AD_____

Award Number: DAMD17-00-C-0012

TITLE: Protection Against the Acute and Delayed Toxicities of Sulfur Mustard

PRINCIPAL INVESTIGATOR: Zdenka Matijasevic, Ph.D.

CONTRACTING ORGANIZATION: University of Massachusetts Medical Center
Worcester, MA 01655

REPORT DATE: April 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2003	Final (15 Mar 2000 - 14 Mar 2003)

4. TITLE AND SUBTITLE
Protection Against the Acute and Delayed Toxicities of Sulfur Mustard

5. FUNDING NUMBERS
DAMD17-00-C-0012

6. AUTHOR(S)
Zdenka Matijasevic, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Massachusetts Medical Center
Worcester, MA 01655

E-Mail: Zdenka.matijasevic@umassmed.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Both the acute and delayed toxicities of DNA damaging agents represent the outcome of a race between protective and toxic pathways triggered by DNA damage. In order to identify targets for therapeutic intervention and conditions that can modulate the outcome of exposure to sulfur mustard we sought to investigate pathways and early events involved in cellular responses to SM-induced damage. We have identified two levels of cellular responses where proper intervention may minimize toxicity. The first level directly involves the activity of DNA repair enzymes, while the second one is based on the regulation of cell cycle progression. We have discovered that in contrast to the protective effect of nucleotide excision repair, alkyl adenine DNA glycosylase, the first enzyme in the base excision repair pathway, sensitizes cells to SM exposure. We show that hypothermia provides protection against SM toxicity. The results suggest that two mechanisms account for this protection: i) hypothermia counteracts sensitizing effect of DNA glycosylase in SM-exposed cells and ii) hypothermia induces reversible cell cycle arrest providing more time for repair of lesions before the critical cellular events, DNA replication and mitosis, take place.

14. SUBJECT TERMS
Sulfur Mustard, DNA modification, DNA repair, Hypothermia, Cell Cycle Arrest, p53 protein

15. NUMBER OF PAGES
67

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

TABLE OF CONTENTS Page

TABLE OF CONTENTS (Cont.) **Page**

INTRODUCTION

During the last several years many aspects of cellular responses to stress and mechanisms leading to the cell death have been elucidated (1-4). A better understanding of those highly regulated processes helps us also to better understand the role of sulfur mustard-induced DNA damage in triggering stress response and cell death. The network of processes initiated by DNA damage and culminating in cell death is extremely complex and highly regulated. However, we reason that numerous points within this network of toxic and protective pathways exist where targeted intervention may modulate the outcome of damage. We therefore sought to investigate pathways and early events along these pathways involved in cellular responses to SM-induced DNA damage in order to identify points suitable for therapeutic intervention. We have identified two levels of cellular responses where proper intervention may minimize toxicity. The first level directly involves the activity of DNA repair enzymes, while the second one involves the regulation of cell cycle progression. Our results suggest that certain conditions such as hypothermia may provide protection against SM toxicity by modulating relevant processes at both of these levels.

We have demonstrated that two independent repair pathways, nucleotide excision repair (NER) (5) and base excision repair (BER) (6) are involved in processing of SM-modified DNA. Cells with a defect in NER are very sensitive to SM indicating that this pathway has an important role in protection against SM toxicity. To our surprise, however, we discovered that cells lacking DNA glycosylase, an early enzyme of the BER pathway, are more resistant to SM (7). This sensitizing effect of glycosylase is specific for SM exposure since the glycosylase activity protects the same cells from simple methylating agents such as methyl methane sulfonate (MMS). This finding represents a new lead in searches for the modulators of sulfur mustard toxicity and suggests that the inhibition of a specific step in the base excision repair pathway may diminish both long-term and short-term toxicity.

We have previously found that SM induces a dose-dependent cell cycle arrest (8) and some mammalian cell lines that have lost the ability to arrest in response to exposure are more sensitive to SM. These results suggested that the conditions causing a delay or a reversible arrest of cell cycle progression might improve survival. We then discovered that mild hypothermia provides such conditions; it causes a reversible cell cycle arrest (9) and protects cells from SM toxicity (7). A possible mechanism of protection based on the cell cycle arrest is increased time for the repair of DNA lesions before the initiation of critical cellular events such as DNA replication and mitosis are taking place. Importantly, hypothermia also counteracts sensitizing effect of DNA glycosylase and significantly contributes to the SM resistance through this mechanism.

BODY

BACKGROUND

The Repair Mechanisms Involved in Processing of SM-induced DNA Lesions

DNA crosslinks are considered major toxic lesions in SM-alkylated DNA, and the repair of crosslinks is most likely a major defense against SM toxicity. At the same time, DNA monoadducts can seriously interfere with DNA functions either directly or as precursors of crosslinks, apurinic (AP) sites, or strand breaks, all of which, if unrepaired, can be deleterious. Our results demonstrate that at least two

distinct DNA repair pathways, nucleotide excision repair (NER) and base excision repair (BER), are involved in processing of SM-induced DNA damages.

Nucleotide excision repair is involved in repair of the wide class of helix-distorting lesions that interfere with base pairing and generally obstruct transcription and normal replication (10). The repair of DNA interstrand crosslinks (ICLs) in mammalian cells requires at least some proteins from NER pathway (11), but the mechanism of mammalian ICL repair is still very poorly understood. The protective effect of nucleotide excision repair against SM toxicity is well defined on the basis of our data on cell survival demonstrating high sensitivity to SM of NER-deficient cells and on the basis of host cell reactivation experiments showing decreased repair of SM-damaged plasmid DNA in NER-deficient cells compared to the cells with functional NER. Protection by NER does not seem to be limited to the repair of DNA crosslinks, since NER-deficient cells are also more sensitive to monofunctional sulfur mustard, CEES, which generates only DNA monoadducts. At this point it is not known what specific steps of the NER pathway are involved in the repair of SM lesions and what conditions may improve NER activity and decrease SM toxicity.

Base excision repair has an important role in protecting cells against the lethal and mutagenic effects of simple alkylating agents. However, there are conflicting reports on the involvement of mammalian alkyladenine DNA glycosylase (AAG), an early enzyme of the BER pathway, in protecting cells against cytotoxic effects of bifunctional alkylating agents (12-13). We provide evidence showing that in the same experimental system where the expression of alkyladenine DNA glycosylase clearly protects cells against the simple monofunctional alkylating agent, methyl methane sulfonate (MMS), it sensitizes cells to SM-induced effects. In addition, *E. coli* cells expressing human DNA glycosylase (hAAG) are also more sensitive to another bifunctional alkylating agent, chloroethylnitrosourea, than the cells without hAAG (14). Since the sensitizing effect of DNA glycosylase is manifested in the same cell systems in which glycosylase protects against MMS, this effect may be due either to the presence of lesions not produced by MMS, such as DNA crosslinks or to the more complex monoadducts. We addressed this question using the monofunctional sulfur mustard, chloroethyl ethyl sulfide (CEES), which generates DNA monoadducts similar to those formed by SM but does not form crosslinks. Our results show that glycosylase does sensitize cells to CEES. While it does not rule out glycosylase processing of crosslinks as a sensitizing mechanism, it does indicate that processing of monoadducts contributes substantially to glycosylase-dependent SM sensitization.

The Role of Hypothermia in Protection Against SM Toxicity

Cell response to hypothermia characterized by p53 accumulation and p53-dependent cell cycle arrest (9) together with the growing amount of evidence on cross-talk between the p53 pathway and DNA repair processes (15-16, 4) support our hypothesis on multiple mechanisms responsible for the beneficial effect of low temperature after SM exposure. We demonstrate that hypothermia decreases the cytotoxicity of sulfur mustard for mammalian cells in culture and suggest that this effect is an outcome of several pathways activated in cells in response to combined stress. Although the general, nonspecific slowdown of cellular processes at low temperature may contribute to the beneficial effects of hypothermia, our data suggest that the activation of specific cellular responses may play a more important, direct role in protection.

At least two independent mechanisms seem to contribute to the protective effect of hypothermia. One of them is manifested through the diminished sensitizing effect of DNA glycosylase at low temperature. Since cells deficient in DNA glycosylase still survive better when incubated at low temperature after SM exposure, other mechanisms may also contribute to protection by hypothermia.

We have found that the levels of two proteins involved in cell cycle regulation, p53 and p21, are increased when cells are incubated at 28°C (9). These two proteins have important roles in cells with damaged DNA. In response to DNA damage, the tumor suppressor p53 protein activates transcription of several genes including the cyclin-dependent kinase inhibitor $p21^{WAF-1}$, which is responsible for cell cycle arrest of DNA-damaged cells (17-18). In contrast to the DNA damage response, nothing is known about the mechanisms of accumulation and the roles of p53 and p21 in response to hypothermia. We have found that hypothermia induces a p53-dependent cell cycle arrest (9) and that p53-deficient cells are more sensitive to low SM doses at 28°C than wild type cells. It suggests that p53 modulates survival at low temperatures. Hypothermia-induced cell cycle arrest may provide more time for the repair, and p53 itself may be directly involved in DNA repair (19-20). We show that cell cycle arrest under the conditions of hypothermia requires $p21^{WAF-1}$. Interestingly, hypothermia induces p53-independent p21 expression raising an intriguing question about the actual role of p53 in cell cycle arrest at low temperature.

MATERIALS AND METHODS

Bis-(chloroethyl) sulfide (SM) was supplied by the US Army Institute of Chemical Defense (Aberdeen Proving Ground, MD). Methyl methane sulfonate (MMS) and chloroethyl ethyl sulfide (CEES) were purchased from Aldrich (Milwaukee, WI). Antibiotics and culture media were obtained from Gibco BRL (Gaithersburg, MD). Fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibodies were from Caltag Laboratories, Burlingame, CA. Primary anti-p53 antibodies were obtained from Oncogene Research Products, Boston, MA. Transfection reagent TransFast and the Dual Luciferase Assay kit were from Promega (Medison, WI).

Bacterial Cells

All bacterial strains used in this study were constructed in Michael R. Volkert's laboratory, University of Massachusetts Medical School, (UMMS), Worcester, MA. Their relevant genotypes are listed in Table 1. Cells were grown in liquid Luria-Bertani (LB) medium at 37°C with aeration. Permanent stocks are maintained at –80°C in LB with 10% DMSO.

Table 1. Bacterial strains

Name	*Relevant Genotype*
MV 1161	*arg*E3
MV1273	*arg*E3 *uvrA*
MV1174	arg*E3* alk*A1*
MV1302	arg*E3* alk*A1* uvr*A*
MV3855	*arg*E3 *alk*A1 *tag*A1 *uvr*A
MV4236	arg*E3* alk*A1* tag*A1* uvr*A / pTrc*
MV4237	*arg*E3 *alk*A1 *tag*A1 *uvr*A / pTrc hAAG-1
MV4239	*arg*E3 *alk*A1 *tag*A1 *uvr*A / pTrc hAAG-2
MV4126	*arg*E3 *alk*A1 *tag*A1/pTrc hAAG-1-his_6

Plasmids

HAp53 plasmid expressing wild type p53 with a hemagglutinin (HA) tag transcribed from the p53 promoter was obtained from Alonzo Ross/Stephen N. Jones (UMMS). It contains the *neo*^{r} gene for aminoglycoside phosphotransferase, which renders cells resistant to the antibiotic G418, as well as Amp^r, which confers resistance to ampicillin. Plasmid Waf-1-*luc* obtained from Timothy Kowalik (UMMS) is p53-inducible, with a p21^{WAF-1} promoter attached to the firefly luciferase gene (17) (Figure 1). This plasmid also contains a gene for resistance to chloramphenicol. phRL (Promega, Madison, WI), contains Amp^r and the gene that codes for *Renilla* luciferase, and pGL3 (also from Promega) has Amp^r and the gene for firefly luciferase.

Plasmid Propagation and Purification

Plasmids HAp53, Waf-1-*luc,* phRL and pGL3 were propagated in *E. coli* bacteria. 200 µl of competent *E. coli* cells, strain JM109 (Promega, Madison, WI), were incubated on ice for 20 min with 0.1 µg plasmid DNA, heat-shocked at 37°C for 5 min., and incubated again on ice for 5 min. 1 ml of LB medium was added to the bacteria, which were grown at 37°C for 40 min, then spread onto agar plates containing selective antibiotic (100 µg/ml ampicillin for HAp53, pGL3, and phRL, and 25 µg/ml chloramphenicol for Waf-1) to isolate transformants. The plates were incubated at 37°C overnight. The resultant colonies were streaked onto agar plates with selective medium, and incubated overnight. Individual colonies of resistant bacteria were inoculated into 5 ml of liquid broth with selective antibiotic and grown overnight at 37°C. 500 ml of LB medium with selective antibiotic was inoculated with the transformed *E. coli* and grown overnight at 37°C for plasmid purification.

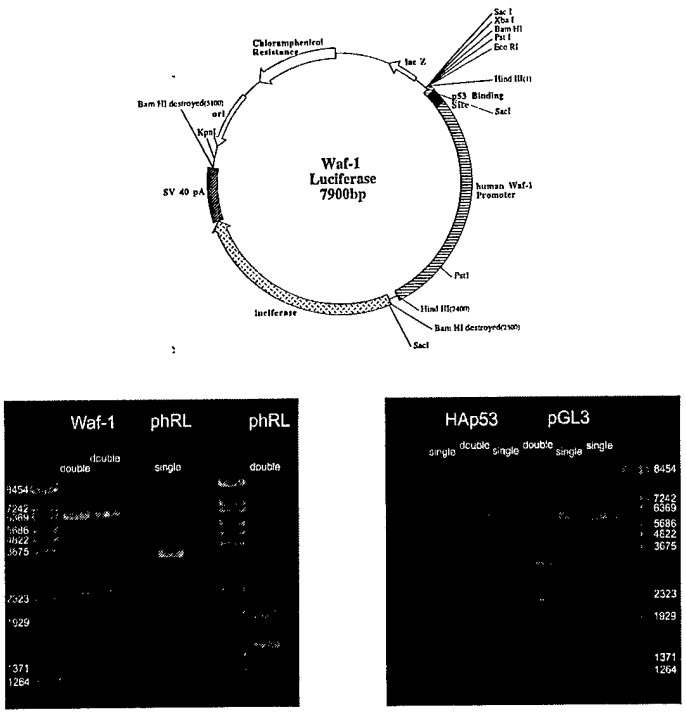


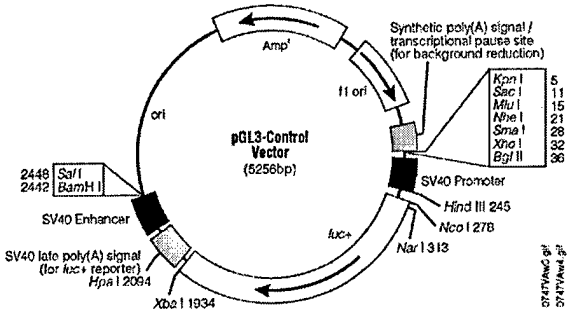
Figure 1. Map of p53-responsive Waf-1-*luc* reporter construct (17) and photographs of agarose gel electrophoresis performed on purified plasmids digested with appropriate restriction enzymes. The sizes (in base pairs) of λ ladder fragments are shown.

