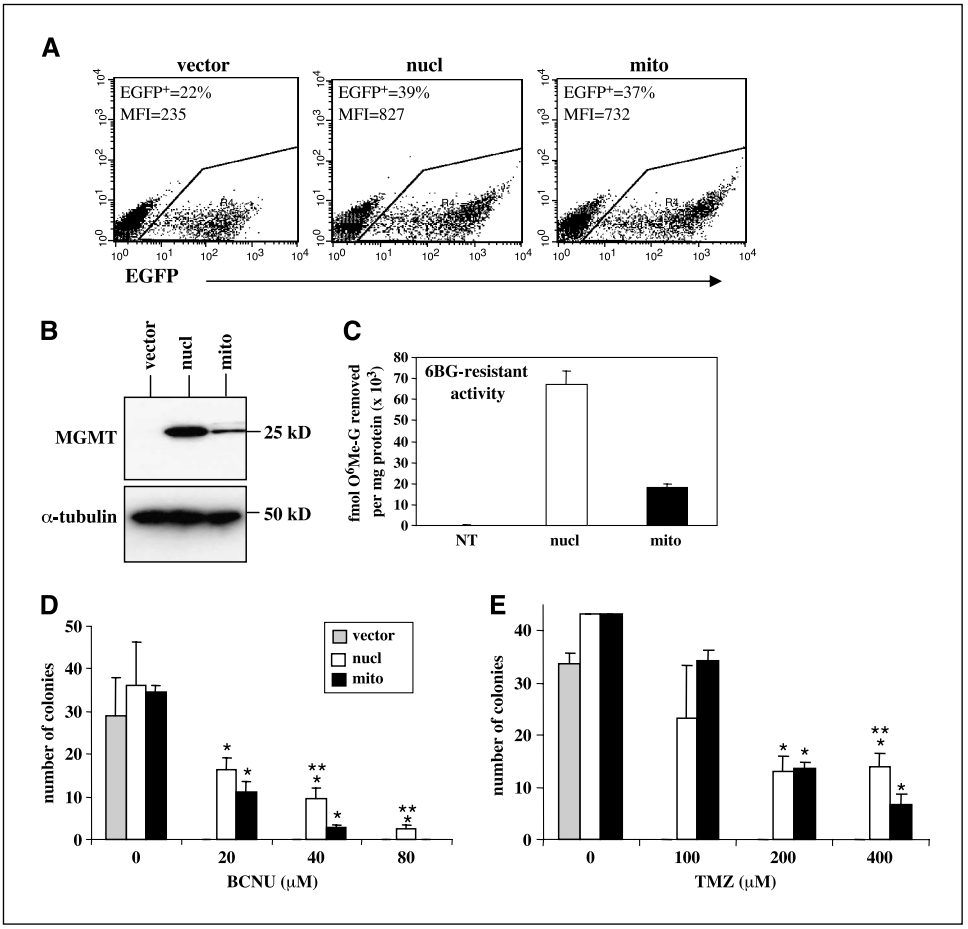

Figure 6. Protection of committed progenitor cells from 6BG/BCNU or 6BG/TMZ by expression of nuclear- or mitochondrial-localized MGMT^{P140K}. Human CD34$^+$ cells derived from G-CSF–mobilized peripheral blood were transduced with retroviruses that express EGFP only (*vector*) or that coexpress nuclear- (*nucl*) or mitochondrial-localized MGMT^{P140K} (*mito*) and EGFP. *A*, flow cytometric analysis of EGFP expression. *B*, Western blot analysis of MGMT^{P140K} expression in vector transduced and nuclear- or mitochondrial-transduced cells. *C*, 6BG-resistant MGMT activity in transduced CD34$^+$ cells. *D*, resistance of colony-forming units to 6BG/BCNU. *E*, resistance of colony-forming units to 6BG/TMZ. *$P < 0.001$ for vector versus mitoP140K or nuclP140K. **$P < 0.01$ for nuclP140K versus mitoP140K. *MFI*, mean fluorescence intensity.

protection of cells by mitochondrial-localized MGMT has not been reported previously in any cell type. The relative efficiency of mitochondrial and nuclear MGMT in protecting against alkylation-induced cell killing depends on the specific alkylating agent and the cell type. Thus, mitochondrial MGMT protects transfected K562 cells more efficiently against BCNU-induced cell killing but equally efficiently against MMS- and TMZ-induced cell killing. This study also shows that simultaneous nuclear and mitochondrial overexpression of MGMT does not provide greater resistance to alkylation-induced cell killing than expression in either one of the two cellular compartments. Similar results were observed when mito-MGMT was expressed in human CD34$^+$ hematopoietic progenitor cells, suggesting that the observations are not unique to K562 cells. Thus, this study supports the hypothesis that mtDNA damage/repair has a significant impact on survival in cells exposed to DNA-alkylating agents.