Plasmids were purified using either the EndoFree Plasmid Mega Kit from Qiagen (Valencia, CA) or the NucleoBond Plasmid Maxi Kit from Clontech (Palo Alto, CA), following the manufacturer's instructions. The *E. coli* culture was centrifuged for 15 min. at 6000 × *g* and the pellet was resuspended with a lysing buffer, then filtered through a QiaFilter Cartridge. The filtrate was then

loaded into the Qiagen-tip or NucleoBond cartridge, which was washed before the plasmid was eluted. Isopropanol was added to precipitate the DNA. The solution was then centrifuged at 15,000×*g*, the supernatant was removed and the DNA pellet was resuspended in TE buffer. Plasmid concentrations were determined by measuring absorbance at 260 nm. Plasmids were verified by restriction enzyme digestion and gel electrophoresis. Plasmid DNA was mixed with enzymes (BamHI and HindIII for pGL3 and phRL, HindIII for Waf-1, and BamHI and EcoRI for HAp53 for double digests), and buffer B (Boehringer Mannheim, Indianapolis, IN), and incubated at 37°C for 1 h. Loading dye was added and samples were loaded onto a 1% agarose gel. The ethidium bromide-stained gels were UV illuminated and photographed (Figure 1).

Plasmid DNA Alkylation

Purified pGL3 DNA containing the firefly luciferase gene was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) at a concentration of 1 μg/μl and incubated with SM or CEES in a SterilchemGard hood at room temperature for 1 h. DNA was precipitated with ethanol, dissolved in TE buffer, and stored at -20°C until used for transfection. Aliquots were analyzed by gel electrophoresis for the level of conversion of supercoiled DNA into the nicked circular form. The results of agarose gel electrophoresis with CEES-alkylated plasmid DNA (Figure 2) show a dose-dependent increase in the levels of conversion. The DNA damage, if unrepaired, is expected to interfere with luciferase expression in cells transfected with damaged plasmids.



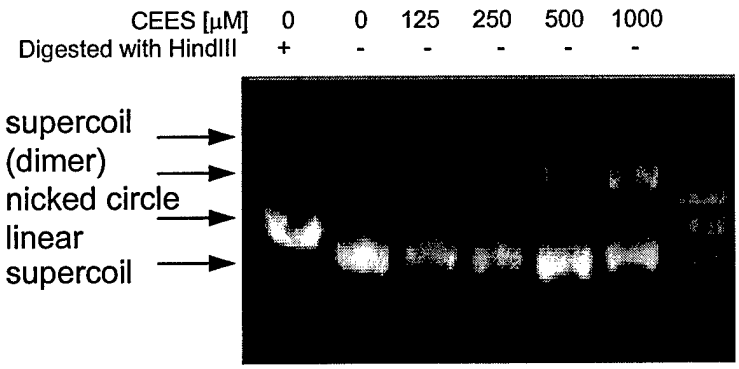


Figure 2. CEES-induced conversion of supercoiled to nicked form of plasmid pGL3 DNA.

Subcloning Glycosylase Gene into Mammalian Expression Vector

For the construction of mammalian vectors expressing human alkyladenine DNA glycosylase, wild type hAAG sequences of the glycosylase isoform 1 and 2 contained in pMV509 and pMV550 plasmids respectively, was used (Figure 3). These plasmids allow expression of hAAG genes from the strong

pTrc promoter and carry the *lacI*^{q} repressor gene to control expression. Their construction and application in our *in vitro* and bacterial studies was described earlier (14, 21).

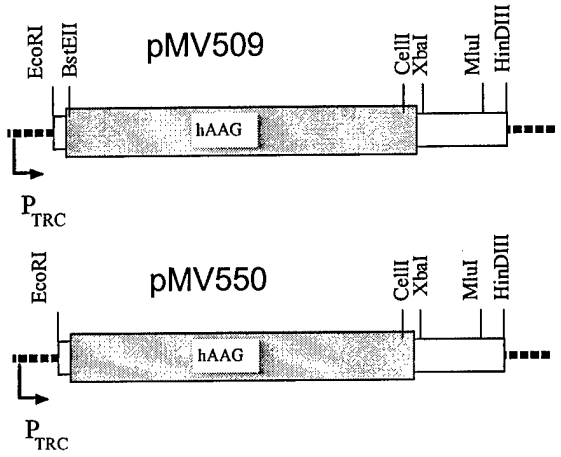


Figure 3. Insert regions of the hAAG1 expression vector pMV509 (top) and the hAAG2 expression vector pMV550 (bottom). The P_{TRC} promoter is indicated by the rightward arrow labeled P_{TRC}, the hAAG coding sequences are indicated by the gray boxes, and the ends of the pTrc99a vector sequences are indicated as heavy dotted lines.

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
pcDNA3.1/BGH reverse priming site: bases 1022-1039
BGH polyadenylation sequence: bases 1028-1252
f1 origin: bases 1298-1726
SV40 early promoter and origin: bases 1731-2074
Neomycin resistance gene (ORF): bases 2136-2930
SV40 early polyadenylation signal: bases 3104-3234
pUC origin: bases 3617-4287 (complementary strand)
Ampicillin resistance gene (*bla*): bases 4432-5428 (complementary strand)
ORF: bases 4432-5292 (complementary strand)
Ribosome binding site: bases 5300-5304 (complementary strand)
bla promoter (P3): bases 5327-5333 (complementary strand)

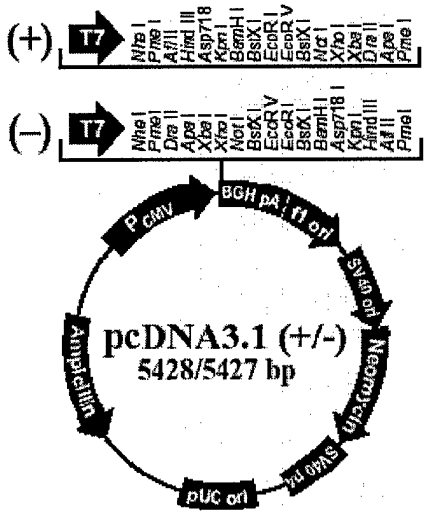


Figure 4. Characteristics of pcDNA expression vector

The pMV509 and pMV550 plasmids were cleaved with EcoRI and XbaI and the fragments carrying hAAG1 or hAAG2 were isolated from the low-melt agarose gels. The eukaryotic expression vector pcDNA3.1 (Invitrogen) (Figure 4) was cleaved in polycloning site with the same enzymes and the linear DNA was treated with the calf intestine alkaline phosphatase (CIP) to prevent vector self-ligation. The hAAG1 or hAAG2 fragment was ligated into the vector and competent *E. coli* cells were transformed with the ligation mixture and plated for selection of ampicillin resistant clones. Mini-lysates were tested by EcoRI XbaI digestion and stocks of plasmids containing isoform hAAG1 and isoform hAAG2 were prepared.

Bacterial Cell Survival and Mutation Frequency

Cells were grown in LB medium to approximately $5x10^8$ cells per ml at 37°C with aeration. Aliquots of cell suspensions were transferred in the SterilchemGARD hood and exposed to different doses of SM or CEES for 60 min. Cell dilutions were prepared in E-salts buffer containing 4% $Na_2S_2O_3$ and plated in triplicate on ESEM media for both total surviving colonies and Arg+ revertants. Plates were incubated for 48h at 37°C and surviving colonies and Arg+ mutants were counted. Survival was

expressed as a percentage of untreated control and mutation frequency was calculated as the number of Arg+ revertants per survivor.

Mammalian Cell Culture

Mouse embryonic fibroblasts (MEF), wild type (APNG+/+) and alkyladenine DNA glycosylase null mutants (APNG-/-), both spontaneously transformed, were a gift from Dr. Rhoderick H. Elder from Paterson Institute for Cancer Research, Manchester, UK (13). Cells were grown in D-MEM/F-12 medium containing L-glutamine (GibcoBRL, Gaithersburg, MD, Cat. # 11320-033) supplemented with fetal bovine serum (FBS) (10%), nonessential amino acids, nucleosides, penicillin and streptomycin. MEFs wild type for p53, p53 null mutants and p21 null mutants, gift from Stephen N. Jones, UMMS (22), were grown on Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FBS and antibiotics.

Mouse embryonic stem (ES) cells, wild type and null mutants for 3-alkyladenine DNA glycosylase, were obtained from Dr. Bevin Engelward (Massachusetts Institute of Technology) (12). SNL76/7 feeder cells were obtained from Dr. Allan Bradley (Baylor College of Medicine, Huston) (23). ES cells were cultured on gelatinize plates with mitotically inactive SNL76/7 feeder cells that express leukemia inhibitory factor (LIF) to prevent ES cells differentiation (23). Feeder cells were grown on (DMEM) supplemented with FBS (10%), glutamine and antibiotics. They were inactivated by mitomycin C (10 μg/ml for 2 h) and kept frozen until use. DMEM media for ES cells growth was supplemented with FBS (15%), glutamine, antibiotics, 2-mercaptoethanol and nonessential amino acids.

Chinese Hamster Ovary *(CHO) cells,* cell lines AA8 (wild type) and UV41 (NER group 4) were obtained from the American Type Culture Collection. These cells were grown at 37°C in monolayer culture in α-modified minimum essential medium supplemented with 10% fetal bovine serum and antibiotics.

Human fibroblasts (AG01522B) were obtained from the Aging Cell Repository, Coriell Institute for Medical Research, (Camden, NJ). Cells were grown as a monolayer in standard minimal essential media (MEM) (GibcoBRL, Gaithersburg, MD, Cat. # 11095-080) supplemented with a nonessential amino acids and vitamins, 15% fetal bovine serum, penicillin and streptomycin.

Human osteosarcoma Saos-2 cells lacking p53 and Rb were obtained from Alonzo Ross, UMMS. Cells were grown in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum at 37°C. All cells were grown in a humidified atmosphere of 5% CO_2.

Cell Proliferation assay by AlamarBlue

AlamarBlue assay for cytotoxicity is based on the reduction of REDOX indicator as a result of cellular metabolic activity from oxidized blue form to reduced red form. AlamarBlue is not toxic to living cells. The reagent is added to the cell media at the final concentration of 10% and cells are incubated for additional 4 h or longer and the absorbance at 570nm and 600nm is determined spectrophotometrically using 96 well plate reader.

Cell Cycle Analysis

Replicative DNA synthesis was determined as 5-bromo-2'-deoxyuridine (BrdU) incorporation. The DNA denaturing conditions were as described by Jones *et al.* (24). Cells were labeled in 13 μM BrdU for 2 h, harvested by trypsinization, fixed with 70% ethanol and stored at -20°C until analysis. Samples were treated with 0.1 N HCl containing 0.5% Triton X-100 for 30 min at room temperature,

boiled for 2 min and rapidly cooled to denature DNA. Cells were then washed twice with PermaCyte solution (BioErgonomics, White Bear Lake, MN), incubated with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibodies for 30 min (1:4 dilution; Caltag Laboratories, Burlingame, CA) and counterstained with propidium iodide. Cell cycle analysis was performed using a Becton-Dickinson FACScan flow cytometer (Mountain View, CA). At least 15,000 events were collected per sample; cell doublets and aggregates were electronically eliminated from analysis. The percentage of cells in each phase of the cell cycle was determined using Modfit software (Verity Software House, Topsham, ME).

Host Cell Reactivation Assay for DNA Repair

The host cell reactivation assay described here has the significant advantage that it can be used to monitor overall DNA repair in intact, growing cells. A plasmid that contains a gene for firefly luciferase was damaged by SM or CEES *in vitro* and transfected into cells that are to be evaluated for repair. Repair of plasmid DNA restores or "reactivates" luciferase expression and the level of increased expression reflects the amount of repair. In order to normalize for variations in the efficiency of transfection and lysis and for the cell number, cells were co-transfected with undamaged pRL reporter plasmid containing Renilla luciferase as an internal standard (Figure 5).

For transfection experiments, cells were plated in 24-well plates at a density of 2 x 10^4 cells/cm^2 and incubated for 24 h at 37°C. Transfection was performed using the liposome-based transfection reagent, TransFast. Optimal conditions for transfection were established at 0.5 μg of plasmid DNA per well, at a charge ratio of transfection reagent to DNA of 1:1, and a ratio of pGL3 to pRL-TK of 10:1. Twenty four hours after plating, the growth media was replaced with 0.2 ml of serum-free media containing the transfection mixture. Cells were incubated for 1.5 h at 37°C, when 1 ml of complete media was added and cells were further incubated for luciferase expression. Cells were lysed with 150 μl per well of Passive lysis buffer provided with the Dual luciferase reporter assay kit (Promega); lysates were stored frozen at -20°C until they were assayed for the luciferase levels.

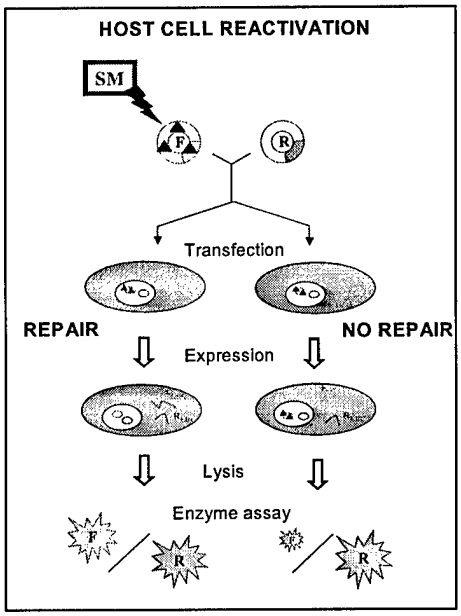


Figure 5. Host cell reactivation assay for monitoring DNA repair using firefly luciferase (F) expression plasmid damaged *in vitro* and transfected into the cells of different repair status. The level of firefly luciferase activity relative to Renilla (R) luciferase activity is a measure for DNA repair normalized for transfection efficiency.

Transient Transfection

Cells were plated at a density of 3 × 10^4/cm^2 in 24-well culture plates and grown at 37°C. Twenty-four hours after plating, cells were transfected with the required amount of plasmid DNA. Typically, 1.1 μg total DNA and 4μl transfection reagent (TransFast, Promega) per well in serum-free medium is used. Cells were incubated at either 37°C or 28°C for required period of time, lysed with 150 μl of Passive Lysis Buffer and stored frozen for the assay.

Luciferase Assay

Luciferase levels are determined using the Dual Luciferase Assay Kit (Promega). The assays for firefly and Renilla luciferase activity are performed sequentially in one reaction tube using 20 µl aliquots of cell lysates. To measure firefly luminescence, cell lysates are added to 100 µl of Luciferase Assay Reagent II and the luminescent signal is monitored by a single-sample luminometer (Monolight 2010, Analytical Luminescence Laboratory, San Diego, CA) with spectral sensitivity over the range from 360 to 620 nm. To measure *Renilla* luminescence, 100 µl of Stop & Glo reagent is added to the mixture to inhibit the firefly reaction and to provide a substrate for the *Renilla* luciferase. A second reading is obtained, and the ratio of firefly to *Renilla* luciferase is calculated.

RESULTS AND DISCUSSION

Repair Mechanisms Involved in Processing of SM-induced DNA Lesions

Nucleotide Excision Repair Protect Cells from Mustard Toxicity

Although DNA crosslinks are considered major toxic lesions in SM-alkylated DNA, DNA monoadducts can seriously interfere with DNA functions either directly or as precursors of crosslinks, apurinic (AP) sites, or strand breaks, all of which, if unrepaired, can be deleterious. Increased sensitivity to SM of NER-deficient mammalian cells in culture indicates that at least some cytotoxic DNA lesions induced by SM are successfully repaired by mammalian NER. It does not indicate, however, which specific lesions are substrates for NER. In order to examine weather NER is involved in processing of mustard-induced DNA monoadducts and the role of NER in survival independently from the effects of crosslinks, we used single-armed sulfur mustard, chloroethyl ethyl sulfide (CEES) which generates only DNA monoadducts, for survival studies with NER-competent and NER-deficient CHO cells.

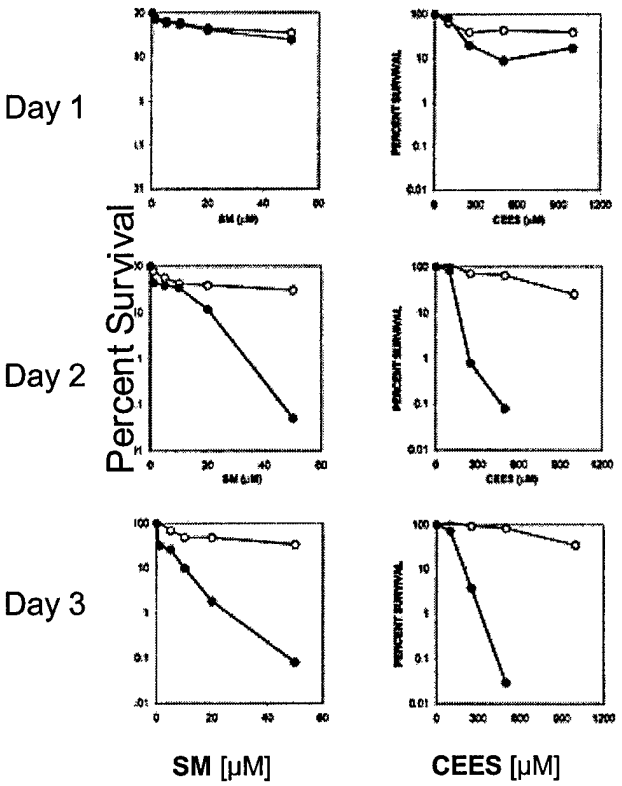


Figure 6. Survival of CHO cells after exposure to SM or CEES. Viable cell numbers were determined by trypan blue exclusion and are expressed as a percent of control on day 1, 2 and 3. Wild type cells (O); NER-deficient cells (●).

The results in Figure 6 demonstrate the importance of NER in protecting cells from cytotoxicity of both SM and CEES. Three days after exposure to 20 µM SM, more than 50% of wild type cells

survive, while NER-deficient cells show a survival of less than 2%. A similar difference is noted after exposure to CEES except that concentrations of CEES more than ten times as great as the SM concentrations are needed to produce the same level of cytotoxicity. Three days after exposure to 300 μM CEES, survival is close to 100% for wild type cells but only about 5% for NER-deficient cells. The difference in cytotoxicity between SM and CEES is assumed to be the result of crosslink formation. In addition to demonstrating that monoadducts are substrate for NER, the results also suggest that at least one of the mustard DNA monoadducts, if unrepaired, represents a lethal lesion for mammalian cells.

Role of Base Excision Repair in SM Toxicity: Studies in Bacterial Cells

Bacterial Alkyladenine DNA Glycosylase Increases Sensitivity to SM

The base excision repair pathways are present in diverse organisms from bacteria to mammalian cells. In all organisms the substrate specificity of the base excision repair pathway is determined by DNA glycosylases. In order to determine whether alkyl adenine DNA glycosylase is able to process biologically relevant alkylation products in SM-exposed cells, we used *E. coli* cells that are either wild type or repair deficient, and measured their survival as a function of SM dose.

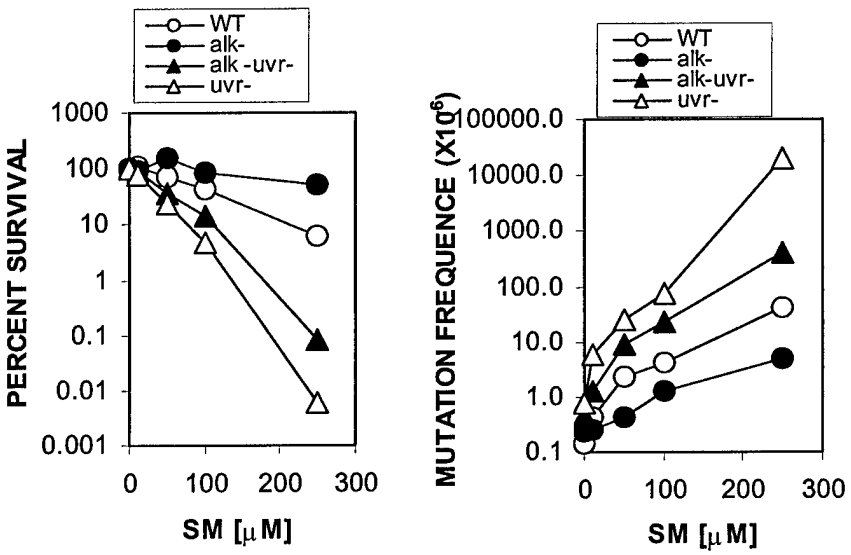


Figure 7. Role of DNA repair in survival and mutation induction in *E. coli* cells exposed to sulfur mustard. Wild type (O), AlkA glycosylase-deficient (●), NER-deficient (△) or glycosylase and NER-deficient (▲) cells were exposed to SM for 30 min and plated for total cell number and for Arg+ revertants. Survival (left panel) was calculated as a percent of untreated control and mutation frequency (right panel) was calculated as a number of revertants among survivors.

Surprisingly, as the results in Figure 7 (left panel) show, *E. coli* cells lacking functional AlkA glycosylase (Alk^-, close symbols) survive better than cells that are wild type for glycosylase (open symbols). In contrast, lack of NER ($uvrA^-$) in *E. coli* cells increases their sensitivity to SM, similar to the effects in CHO cells. This sensitizing effect of AlkA glycosylase function occurs in cells regardless of their NER status; AlkA+ cells are more sensitive to SM than $AlkA^-$ cells whether or not they have functional nucleotide excision repair.

Another potential consequence of DNA damage, induction of mutations, was tested as the reversion of Arg^- into the Arg+ phenotype in cells with different repair background. The results in Figure 7 (right panel) show that, in contrast to NER which protects cells from accumulation of mutations, alkA function increases mutation frequency in *E. coli* above that seen in the AlkA-deficient strain.

Isoforms of Human DNA Glycosylase Expressed in E. coli

In order to study the effects of human alkyl adenine DNA glycosylase (hAAG) in SM exposed cells, cloned isoforms of hAAG that can be expressed from an IPTG-inducible promoter were introduced into *alkA tagA uvrA E. coli* cells by transformation. Human alkyl adenine DNA glycosylase has been shown to exist in two isoforms and both are expressed in wild type mammalian cells. The two forms are produced by alternative splicing and differ with respect to exon 1 (25). Both isoforms can complement methyl methansulfonate (MMS) sensitivity of *E. coli* cells deficient in Alk and Tag glycosylases (14). In order to confirm that human glycosylase is expressed under the conditions of our experiment, we measured survival after the exposure to MMS in parallel with SM experiment. The results in Figure 8, left panel, show that hAAG is IPTG-inducible and that it protects *E. coli* cells from MMS toxicity. However, under the same experimental conditions, expression of hAAG increases sensitivity of *E. coli* cells to sulfur mustard (Figure 8, right panel). This effect is more pronounced in cells containing isoform 2 (hAAG2) than isoform 1 (hAAG1) glycosylase.

The results described so far indicate that neither bacterial nor human DNA glycosylase protect bacteria from SM toxicity. However, increased sensitivity of cells expressing glycosylase compared to the glycosylase-deficient cells indicate that human and bacterial glycosylases do act on SM lesions, but process them in a manner that increases toxicity. There are several possible explanations for this. First, glycosylases, especially human glycosylase that is expressed from multicopy plasmids, may be hyperactive and may process lesions too rapidly, thereby overwhelming subsequent steps in DNA repair. This possibility is ruled out by the results from MMS experiments. These studies suggest that the highly expressed human glycosylase does not overwhelm the subsequent repair steps when DNA is modified by methylation. In both SM and MMS treated cells, a glycosylase-performed repair step would result in the production of abasic sites (AP sites). The AP endonucleases, DNA polymerase and ligase, required for processing of the abasic sites appear to be capable of accomplishing the repair process on MMS-modified substrate. In fact, the rate-limiting step in base excision repair appears to be the glycosylase step, since the highly expressed hAAG provides a high level of protection against MMS damage.

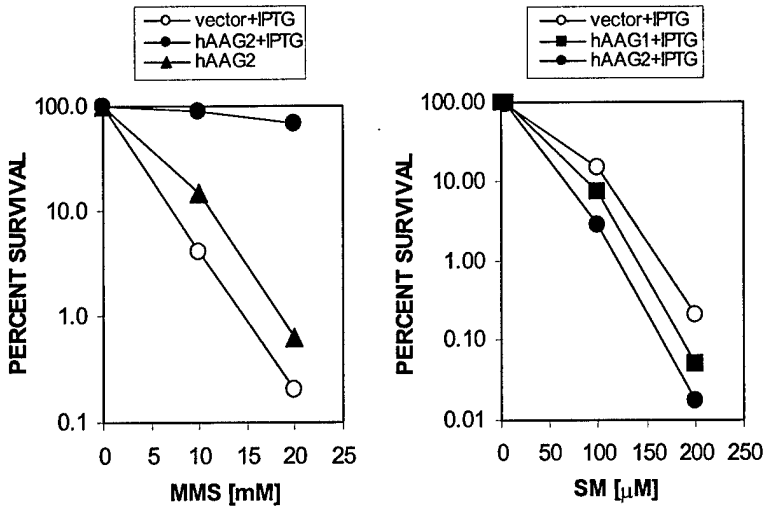


Figure 8. Effects of cloned human DNA glycosylase on survival of *E. coli* cells exposed to MMS or SM. Cloned human alkyl adenine DNA glycosylase hAAG1 (■), hAAG2 (●) or cloning vector only (O) were introduced into the *E. coli* cells deficient for bacterial glycosylase and NER (uvrA$^-$ alkA$^-$ tagA$^-$). Left panel: hAAG provides full protection against MMS toxicity in IPTG-induced cells (●) and slightly increases resistance in un-induced cells (▲). Right panel: both hAAG1 and hAAG2 isoforms increase sensitivity of E. coli cells to SM.

Second, ineffective repair by glycosylase may interfere with the repair by other, more effective, repair processes such as NER. If sensitization derives from the interference with NER, then we should not see sensitization by glycosylase expression in NER-defective cells. The results in Figures 7 rule out such possibility, at least for the bacterial glycosylase, since both NER+ and NER$^-$ cells manifest increased sensitivity to SM treatment by glycosylase expression.

Third, glycosylase may convert SM lesions to repair intermediates that are more toxic than the primary DNA lesion. The unrepaired AP sites represent highly toxic and mutagenic lesions, however, they are also formed by DNA glycosylase during the repair of MMS-modified bases suggesting that the sensitizing step and the lesion responsible for sensitization are unique to SM-modified DNA. It is possible that glycosylase initiates repair and either is unable to dissociate, or AP site processing can not take place, due to the effect of neighboring DNA crosslinks. Alternatively, glycosylase may "unhook" a certain fraction of SM-induced crosslinks generating more toxic intermediates. It is also possible that the mammalian glycosylase can repair SM lesions, but requires additional factors, not present in bacterial cells, for processing.

Mustard Monoadducts Contribute to Sensitizing Effect of DNA Glycosylase

Different DNA lesions and mechanisms could account for sensitization. Although DNA monoadducts are natural substrates for glycosylase, it is possible that DNA crosslinks are responsible for sensitization. For example, glycosylase may "unhook" a certain fraction of SM-induced crosslinks, generating more toxic intermediates, or alternatively, DNA crosslinks may have an inhibitory effect on the repair of the neighboring monoadducts by glycosylase. In order to address the question of whether monoadducts contribute to the increased lethal and mutagenic effects in cells with functional DNA glycosylase, we used monofunctional sulfur mustard, chloroethyl ethyl sulfide (CEES), which generates DNA monoadducts similar to those formed by SM, but does not form DNA crosslinks. CEES effects on bacterial cells with or without endogenous DNA glycosylase are compared in Figure 9. The CEES doses tested (from 0.1 to 2.0 mM) were highly mutagenic, yet did not cause cytotoxicity. The mutation frequency was higher in cells deficient in nucleotide excision repair, indicating a protective role of this repair against mutagenic monoadducts. In contrast, the presence of functional DNA glycosylase, similar to results with SM, significantly increased mutation frequency after exposure to CEES. This sensitizing effect of glycosylase is not dependent on NER; DNA glycosylase increased mutagenesis in both NER-wild type and NER-deficient cells. The results strongly suggest that the sensitizing effect of DNA glycosylase on SM-exposed cells is largely due to its processing of monoadduct(s) in DNA.