MGMT plays an important biological role in directly repairing O^6-methylguanine in DNA. MGMT has been studied extensively because of its potential usefulness in gene therapy and/or to protect against nontarget effects during cancer chemotherapy (1, 4, 7). Normal hematopoietic cells including K562 cells express a low level or completely lack endogenous MGMT, making hematopoietic cells particularly sensitive to depletion in cancer patients treated with alkylating agents. Previous studies showed that overexpression of MGMT in human or murine hematopoietic cells increases cellular resistance to BCNU (1, 4, 39, 40) and TMZ (7, 8, 41–43). These studies focused exclusively on overexpression of MGMT in the nuclear compartment (1, 4, 7, 24) and gave little explicit consideration to the potential involvement of mtDNA damage/repair in survival after exposure to DNA alkylating agents.

Studies of mtDNA repair show the presence of base excision repair and nucleotide excision repair pathways that share

Table 1. Theoretical assessment of progenitor protection—nuclP140K versus mitoP140K

Drug treatment	No. of progenitors protected based on normalized P140K activity	
	NuclP140K*	MitoP140K
6BG/BCNU (μmol/L)		
20	18 ± 2	48 ± 2
40	12 ± 2	16 ± 1
80	4 ± 1	ND
6BG/TMZ (μmol/L)		
100	22 ± 11	128 ± 3
200	14 ± 3	56 ± 2
400	12 ± 3	32 ± 2

Abbreviation: ND, none detected.
*NuclP140K activity was set at 100%. Because mitoP140K activity was 4-fold lower, the number of colony-forming units protected in the mitoP140K groups was multiplied by 4.

significant similarity to nuclear base excision repair and nucleotide excision repair (15, 44). There is some evidence for reversal of O^6-methylguanine lesions by direct repair in mitochondria (45), but there is no definitive evidence for expression of MGMT in the mitochondrial compartment (46). Nevertheless, it is clear that mtDNA is susceptible to alkylation and oxidative DNA damage (20, 47–49) and this damage is thought to have significant consequences for survival at the cellular level as well as health consequences for animal species including mammals. Numerous human diseases (i.e., diabetes, ischemic heart disease, Parkinson's disease, Alzheimer's disease) as well as normal aging are associated with mutations in mtDNA (15, 50, 51). These observations are consistent with the present study, which suggests that increased DNA repair potential in the mitochondria increases survival of K562 cells exposed MMS, BCNU, or TMZ.

The results presented here raise a number of questions for future studies. For example, does overexpression of MGMT in mitochondria enhance the survival of a specific subpopulation of cells?; if yes, then does the DNA repair capacity of this subpopulation differ from other subpopulations and how? Does overexpression of MGMT in mitochondria decrease the number of persistent DNA lesions or the mutation rate in mtDNA and/or nuclear DNA of cells exposed to alkylating agents? It is also interesting to note that low level expression of mitochondrial $MGMT^{P140K}$ in hematopoietic progenitor cells efficiently protects cells against alkylation-induced cell killing. The reason for this high efficiency is not yet known.

High-dose treatment with chemotherapeutic alkylating agents is associated with negative off-target effects on hematopoietic cells (1, 2, 4), such that the primary dose-limiting toxicity for most alkylating agent chemotherapeutics is bone marrow suppression. This problem has led clinical researchers to explore whether hematopoietic cells can be engineered with increased resistance to alkylating agent–induced cell killing (4, 7, 24, 37, 38, 52, 53). In some cases, autologous $CD34^+$ hematopoietic stem cells were isolated, expanded, and engineered for higher drug resistance (12). Such cells could potentially be reintroduced into a cancer patient before dosing with chemotherapeutic alkylating agents. The studies presented here provide useful information to guide future experiments along these lines. In particular, it may be appropriate to engineer recipient hematopoietic cells to express MGMT (or other relevant DNA repair enzymes) in both nuclear and mitochondrial cellular compartments. This approach would maximize cellular resistance to alkylating agent chemotherapy. It should be emphasized that the results presented here show the feasibility of engineering primary hematopoietic precursor cells for increased resistance to akylating agent–induced cell killing, laying the groundwork for future studies along these lines. One such study will evaluate the *in vivo* and *in vitro* impact of dual expression of nuclear and mitochondrial $MGMT^{P140K}$ in hematopoietic $CD34^+$ cells. Additional future studies might explore the impact of mitochondrial targeting of other base excision repair enzymes on cell survival during alkylation chemotherapy or other chemotherapeutic protocols with other types of drugs.

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