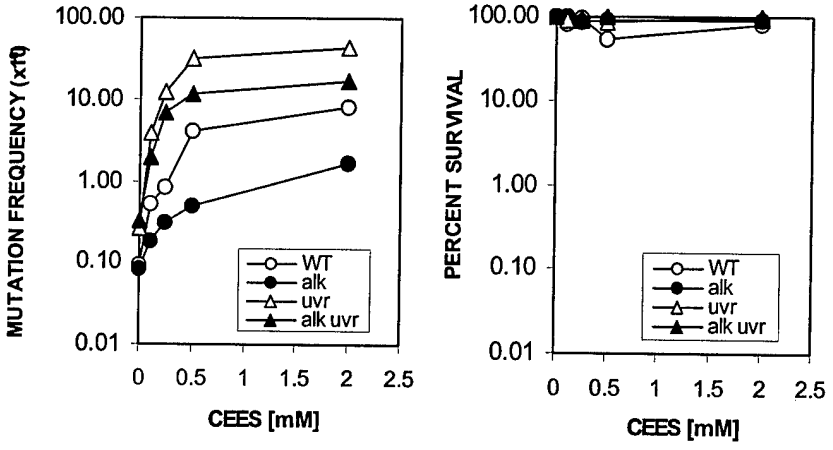


Figure 9. Effect of DNA repair on survival and mutation induction in repair deficient *E. coli* cells exposed to 2-chloroethyl ethyl sulfide (CEES). Wild type (○), AlkA glycosylase-deficient (●), NER-deficient (△) or glycosylase and NER-deficient (▲) cells were exposed to CEES for 60 min and plated for total cell number and for Arg+ revertants. Mutation frequency (left panel) was calculated as the number of revertants among survivors and survival (right panel) was calculated as a percentage of untreated control.

The CEES doses used in these experiments did not interfere with bacterial survival, which may be affected only at higher levels of monoadduct formation in DNA or limited to DNA crosslinks, in which case it would be unique to bifunctional mustards. Effects of higher CEES doses on bacterial cells with or without endogenous DNA glycosylase are compared in Figure 10. The presence of a functional DNA glycosylase significantly increases both killing (Figure 10, left panel) and mutation frequency (Figure 10, right panel) in *E. coli* cells exposed to CEES. This sensitizing effect of glycosylase is not dependent on NER; DNA glycosylase increases mutagenesis and decreases survival in both NER-wild type and NER-deficient cells. The results strongly suggest that the sensitizing effect of DNA glycosylase on both survival and mutagenesis in SM-exposed cells is, at least in part, due to the presence of mustard monoadducts in DNA.

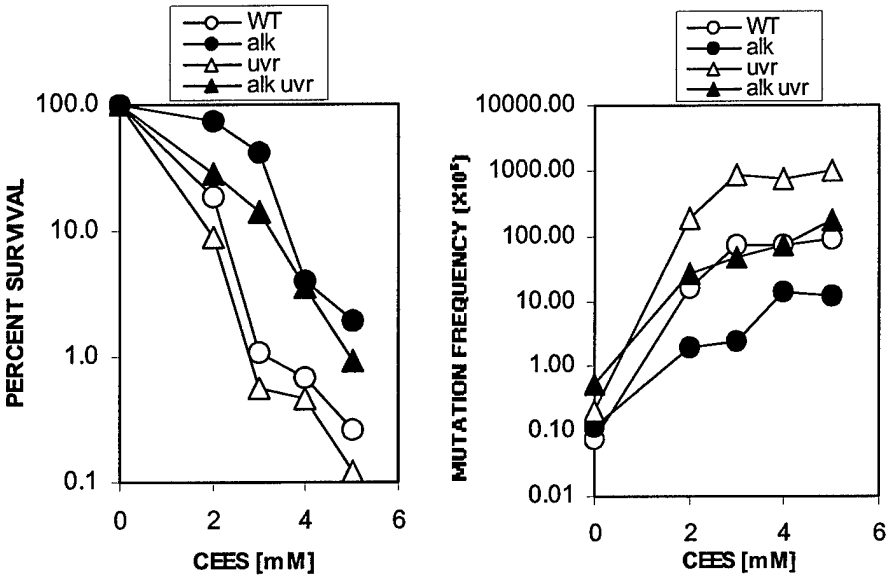


Figure 10. Effect of DNA repair on survival and mutation induction in *E. coli* cells exposed to 2-chloroethyl ethyl sulfide (CEES). Wild type (○), AlkA glycosylase-deficient (●), NER-deficient (△) or glycosylase and NER-deficient (▲) cells were exposed to CEES for 60 min and plated for total cell number and for Arg+ revertants. Survival (left panel) was calculated as a percentage of untreated control and mutation frequency (right panel) was calculated as the number of revertants among survivors.

Role of Base Excision Repair in SM toxicity: Studies in Mammal Cells

Mammalian Glycosylase Sensitizes Cells to SM Toxicity

In order to examine whether the sensitization to SM by DNA glycosylase also occurs in mammalian cells we performed survival studies with wild type and 3-alkyl adenine DNA glycosylase (AAG) null mutants of mouse embryonic stem (ES) cells. The results in Figure 11 (left panel) demonstrate protective effect of glycosylase against the methylating agent MMS toxicity. However, when cells are exposed to SM (right panel), the presence of glycosylase function in wild type cells (WT, open symbols) has a similar effect as it has in bacterial cells, it increases sensitivity to SM; lack of AAG in null mutant cells (AAG-/-, close symbols) make cells more resistant to SM.

Therefore, the results with ES cells indicate that the sensitizing effect of alkyl adenine DNA glycosylase on SM toxicity is not unique for bacterial cells, but also occurs at least in some undifferentiated mammalian cells. It also suggests that it may be possible to manipulate, diminish or inhibit, glycosylase activity in order to both enhance survival and reduce mutagenic effect after SM exposure.

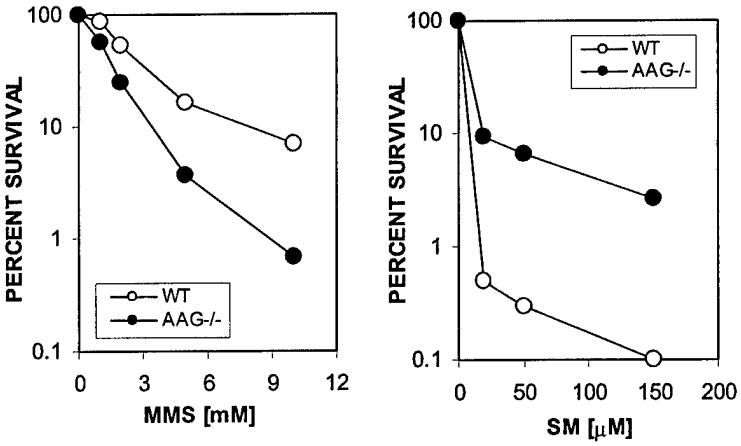


Figure 11. Effect of DNA glycosylase on survival of mouse embryonic stem cells exposed to MMS or SM. Mouse embryonic stem cells, wild type (WT, O) or DNA glycosylase null mutants (AAG-/-, ●) were exposed to MMS (left panel) or to SM (right panel) for 1h and incubated at 37°C for 2 days. Cell viability was determined by trypan blue exclusion assay. While presence of glycosylase protects cells from toxic effects of MMS, it increases their sensitivity to SM.

Mammalian Glycosylase Sensitizes Cells to Mustard Monoadducts

In order to examine whether the glycosylase activity effects survival of mammalian cells exposed to CEES we performed survival studies with wild type (APNG+/+) and DNA glycosylase deficient (APNG-/-) mouse embryonic fibroblasts. Cells were exposed to increasing doses of CEES for one hour and incubated at 37°C. Survival was determined by trypan blue exclusion assay as a function of time and CEES dose. Figure 12 shows the survival as a function of time of APNG + or APNG - cells exposed to different doses of CEES. The results clearly show the disadvantage of the presence of DNA glycosylase for the survival and recovery after the exposure to CEES. While cells deficient in glycosylase (APNG -/-) show the dose-dependent recovery from all CEES doses except the highest one (1500 μM), even much lower doses (1000, 750, and 500 μM) are cytotoxic for cells containing functional glycosylase.

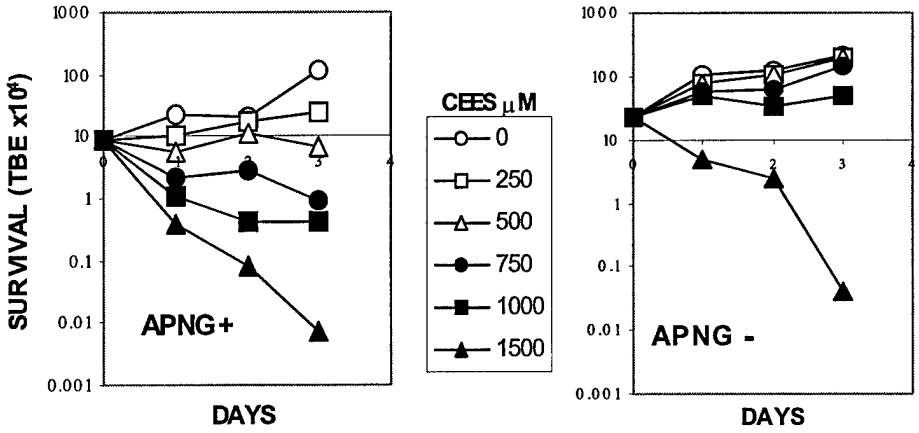


Figure 12. Effect of DNA glycosylase on survival after exposure to CEES. Wild type (APNG +) and glycosylase-deficient (APNG -) mouse embryo fibroblasts were exposed to increasing doses of CEES for 1 hour and incubated at 37°C. Cell survival as determined by trypan blue exclusion assay was plotted as a function of time post exposure

In Figure 13 survival of glycosylase-deficient cells (close circles) three days after exposure to CEES is directly compared to the survival of cells with functional glycosylase (open circles). The results clearly demonstrate tenfold increased resistance to CEES of cells lacking 3-alkyladenine DNA glycosylase.

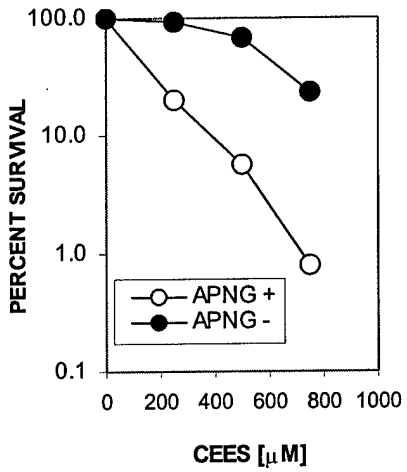


Figure 13. Sensitizing effect of DNA glycosylase on CEES toxicity in mammalian cells. Wild type (APNG +) and DNA glycosylase-deficient (APNG-) mouse embryo fibroblasts were exposed to increasing doses of CEES for one hour and incubated at 37°C for three days. Survival determined by trypan blue exclusion assay was expressed as a percent of untreated control and plotted as a function of CEES dose.

DNA Glycosylase Interferes with Repair of SM- and CEES-damaged DNA

In order to determine the effects of mammalian DNA glycosylase on the repair of mustard-induced DNA damage we applied the host cell reactivation approach that we already successfully used in our studies on nucleotide excision repair. This approach is based on the comparison of the levels of damaged DNA reactivation among the cells of different DNA repair status (See: Material and Methods).

Repair of DNA damage restores or "reactivates" expression from the reporter gene and the difference in the levels of expression reflects the difference in DNA repair capacity between the two cell lines. Figure 14 shows the results of experiments where undamaged or SM-damaged firefly luciferase reporter plasmid was transfected into the wild type (APNG+) or glycosylase deficient (APNG-) mouse embryonic fibroblasts. Cells were incubated at 37°C for 30 h after transfection, lysed and assayed for the levels of luciferase expression. The results show that in cells with functional DNA glycosylase (APNG+), reactivation of plasmid DNA exposed to higher SM doses is significantly lower than in cells deficient in DNA glycosylase (APNG-) suggesting that glycosylase interferes with restoration of the functional integrity of SM-damaged DNA. This is in agreement with the survival data demonstrating a sensitizing effect of DNA glycosylase for SM-exposed cells.

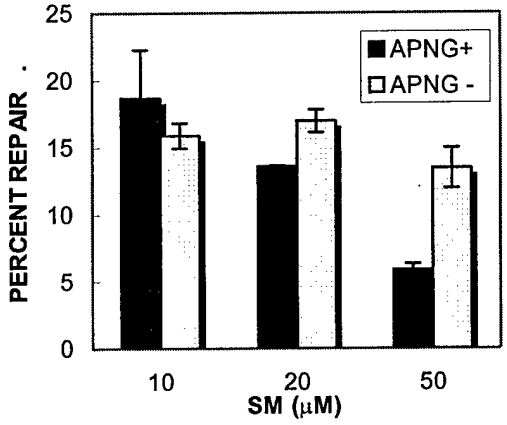


Figure 14. Effect of DNA glycosylase on luciferase expression from SM-damaged reporter plasmids. Wild type cells (■) and APNG-deficient cells (□) were cotransfected with either the SM-damaged or undamaged firefly luciferase reporter plasmids and with undamaged Renilla luciferase reporter plasmids. Cells were incubated at 37°C and lysed. Firefly luciferase activity was normalized to correct for variations in transfection efficiency using Renilla luciferase as an internal standard. Firefly luciferase activity in cells transfected with damaged plasmid, expressed as a percent of activity in cells with undamaged plasmid, represents the extent of DNA repair for the given cell line.

The results with the monofunctional sulfur mustard, chloroethyl ethyl sulfide (CEES) have shown that the presence of a functional DNA glycosylase significantly increases sensitivity of both *E. coli* and mammalian cells to CEES. Those results indicate that mustard monoadducts are involved in the sensitization by glycosylase and that this effect is independent of the presence of crosslinks in DNA. The results of luciferase assay in Figure 15 show that, compared to the SM-damaged DNA, interference with repair is even more pronounced when CEES-damaged DNA is introduced into the APNG + cells. While luciferase expression decreases with the increased levels of plasmid alkylation in both cell lines, plasmid reactivation is more than four-fold higher in the absence of DNA glycosylase (APNG - cells) regardless of the level of alkylation. For example, while APNG- cells reactivate plasmid exposed to 125 μM CEES to the level of undamaged control, reactivation of the same plasmid in APNG+ cells is less than 25% of control. These results suggest that glycosylase activity actually prevents the repair of mustard monoadducts either by generating repair intermediates that effect DNA transactions such as transcription more than the original lesion or by interfering with other relevant repair pathway(s) such as the nucleotide excision repair.

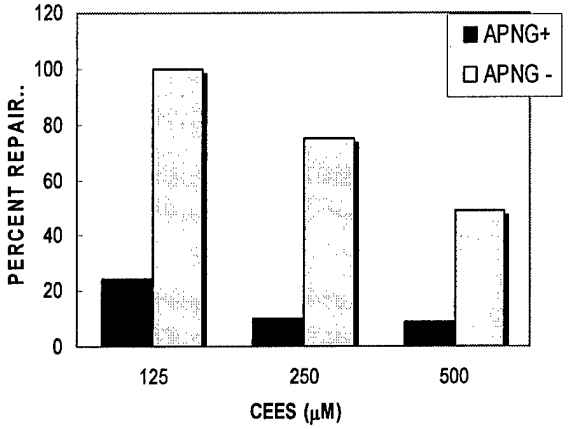


Figure 15. Effect of DNA glycosylase on luciferase expression from CEES- damaged reporter plasmids. Wild type cells (APNG+, ■) and glycosylase-deficient cells (APNG-, □) were cotransfected with either the CEES-damaged or undamaged firefly luciferase reporter plasmids. Cells were incubated at 37°C for 72h and lysed. Firefly luciferase activity was normalized to correct for variations in transfection efficiency using Renilla luciferase as an internal standard. Firefly luciferase activity in cells transfected with damaged plasmid, expressed as a percent of activity in cells with undamaged plasmid, represents the extent of DNA repair for the given cell line.

Subcloning of Human DNA Glycosylase into the Mammalian Expression Vector

In order to determine the effect of DNA glycosylase on sulfur mustard toxicity in an isogenic background and to generate constructs for the future studies on the mechanisms of sensitization by glycosylase, we have subcloned human DNA glycosylase into the mammalian expression vector.

The human alkyladenine DNA glycosylase *(hAAG)* gene exists in two known isoforms that result from alternative splicing, incorporating either the 13 amino acid exon 1a (hAAG1), or the 8 amino acid exon 1b (hAAG2) (26, 27). Both isoforms of hAAG appear to be expressed in all tissues examined (27, 25). DNA glycosylase isoforms *hAAG1* and *hAAG2* (26, 21) were subcloned into the eukaryotic expression vector pcDNA3.1, plasmids were propagated in *E. coli* and plasmid DNA was purified as described in Material and Methods. Glycosylase deficient mouse embryo fibroblasts were transfected with either pcDNA3.1 vector, vector containing glycosylase isoform 1 (*hAAG1*) or isoform 2 (*hAAG2*) at two different levels of plasmid DNA. Cells were exposed to sulfur mustard and survival was determined by the alamarBlue assay. The results in Figure 16 show the percent survival of transfected cells exposed to 250 μM sulfur mustard compared to untreated control transfected under the same conditions.

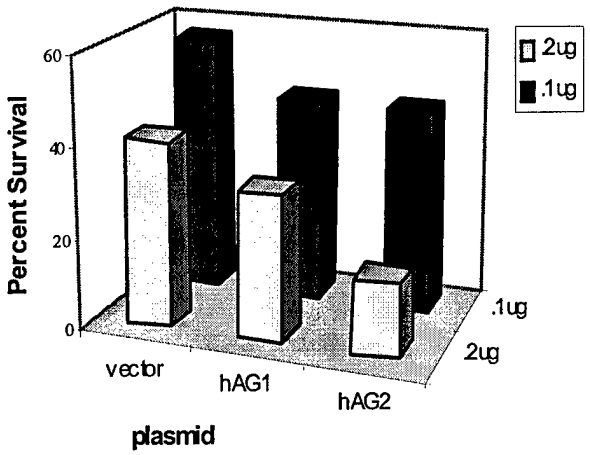


Figure 16. Effect of DNA glycosylase on survival after exposure to SM. Mouse embryo fibroblasts null mutant for DNA glycosylase were plated in 48 well plates at the density 2.5 x 10^4 cells/cm^2, incubated for 6 h at 37°C and transiently transfected with 0.1 (dark bars) or 0.2 (light bars) μg per well of vector, vector containing gene for glycosylase isoform hAAG1 (hAG1) or isoform hAAG2 (hAG2). 18 h after transfection cells were exposed to SM for 1 h. Survival was determined by the AlamarBlue assay 40 h after the exposure.

The presence of glycosylase sensitizes cells to SM and the sensitizing effect is more pronounced in the population of cells transfected with the higher amount of plasmid DNA (0.2 compared to 0.1 μg per well). Also, the hAAG2 glycosylase isoform appears to have a stronger effect, at least at 0.2 μg DNA per well, than the hAAG1 isoform.

The Role of Hypothermia in Protection against SM Toxicity

Hypothermia Arrests Cell Cycle Progression of Mammalian Cells in Culture

In contrast to the heat shock, very little is known at the molecular level about the response of mammalian cells to cold. We have demonstrated (9) that hypothermia represents a p53-inducing stress condition and that lowering temperature to 28°C causes reversible growth arrest in population of normal human fibroblasts. FACS analysis of DNA content per cell demonstrated that this growth arrest is due to the cell cycle arrest that occurs at 28°C (9).

The 5-bromo-2'-deoxiuridine (BrdU) incorporation assay was used to compare the extent of DNA synthesis in undamaged normal human fibroblasts at 37°C and 28°C. The results in Figure 17 show that the entrance into the S phase is delayed for at least two days when cells are incubated at 28°C. The effect of low temperature on cell cycle in damaged cells is likely to be dependent on a number of factors such as the cell cycle stage at the time of treatment, effects of treatment itself on cell cycle progression, and others. Providing low temperature does not compromise protective pathways or intensify toxic ones, the delay in progression is likely to be beneficial for survival by allowing more time for the repair of damaged DNA before the onset of DNA synthesis and mitosis.

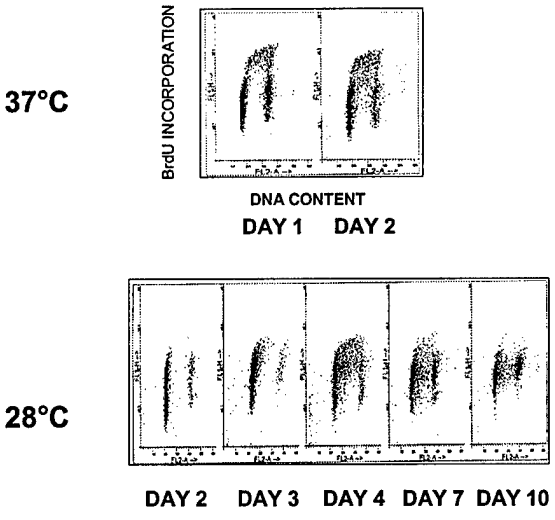


Figure 17. Effect of hypothermia on DNA synthesis in normal human fibroblasts. Cells were incubated at 37°C or 28°C and labeled with BrdU for 2 h. After staining with anti-BrdU-FITC and propidium iodide, cells were analyzed by flow cytometry. . PI fluorescence (x-axis) visualizes DNA content per cell and FITC fluorescence (y-axis) the BrdU incorporation. The BrdU incorporation is delayed in cells at 28°C for at least two days.

Hypothermia Protects Mammalian Cells from SM Toxicity

We have shown that nucleotide excision repair (NER) protects cells from SM toxicity. In order to examine whether low temperature compromises protective effect of NER, wild type and NER-deficient CHO cells were exposed to SM and incubated for two days at either 37°C or 28°C (Figure 18). The results show that both cell lines survive much better when incubated at low temperature after SM exposure and that NER provides additional protection at 28C. It indicates that low temperature protects mammalian cells from SM toxicity and does not compromise NER repair activity in SM-exposed cells.

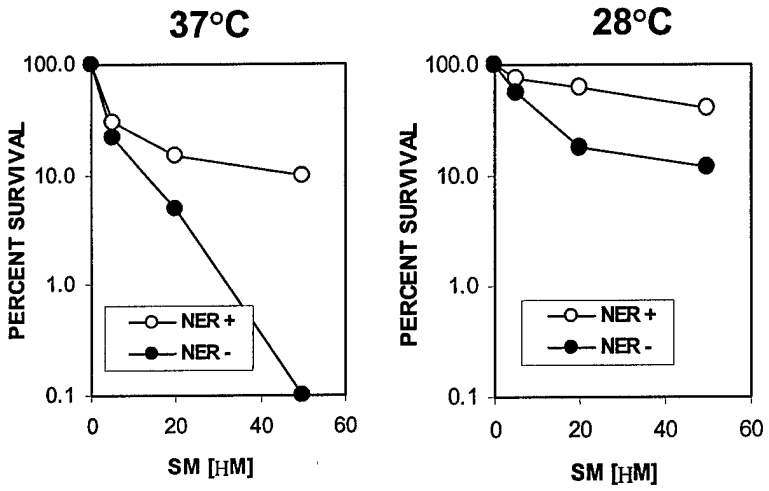


Figure 18. Hypothermia increases survival and does not compromise NER activity in SM-exposed cells. CHO cells wild type (O) or NER-deficient (●) were exposed to SM and incubated at either 37°C (left panel) or 28°C (right panel) for two days when survival was determined by trypan blue assay.

Hypothermia Counteracts Sensitizing Effect of DNA Glycosylase

In order to investigate the effect of hypothermia on DNA glycosylase activity, mouse embryonic stem cells, wild type or DNA glycosylase null mutants were exposed to SM and then incubated at either 37°C or 28°C. The results in Figure 19 confirm sensitizing effect of glycosylase at 37°C and clearly demonstrate a protective effect of post-exposure incubation at 28°C on cells with functional DNA glycosylase (AAG +). Increased survival may be the result of either increased (or prolonged) activity of protective pathway(s) or weakened activity of toxic pathway(s). Compared to the wild type, glycosylase-deficient cells (AAG-) survive better at both temperatures and the beneficial effect of low temperature is much less pronounced than in the wild type cells. The results strongly suggest that at least one component of protection from toxicity of SM by hypothermia is due to the modulation of repair pathway initiated by DNA glycosylase.

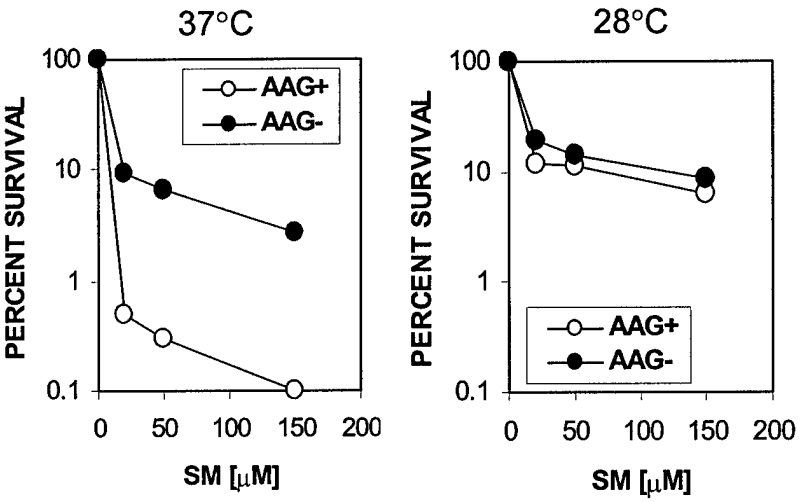


Figure 19. Hypothermia protects mammalian cells from SM toxicity by diminishing sensitizing effect of DNA glycosylase activity. Mouse embryonic stem cells, wild type (O) or DNA glycosylase null mutants (●) were exposed to SM for 1h and then incubated at either 37°C (left panel) or 28°C (right panel) for two days. Cell viability was determined by trypan blue exclusion assay.

Mechanisms of Cellular Response to Hypothermia

Role of p21^{WAF-1} in Cell Cycle Arrest by Hypothermia

Hypothermia ameliorates toxic effects of SM. We believe that at least two mechanisms are involved; one is related to the weakening of the sensitizing effect of DNA glycosylase and the other is based on the cell cycle arrest at low temperature (7). Hypothermia arrests cell cycle progression in a p53-dependent manner (9, 28) and therefore we expect that p53 has the key role in cell cycle-related protective effects of hypothermia.

In DNA damage-induced cell cycle arrest, p53 transcriptionally activates expression of the cyclin-dependent kinase inhibitor, p21 protein. This protein inhibits the transition from G1 to S phase of the cell cycle by preventing phosphorylation of Rb protein. In order to determine whether or not p21 is involved in the cell cycle arrest that occurs in mouse and human cells at low temperature (9), we examined cell cycle progression as BrdU incorporation in p21 null mutant mouse embryonic fibroblasts incubated at 28°C. The results in figure 20 show that in contrast to wild type mouse embryo fibroblasts that arrest at 28°C, p21 null mutants, continue to incorporate BrdU and are similar to p53 null mutants in this regard. This suggests that in addition to p53, p21 protein is required for cell cycle arrest by hypothermia.

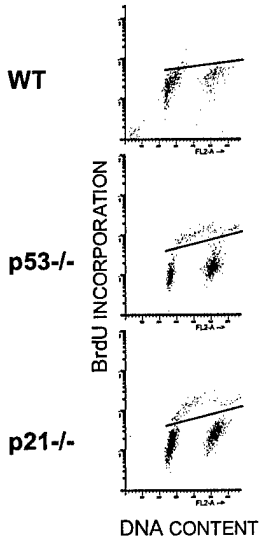


Figure 20. Effect of p53 and p21 status on cell cycle progression at low temperature. Mouse embryo fibroblasts were incubated at 28°C for 45h, labeled with BrdU for 2h and stained with anti-BrdU-FITC and propidium iodide for FACS analysis. Cell cycle arrest occurs in cells with wild type p53 but not in p53- and p21-deficient cells. PI fluorescence (x-axis) visualizes DNA content per cell and FITC fluorescence (y-axis, above the line) the BrdU incorporation. In contrast to the p53- and p21-deficient cells, there are only two subpopulations in wild type cells: G1 (events on the left) and G2 (events on the right, double fluorescence intensity indicating twice as much DNA); there are no S-phase cells (between G1 and G2) that actively replicate DNA and incorporate BrdU.

Expression of p21 at Low Temperature

We have shown that hypothermia increases levels of p21 in normal human fibroblasts (9) and that cell cycle arrest at low temperature requires p21 (Figure 20). We then addressed the questions: is p21 expression activated at 28°C and, if it is activated, does p53 regulate its expression? Human Saos-2 cells deficient in p53 were transiently transfected with p53 expressing plasmid and co-transfected with p53-responsive p21^{WAF-1}- *luc* reporter plasmid containing the p21^{WAF-1} promoter upstream of the gene for firefly luciferase. This plasmid allows for the determination of p21 expression by measuring the levels of luciferase in cell lysates. Figure 21 shows p21 expression in cells incubated at either 37°C or 28°C in the presence or absence of p53. Luciferase levels were determined 72h after transfection. The results show that low temperature indeed induces expression from the p21 promoter, and that this expression is taking place in cells both with and without p53, although the levels of expression are higher in the presence of p53.

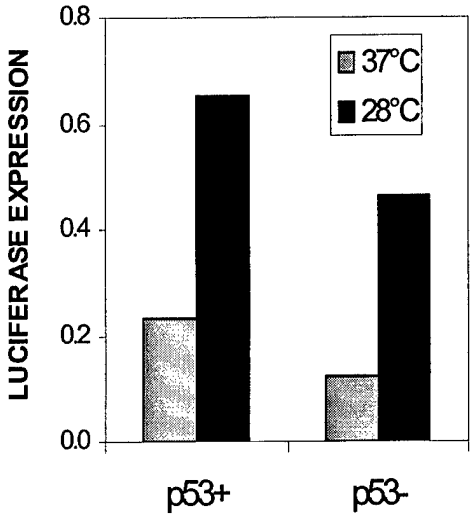


Figure 21. Effect of temperature on expression from p21^{WAF-1} promoter in the presence or absence of p53. Human Saos-2 cells deficient in p53 or stably transfected with p53 were transiently transfected with p21^{WAF-1}-luc reporter plasmid. p21^{WAF-1} expression was measured as firefly luciferase levels 72 h after transfection.

These results demonstrate that hypothermia activates expression of p21^{WAF-1} and suggest that p53 may have a role other than p21 activation in cell cycle arrest at low temperature. p53-independent p21 expression was described for some tumor cell lines following serum stimulation (29) and for normal tissues as p53-independent compartmentalization (30) and TGF-β-regulated growth control (31). Hypothermia-induced regulatory pathways of p21 expression require further investigation.

Cell Survival under the Conditions of Mild Hypothermia

Hypothermia arrests cell cycle progression in a p53-dependent manner (9) and this cell cycle arrest may contribute to the protective effect of hypothermia against SM toxicity. We addressed the questions, 1) how do the undamaged mammalian cells tolerate conditions of prolonged hypothermia and 2) whether or not cellular p53 status has a role in survival under such conditions.

To test this, p53 wild type and p53 null mutant mouse embryo fibroblasts were incubated at 37°C and 28°C, and viability was determined by TBE assay. As shown in Figure 22, p53 wild type cells maintain a constant viable cell number with very few dead cells in the population for the duration of the experiment (two weeks). In contrast, survival of p53 deficient cells is compromised by prolonged periods of hypothermia. Although total cell numbers increase during the first several days at 28°C, the number of dead cells in p53-/- population increases over time and, at later time points, exceeds that of viable cells in the population.

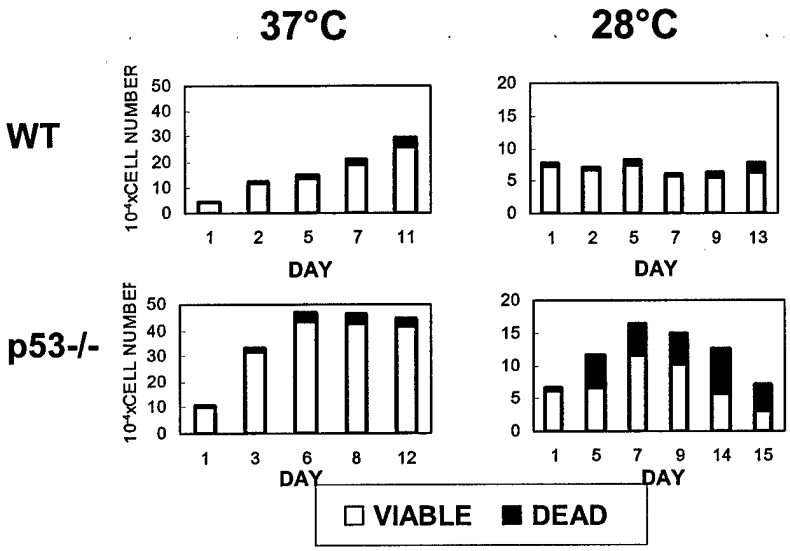


Figure 22. Loss of p53 function increases the level of cell death under hypothermic conditions. Mouse embryo fibroblasts, wild type or p53-null mutants, were incubated at 37°C or 28°C and viability was determined by the trypan blue exclusion assay. Dead cells fraction of total number is shown as closed bars; viable cells fraction as open bars.

The results demonstrate that healthy p53 wild type cells tolerate prolonged periods of low temperature better than p53-deficient cells. The increased tolerance to prolonged hypothermia correlates with the ability to arrest/delay cell cycle progression and could be due to the p53-dependent activation of the G2/M checkpoint at 28°C. Low temperatures compromises tubulin binding to centrosomes (32) that may trigger the p53-dependent G2/M cell cycle arrest. Further studies are needed in order to confirm a p53-dependent nature of hypothermia-induced G2/M arrest and the protective effect of this arrest on cell survival.

KEY RESEARCH ACCOMPLISHMENTS

We have found that:

- Nucleotide excision repair (NER) protects both mammalian and bacterial cells against toxic effects and, at least bacterial cells, against the mutagenic effects of sulfur mustard (SM).
- Cytotoxic mustard monoadducts are substrate for NER.
- In contrast to the protective effect of NER, bacterial alkyladenine DNA glycosylase, the first enzyme on the base excision repair pathway, increases sensitivity to toxic and mutagenic effects of SM.
- Sensitizing effect of DNA glycosylase occurs, at least in bacterial cells, regardless of their NER status.
- While cloned isoforms of human DNA glycosylase complement the sensitivity of glycosylase-deficient *E. coli* cells to methylating agent MMS, they sensitize cells to SM toxic effects.
- Sensitizing effect of DNA glycosylase is not limited to bacterial cells; mouse embryonic stem cells and fibroblasts with functional DNA glycosylase are more sensitive to SM than the isogenic cells lacking glycosylase activity.
- Presence of mustard monoadducts in DNA is responsible for the sensitizing effect of DNA glycosylase.
- DNA glycosylase interferes with the repair of SM and CEES damaged DNA.
- Hypothermia (28°C) significantly delays the initiation of replicative DNA synthesis in normal human fibroblasts.
- Hypothermia protects mammalian cells from SM toxicity.
- Hypothermia does not compromise nucleotide excision repair function in SM-exposed cells.
- Hypothermia counteracts sensitizing effect of DNA glycosylase in SM-exposed cells
- Cell cycle arrest at low temperature requires the presence of both tumor suppressor p53 and kinase inhibitor $p21^{WAF-1}$ in cell.

- Expression of $p21^{WAF-1}$ is induced under the conditions of hypothermia.
- Hypothermia activates expression of $p21^{WAF-1}$ in p53-independent manner.
- p53 deficient cells have reduced tolerance to prolonged periods of hypothermia.

REPORTABLE OUTCOMES

Publications and Presentations

Matijasevic, Z., J. E. Snyder, and D.B.Ludlum. 2000. Hypothermia as a model for selective protection of normal cells from the toxicity of antitumor agents. Proc. Am. Assoc. Cancer Res. 41, 250-251.

Matijasevic, Z., M. Precopio, J. E. Snyder, and D. B. Ludlum. Repair of sulfur mustard-induced DNA damage in mammalian cells measured by a host-cell reactivation assay. US Army Medical Defense Bioscience Review, 4 June – 9 June, 2000.

Matijasevic, Z., Powers, C.M., and. Volkert, M.R. Alkylpurine DNA glycosylase sensitizes cells to sulfur mustard toxicity. Gordon Research Conference, New London, NH, August 12-17, 2001.

Matijasevic, Z., Precopio, M., Snyder, J. E. and. Ludlum, D. B. 2001. Repair of sulfur mustard-induced DNA damage in mammalian cells measured by a host-cell reactivation assay. Carcinogenesis, *22*: 661-664.

Powers, C.M., Santandrea, A.M., Volkert, M. R., and Matijasevic, Z. Alkyladenine DNA glycosylase sensitizes cells to sulfur mustard toxicity. Proceedings of the US Army Medical Defense Bioscience Review 2002, 749-757.

Bonnano, K.C., Wyrzykowski, J., Chong, W., Matijasevic, Z. and Volkert, M.R. Alkylation resistance of *E. coli* cells expressing different isoforms of human alkyladenine DNA glycosylase (hAAG). DNA Repair *1:* 507-516, 2002.

Matijasevic, Z. Selective protection of non-cancer cells by hypothermia. Anticancer Res. *22:* 3267-3272, 2002.

Li, Q., Wright, S. E., Matijasevic, Z., Chong, W., Ludlum, D. B., and Volkert, M. R The role of human alkyladenine glycosylase in cellular resistance to the chloroethylnitrosoureas. Carcinogenesis *24:* 589-593, 2003.

Personnel Receiving Contract Support

Zdenka Matijasevic, Ph.D.	Principal Investigator
Michael Volkert, Ph.D.	Co-investigator
Christine Powers	Research Associate

Alida Santandrea	Research Assistant
Anita Fenton	Research Associate
Karen Sargent	Research Assistant

CONCLUSIONS

By investigating natural pathways involved in cellular responses to SM-induced DNA damage, we were successful in identifying two levels of response where the proper intervention may modulate the outcome of damage and minimize toxicity. The first level directly involves the activity of DNA repair enzymes, while the second level involves the regulation of cell cycle progression.

Two DNA repair pathways involved in processing of SM-damaged DNA have opposite effects on survival and mutagenesis of exposed cells. While the nucleotide excision repair (NER) protects cells, base excision repair, due to the activity of DNA glycosylase, sensitizes cell to SM. These findings clearly set the directions for possible intervention: conditions that, either separately or in concert, enhance the effects of NER and attenuate sensitizing effect of DNA glycosylase, should provide protection against SM toxicity.

We have determined one type of DNA lesions, mustard monoadducts, that are involved in glycosylase-mediated sensitization and identified one set of post-exposure conditions, low temperature, that counteracts sensitizing effects of glycosylase and increases survival. Future studies that would shed more light into both, mechanisms of sensitization by glycosylase and role of NER in repair of SM-induced crosslinks, would allow us to explore other avenues of intervention that directly involve processing of DNA damage.

Intervention at the level of cell cycle regulation is exemplified by our results with hypothermia suggesting that the arrest or delay in cell cycle progression has beneficial effects on survival after SM exposure. Mechanisms of protection by low temperature may be based on increased time available for the repair of lesions before the initiation of critical cellular events such as DNA replication and mitosis. However, cell responses to hypothermia appear to be very complex and other mechanisms of protection may be involved.

We were the first to identify two proteins, p53 and p21, whose levels are increased by hypothermia and both proteins are required for the cell cycle arrest at low temperature. Our finding of p53-independent expression of p21 at low temperature raises an intriguing question of the p53 role in cell cycle arrest at low temperature. It is likely that p53 has an additional role in response to combined stress, either in apoptotic or DNA repair branches of its functional network. Better understanding of cellular response to low temperature would help us to determine specific processes as a potential target(s) for intervention.

REFERENCES

1. Smith, M.L., and Fornace, A.J. p53-mediated protective responses to UV irradiation. Proc. Natl. Acad. Sci. USA *94*: 12255-12257, 1997.

2. Agarwal, M.L., Taylor, W.R., Chernov, M.V., Chernova, O.B., and Stark, G.R. The p53 network. J. Biol. Chem. *273*: 1-4, 1998.

3. Engelward, B.P., Allan, J.M., Dreslin, A.J., Kelly, J.D., Wu, M.M., Gold, B., and Samson, L.D. A chemical and genetic approach together define the biological consequences of 3-methyladenine lesions in the mammalian genome. J. Biol. Chem. *273*: 5412-5418, 1998.

4. Rich, T., Allen, R.L., Wyllie, A.H. Defying death after DNA damage. Nature. *12*:407:777-783, 2000.

5. Matijasevic, Z., Precopio, M.L., Snyder, J.E., and Ludlum, D.B. Repair of sulfur mustard-induced DNA damage in mammalian cells measured by a host cell reactivation assay. Carcinogenesis, *22:* 661-664, 2001.

6. Matijasevic, Z., Stering, A., Niu, T.-Q., Austin-Ritchie, P., and Ludlum, D.B. Release of sulfur mustard-modified DNA bases by Escherichia coli 3-methyladenine DNA glycosylase II. Carcinogenesis *17*: 2249-2252, 1996.

7. Powers, C.M., Santandrea, A.M., Volkert, M. R., and Matijasevic, Z. Alkyladenine DNA glycosylase sensitizes cells to sulfur mustard toxicity. Proceedings of the US Army Medical Defense Bioscience Review, 749-757, 2002.

8. Matijasevic, Z., Stering, A., and Ludlum, D.B. Cellular responses to sulfur mustard alkylation. Proc. Am. Assoc. Cancer Res. *38*: 132, 1997.

9. Matijasevic, Z., Snyder, J.E., and Ludlum, D.B. Hypothermia causes a reversible, p53-mediated cell cycle arrest in cultured fibroblasts. Oncol. Res. *10:* 605-610, 1998.

10. Hoeijmakers, J.H. Genome maintenance mechanisms for preventing cancer. Nature *411*: 366-374, 2001.

11. Dronkert, M.L., and Kanaar, R. Repair of DNA interstrand cross-links. Mutat. Res. *486*: 217-247, 2001.

12. Engelward, B.P., Dreslin, A., Christensen, J., Huszar, D., Kurahara, C., and Samson, L. Repair-deficient 3-methyladenine DNA glycosylase homozygous mutant mouse cells have increased sensitivity to alkylation-induced chromosome damage and cell killing. EMBO J. *15:* 945-952, 1996.

13. Elder, H.R., Jansen, J.G., Weeks, R.J., Willington, M.A., Deans, B., Watson, A.J., Mynett, K. J., Bailey, J.A., Cooper, D.P., Rafferty, J. A., Heeran, M. C., Wijnhoven, S. W.P., van Zeeland, A.A., and Margison, G.P. Alkylpurine-DNA-*N*-Glycosylase knockout mice show increased susceptibility to induction of mutations by methyl methanesulfonate. Mol. Cell. Biol. *18*: 5828-5837, 1998.

14. Bonnano, K.C., Wyrzykowski, J., Chong, W., Matijasevic, Z. and Volkert M.R. Alkylation resistance of *E. coli* cells expressing different isoforms of human alkyl adenine DNA glycosylase (hAAG) (*in press*).

15. Beneke, R., Geisen, C., Zevnik, B., Bauch, T., Muller, W.U., Kupper, J.H., Moroy, T. DNA excision repair and DNA damage-induced apoptosis are linked to Poly(ADP-ribosyl)ation but have different requirements for p53. Mol. Cell. Biol. *20:* 6695-6703, 2000.

16. Offer, H., Zurer, I., Banfalvi, G., Reha'k, M., Falcovitz, A., Milyavsky, M., Goldfinger, N., Rotter, V. p53 modulates base excision repair activity in a cell cycle-specific manner after genotoxic stress. Cancer Res. *61:* 88-96, 2001.

17. El-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. Cell *75*: 817-825, 1993.

18. Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. The p21 cdk-interacting protein CIP1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*: 805-816, 1993.

19. Ford, J.M., and Hanawalt, P.C. Expression of Wild-type p53 is required for efficient global genomic nucleotide excision repair in UV-irradiated human fibroblasts. J. Biol. Chem. *272*: 28073-28080, 1997.

20. Lloyd, D.R., and Hanawalt, P.C. p53-dependent global genomic repair of benzo[a]pyrene-7,8-diol-9,10-epoxide adducts in human cells. Cancer Res. *60*: 517-521, 2000.

21. Li, Q., Wright, S. E., Matijasevic, Z., Chong, W., Ludlum, D. B., and Volkert, M. R The role of human alkyladenine glycosylase in cellular resistance to the chloroethylnitrosoureas. Carcinogenesis *24:* 589-593, 2003.

22. Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery Jr., C.A., Butel, J.S., and Bradley, A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*: 215-221, 1992.

23. McMahon, A.P., and Bradley, A., Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. Cell, *62*, 1073-1085, 1990.

24. Jones, S. N., Sands, A. T., Hancock, A. R., Vogel, H., Donehower, L. A., Linke, S. P., Wahl, G. M., and Bradley, A. The tumorigenic potential and cell growth characteristics of p53-deficient cells are equivalent in the presence or absence of Mdm2. Proc. Natl. Acad. Sci. USA 93:14106-14111; 1996.

25. Pendlebury, A., Frayling, I.M., Santibanez Koref, M.F., Margison, G.P., and Rafferty, J.A. Evidence for the simultaneous expression of alternatively spliced alkylpurine *N*-glycosylase transcripts in human tissues and cells. Carcinogenesis *15*: 2957-2960, 1994.

26. Vickers, M.A., Vyas, P., Harris, P.C., Simmons, D.L., and Higgs, D.R. Structure of the human 3-methyladenine DNA glycosylase gene and localization close to the 16p telomere. Proc. Natl. Acad. Sci. U.S.A. *90:* 3437–3441, 1993.

27. O'Connor, T.R. Purification and characterization of human 3-methyl-adenine-DNA glycosylase. Nucleic Acids Res. *21*: 5561-5569, 1993.

28. Matijasevic, Z. Selective protection of non-cancer cells by hypothermia. Anticancer Res. *22:* 3267-3272, 2002.

29. Zeng, Y.X., and el-Deiry, W.S. Regulation of p21WAF1/CIP1 expression by p53-independent pathways. Oncogene *12*: 1557-1564, 1996.

30. el-Deiry, W.S., Tokino, T., Waldman, T., Oliner, J.D., Velculescu, V.E., Burrell, M., Hill, D.E., Healy, E., Rees, J.L., Hamilton, S.R., et al. Topological control of p21WAF1/CIP1 expression in normal and neoplastic tissues. Cancer Res. *55*: 2910-2919, 1995.

31. Moustakas, A., Pardali, K., Gaal, A., and Heldin, C.H. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. Immunol. Lett. *82*: 85-91, 2002.

32. Méda, P., Chevrier, V., Eddé, B., and Job, D. Demonstration and analysis of tubulin binding sites on centrosomes. Biochemistry *36*: 2550-2558, 1997.

APPENDICES

Abbreviations

AAG	alkyladenine glycosylase
AP	apurinic/apyrimidinic
APNG	alkylpurine glycosylase
BER	base excision repair
BrdU	5-bromo-2'-deoxyuridine
CEES	chloroethyl ethyl sulfide
CHO	Chinese hamster ovary
CL	crosslink
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HA	hemagglutinin
hAAG	human alkyladenine glycosylase
MEF	mouse embryonic fibroblasts
MEM,	minimal essential media
MMS	methyl methane sulfonate
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
NER	nucleotide excision repair
NHF	normal human fibroblasts
PI	propidium iodide
SM	sulfur mustard
TBE	trypan blue excluding assay
WT	wild type

Manuscripts

Matijasevic, Z., Precopio, M., Snyder, J. E. and. Ludlum, D. B. 2001. Repair of sulfur mustard-induced DNA damage in mammalian cells measured by a host-cell reactivation assay. Carcinogenesis, *22*: 661-664.

Powers, C.M., Santandrea, A.M., Volkert, M. R., and Matijasevic, Z. Alkyladenine DNA glycosylase sensitizes cells to sulfur mustard toxicity. Proceedings of the US Army Medical Defense Bioscience Review 2002, 749-757.

Bonnano, K.C., Wyrzykowski, J., Chong, W., Matijasevic, Z. and Volkert, M.R. Alkylation resistance of *E. coli* cells expressing different isoforms of human alkyladenine DNA glycosylase (hAAG). DNA Repair *1:* 507-516, 2002.

Matijasevic, Z. Selective protection of non-cancer cells by hypothermia. Anticancer Res. *22:* 3267-3272, 2002.

Li, Q., Wright, S. E., Matijasevic, Z., Chong, W., Ludlum, D. B., and Volkert, M. R The role of human alkyladenine glycosylase in cellular resistance to the chloroethylnitrosoureas. Carcinogenesis *24:* 589-593, 2003.

Carcinogenesis vol.22 no.4 pp.661–664, 2001

Repair of sulfur mustard-induced DNA damage in mammalian cells measured by a host cell reactivation assay

Z.Matijasevic[1], M.L.Precopio, J.E.Snyder and D.B.Ludlum

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01655-0126, USA

[1]To whom correspondence should be addressed

DNA damage is thought to be the initial event that causes sulfur mustard (SM) toxicity, while the ability of cells to repair this damage is thought to provide a degree of natural protection. To investigate the repair process, we have damaged plasmids containing the firefly luciferase gene with either SM or its monofunctional analog, 2-chloroethyl ethyl sulfide (CEES). Damaged plasmids were transfected into wild-type and nucleotide excision repair (NER) deficient Chinese hamster ovary cells; these cells were also transfected with a second reporter plasmid containing *Renilla* luciferase as an internal control on the efficiency of transfection. Transfected cells were incubated at 37°C for 27 h and then both firefly and *Renilla* luciferase intensities were measured on the same samples with the dual luciferase reporter assay. Bioluminescence in lysates from cells transfected with damaged plasmid, expressed as a percentage of the bioluminescence from cells transfected with undamaged plasmid, is increased by host cell repair activity. The results show that NER-competent cells have a higher reactivation capacity than NER-deficient cells for plasmids damaged by either SM or CEES. Significantly, NER-competent cells are also more resistant to the toxic effects of SM and CEES, indicating that NER is not only proficient in repairing DNA damage caused by either agent but also in decreasing their toxicity. This host cell repair assay can now be used to determine what other cellular mechanisms protect cells from mustard toxicity and under what conditions these mechanisms are most effective.

Introduction

Sulfur mustard, bis-(2-chloroethyl) sulfide (SM), is a bifunctional alkylating agent that has cytotoxic, mutagenic and vesicant properties, and is considered carcinogenic by the IARC (1). Sulfur mustard interacts with cellular DNA to form the cross-link, di-(2-guanin-7-yl-ethyl)-sulfide, and two monoadducts, 7-(2-hydroxyethylthioethyl) guanine (HETEG) and 3-(2-hydroxyethylthioethyl) adenine (HETEA) (reviewed in ref. 2). DNA modification by SM has been shown to interfere with replication and transcription and is probably responsible for its various toxicities (3–5).

Since the resistance of *Escherichia coli* cells to the lethal effect of SM correlates with their ability to remove cross-links, it has generally been assumed that the formation of DNA cross-links is a major cause of SM toxicity (3,6). However, the toxicity and vesicating properties of monofunctional derivatives of sulfur mustard, such as 2-chloroethyl ethyl sulfide (CEES), which generate similar monoadducts but do not form cross-links (2), suggest that monoadducts also contribute to the biological effects of sulfur mustard.

Previously, the cellular repair of SM-damaged DNA has been demonstrated either by measuring the disappearance of alkyl groups from DNA (7,8) or by monitoring the occurrence of non-semiconservative DNA synthesis ('repair synthesis') in cells exposed to sulfur mustard (9). The removal of DNA cross-links specifically has been demonstrated by several investigators in both *E.coli* and mammalian cells (3,8,10–12).

However, the specific cellular repair pathways and enzymes that act on SM-induced DNA adducts have not been completely established. *In vitro* studies have shown that bacterial 3-alkyl adenine DNA glycosylase II releases both of the SM monoadducts, 7HETEG and 3HETEA, from SM-modified DNA indicating that base excision repair may play a role in repairing sulfur mustard lesions (13). The involvement of another repair pathway in eukaryotic organisms, nucleotide excision repair (NER), has been suggested by the study of Kircher *et al.* (14) who have shown that yeast mutants deficient in nucleotide excision repair are much more sensitive to sulfur mustard than wild-type cells.

Although the biochemical studies mentioned above indicate that DNA repair processes act on SM-modified DNA, they do not demonstrate whether or not the damaged DNA has been restored to a functional state. To address this issue, other investigators have used a variety of host cell reactivation assays to demonstrate functional repair of DNA after damage by antitumor agents (15–18). In this manuscript, we describe the use of a dual luciferase reporter assay to demonstrate cellular repair of mustard-induced DNA damage.

We have first established that NER-competent Chinese hamster ovary cells are more able to withstand the toxic effects of SM than are NER-deficient cells. Then, using the dual luciferase host cell reactivation assay, we have shown that NER-competent cells are able to repair SM-damaged reporter plasmid and bring luciferase expression from the plasmid to higher levels than in NER-deficient cells, thus correlating DNA repair with SM toxicity.

We have also used the host cell reactivation assay to examine cellular repair of the damage caused by the single armed mustard, CEES. As shown in Figure 1, CEES forms monoadducts that are very similar to those formed by SM, thus allowing the effects of monoadducts on survival and repair to be examined independently from the effects of cross-links. These studies have shown that the toxicity of CEES is also decreased in NER-competent cells in comparison with NER-deficient cells. Luciferase expression from plasmid damaged by CEES is enhanced in NER-competent cells compared with NER-deficient cells, indicating that the monofunctional mustard adducts formed by CEES are cytotoxic and are also substrates for NER. From these results, we conclude that this

Abbreviations: CEES, 2-chloroethyl ethyl sulfide; HETEA, 3-(2-hydroxyethylthioethyl) adenine; HETEG, 7-(2-hydroxyethylthioethyl) guanine; NER, nucleotide excision repair; SM, sulfur mustard, bis-(2-chloroethyl) sulfide.

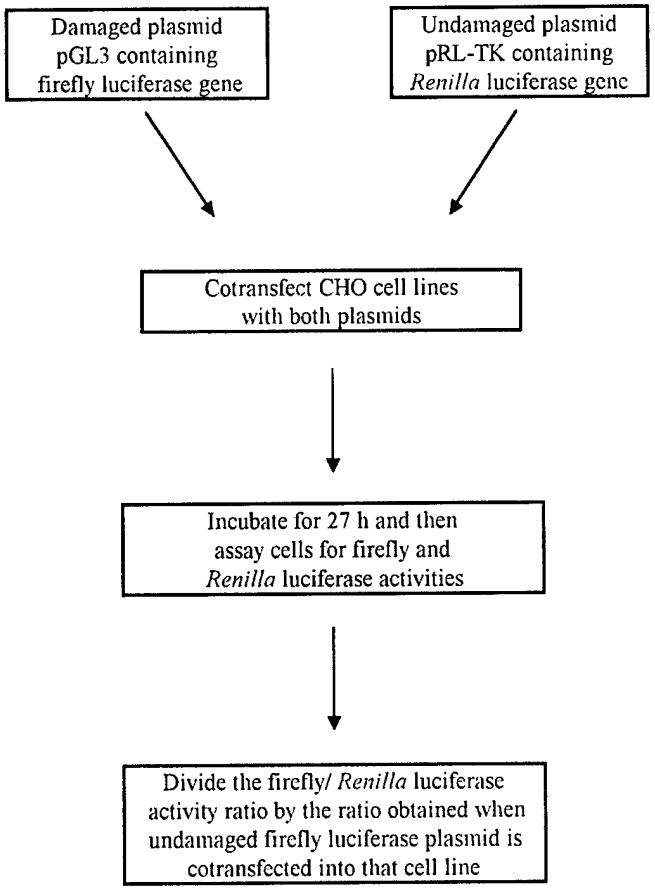


Fig 3. Scheme for monitoring DNA repair using damaged luciferase reporter gene in CHO cells. The level of firefly luciferase activity relative to *Renilla* luciferase activity is a measure of how efficiently the damaged firefly luciferase gene has been repaired.

Results

The data in Figure 2 show that CHO cells exposed to either SM or CEES are protected from cytotoxicity by the NER mechanism. Referring to data on day 3 (D3), >50% of NER-competent cells survive exposure to 20 μM SM while fewer than 2% of the NER-deficient cells survive. A similar difference is noted after exposure to CEES except that concentrations of CEES >10-fold greater than those of SM are required to produce the same level of cytotoxicity. Again at day 3, survival for wild-type cells is close to 100% after exposure to 300 μM CEES, but only ~5% for NER-deficient cells.

Based on the assumption that unrepaired DNA damage is responsible for SM cytotoxicity, we would expect that the increased survival shown in Figure 2 would be accompanied by an increase in cellular repair of DNA. This has been confirmed by measuring cellular DNA repair directly with the host cell repair assay as described below. In this assay, a plasmid that contains a gene for firefly luciferase is damaged by SM or CEES and then transfected into the cells that are to be evaluated for repair. Cells that can repair the DNA damage will express the luciferase gene at a higher level (i.e. the cells will 'reactivate' the gene). This results in an increased intensity of firefly luciferase bioluminescence that can be used to determine the extent of repair.

To compensate for variations in the efficiency of transfection and other experimental variables, luciferase reporter gene assays are usually run using dual transfection with DNA from

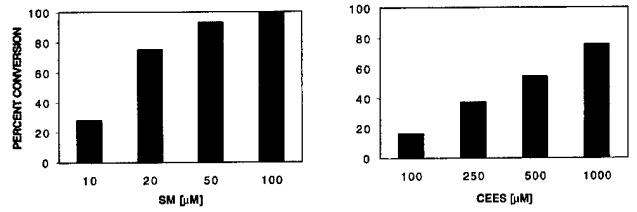


Fig 4. DNA damage caused by SM and CEES. The percent conversion of the firefly luciferase plasmid to Form II is plotted *versus* the concentration of mustard to which it was exposed.

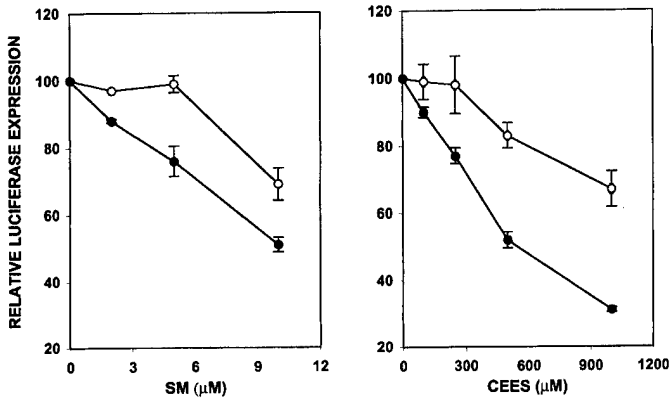


Fig 5. Relative luciferase expression in wild type (○) and NER-deficient cells (●); values are means ± SD from three to five separate assays. See text for details.

two plasmids, one containing the damaged firefly luciferase gene and the other (pRL) containing an undamaged *Renilla* luciferase gene. The intensity of firefly luciferase enzyme activity can then be compared with the level of *Renilla* luciferase activity as a control. This protocol is shown in Figure 3.

The firefly luciferase gene was damaged as described in the Materials and methods by exposing plasmid pGL3 to either SM or CEES *in vitro*. Damaged plasmid was then recovered and used in the host cell reactivation assays as shown in Figure 3.

Damage to plasmid DNA can be detected by measuring the conversion of the supercoiled form of the plasmid (Form I) to the nicked circular form (Form II). We assume that the extent of DNA damage that causes this conversion parallels the DNA damage that interferes with expression of the luciferase gene. Accordingly, we have measured conversion of the plasmid to Form II as an indication of the DNA damage caused to the firefly luciferase gene by SM and CEES. The level of conversion of Form I to Form II is shown in Figure 4. As expected, both SM and CEES convert Form I to Form II, but it takes an ~10-fold higher concentration of CEES to cause as much damage as is caused by a given concentration of SM.

The host cell reactivation data in Figure 5 show the extent to which this damage was repaired in the two different cell lines. In these experiments, plasmid containing damaged firefly luciferase gene was transfected separately into either wild-type or NER-deficient CHO cells. As shown in Figure 5, wild-type cells can return luciferase expression to normal if the plasmids are damaged with low concentrations of SM or CEES. In contrast, NER-deficient cells show much less repair, and levels of firefly luciferase expression fall off as the concentrations of SM or CEES are raised.

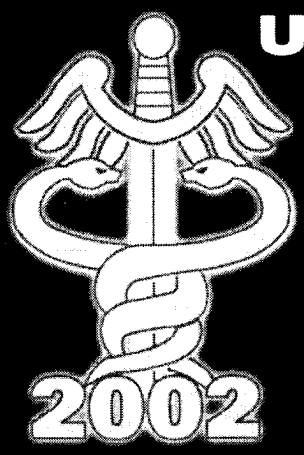
It is possible that some of the damage to the luciferase gene

US Army Medical Research and Materiel Command

US Army Medical Research Institute of Chemical Defense

Bioscience Review

US Army Medical Defense



2002 Proceedings

close open

Alkyladenine DNA Glycosylase Sensitizes Cells To Sulfur Mustard Toxicity

Christine M. Powers, Alida M. Santandrea, Michael R.Volkert, and **Zdenka Matijasevic**

University of Massachusetts Medical School, Worcester, MA

ABSTRACT

DNA crosslinks are considered major toxic lesions in sulfur mustard-alkylated DNA, and the repair of crosslinks is most likely a major defense against sulfur mustard (SM) toxicity. However, DNA monoadducts can also seriously interfere with DNA functions either directly or as precursors of crosslinks, apurinic sites, or strand breaks, all of which, if unrepaired, can be deleterious. Our results suggest that at least two distinct DNA repair pathways, nucleotide excision repair (NER) and base excision repair (BER), are involved in the processing of SM-induced DNA damages. Increased sensitivity to SM of NER-deficient mammalian cells in culture indicates that at least some cytotoxic DNA lesions induced by SM are successfully repaired by mammalian NER. The results from the host cell reactivation assay using a plasmid damaged by either SM or its monofunctional analog, 2-chloroethyl ethyl sulfide, demonstrate a role for NER in the repair of mustard-monoadducts. The unexpected finding, however, is that in contrast to the protective effect of NER, expression of alkyadenine-DNA glycosylase, the first enzyme in the BER pathway, renders bacterial cells more sensitive to both toxic and mutagenic effects of sulfur mustard. The sensitizing effect of DNA glycosylase activity is not unique to bacterial cells; it also occurs in mammalian cells exposed to SM. This finding represents a new lead in the search for the modulators of sulfur mustard toxicity and suggests that the inhibition of a specific step in the base excision repair pathway may diminish both long- and short-term toxicity. Our recent data strongly suggest that the decrease in glycosylase activity at low temperature may contribute to the protective effect of hypothermia against SM toxicity. We discuss possible mechanisms responsible for the glycosylase-mediated sensitization.

This work was supported by the U.S. Army Medical Research and Materiel Command under contract DAMD17-00-C-0012.

INTRODUCTION

Since DNA damage represents the initial event in sulfur mustard (SM) toxicity, we sought to first identify pathways involved in cellular responses to SM-induced DNA damage and in protection from its toxicity. We have demonstrated that two independent repair pathways, nucleotide excision repair (NER) [1] and base excision repair (BER) [2] are involved in processing of SM-modified DNA. The protective effect of nucleotide excision repair against SM toxicity is well defined on the basis of survival data demonstrating high sensitivity to SM of NER-deficient cells and on the basis of host cell reactivation experiments showing decreased repair of SM-damaged plasmid DNA in cells with compromised NER [1]. Protection by NER does not seem to be limited to the repair of DNA crosslinks, since NER-deficient cells are also more sensitive to monofunctional sulfur mustard, CEES, which generates only DNA monoadducts. At this point it is not known what specific steps of the NER pathway are involved in the repair of SM lesions and what conditions may improve NER activity and decrease SM toxicity. Base excision repair has an important role in protecting cells against the lethal and mutagenic effects of simple alkylating agents [3]. However, there are conflicting reports on the involvement of mammalian alkyladenine DNA glycosylase (AAG), the first enzyme of the BER pathway, in protection against cytotoxic effects of bifunctional alkylating agents [4], [5]. Here we show that in the same experimental system where the expression of alkyl adenine DNA glycosylase clearly protects cells against the methylating agent methyl methane sulfonate (MMS), it sensitizes cells to SM-induced effects. This sensitizing effect of glycosylase also occurs in cells exposed to monofunctional sulfur mustard CEES. While these results do not rule out the possible role of DNA crosslinks in glycosylase-mediated sensitization, they do indicate that the processing of SM monoadducts contributes to the phenomenon.

The discovery that the activity of a specific enzyme increases SM toxicity encourages search for the conditions that could modulate this activity. We have previously demonstrated that hypothermia induces reversible cell cycle arrest in mammalian cells in culture [6] and this p53-dependent response is probably one of the mechanisms how hypothermia protects cells from SM toxicity. Here we show that hypothermia also abrogates glycosylase-mediated sensitization of SM-exposed cells suggesting that the modulation of glycosylase activity represents another mechanism of protection at low temperature.

MATERIALS AND METHODS

Bis-(chloroethyl) sulfide (SM) was supplied by the US Army Institute of Chemical Defense (Aberdeen Proving Ground, MD). Methyl methane sulfonate (MMS) and 2-chloroethyl ethyl sulfide (CEES) were purchased from Aldrich (Milwaukee, WI). Antibiotics were obtained from Gibco BRL (Gaithersburg, MD). Luciferase reporter plasmids pGL3 and pRL-TK, transfection reagent TransFast and Dual Luciferase assay kit were purchased from Promega.

Bacterial Cells

All bacterial strains used in this study were constructed in author's (M.R.V.) laboratory, University of Massachusetts Medical School, Worcester, MA. Their relevant genotypes are: MV1161, *arg*E3; MV1273, *arg*E3 *uvrA*; MV1174, *arg*E3 *alk*A1; MV1302, *arg*E3 *alk*A1 *uvr*A. Cells were grown in liquid Luria-Bertani (LB) medium at 37°C with aeration. Permanent stocks are maintained at –80°C in LB with 10% DMSO.

Bacterial Cell Survival and Mutation Frequency

Cells were grown in LB medium to approximately $5x10^8$ cells per ml at 37°C with aeration. Aliquots of cell suspensions were transferred in the SterilchemGARD hood and exposed to different doses of SM or CEES for 60 min. Cell dilutions were prepared in E-salts buffer containing 4% $Na_2S_2O_3$ and plated in triplicate on ESEM media for both total surviving colonies and Arg+ revertants. Plates were incubated for 48h at 37°C and surviving colonies and Arg+ mutants were counted. Survival was expressed as a percentage of untreated control and mutation frequency was calculated as the number of Arg+ revertants per survivor.

Mammalian Cell Culture

Mouse embryonic fibroblasts (MEF), wild type (APNG+/+) and alkyladenine DNA glycosylase- null mutants (APNG-/-), both spontaneously transformed, were a gift from Dr. Rhoderick H. Elder from Paterson Institute for Cancer Research, Manchester, UK [5]. Cells were grown in D-MEM/F-12 medium containing L-glutamine (GibcoBRL, Gaithersburg, MD) supplemented with fetal bovine serum (10%), nonessential amino acids, nucleosides, penicillin and streptomycin.

Mouse embryonic stem (ES) cells, wild type and null mutants for 3-alkyladenine DNA glycosylase, were obtained from Dr. Bevin Engelward (Massachusetts Institute of Technology) [4]. SNL76/7 feeder cells were obtained from Dr. Allan Bradley (Baylor College of Medicine, Huston) [7]. ES cells were cultured on gelatinize plates with mitotically inactive SNL76/7 feeder cells that express leukemia inhibitory factor (LIF) to prevent ES cells differentiation [7]. Feeder cells were grown on Dulbecco's modified Eagle's medium (DMEM) supplemented with FBS (10%), glutamine and antibiotics. They were inactivated by mitomycin C (10 μg/ml for 2 h) and kept frozen until use. DMEM media for ES cells growth was supplemented with FBS (15%), glutamine, antibiotics, 2-mercaptoethanol and nonessential amino acids.

Mammalian Cells Survival

Cells were plated in 12- or 6-well plates at a density of 2 x 10^4 cells/cm^2. After 24 h the medium was replaced with fresh medium containing the indicated concentrations of SM or MMS; dilute solutions of these compounds in absolute alcohol were prepared immediately before treatment. Cells were exposed to the chemicals for 1 h at room temperature in a SterilchemGard hood and then incubated in fresh medium at 37°C or 28°C. At the indicated times, cell viability was determined by the trypan blue exclusion assay. In order to include cells that may have been detached from the monolayer, the medium above the monolayer was collected and centrifuged prior to trypsinization, and the pellet was combined with cells detached by trypsin.

Host Cell Reactivation Assay for DNA Repair

This assay measures repair as the restoration of luciferase expression from *in vitro*-damaged plasmids after the transfection into mammalian cells in culture. Purified pGL3 DNA containing the firefly luciferase gene was dissolved in TE buffer at a concentration of 1 μg/μl and incubated with SM in a SterilchemGard hood at room temperature for 1 h. DNA was precipitated with ethanol, dissolved in TE buffer, and stored at -20°C until used for transfection. Aliquots were analyzed by gel electrophoresis for the level of conversion of supercoiled DNA into the nicked circular form. In order to normalize for variations in the efficiency of transfection and lysis and for the cell number, cells were co-transfected with undamaged pRL reporter plasmid containing Renilla luciferase as an internal standard.

For transfection experiments, cells were plated in 24-well plates at a density of 2 x 10^4 cells/cm^2 and incubated for 24 h at 37°C. Transfection was performed in serum-free media using the liposome-based transfection reagent (Promega). Optimal conditions for transfection were established at 0.5 μg of plasmid DNA per well, at a charge ratio of transfection reagent to DNA of 1:1, and a ratio of pGL3 to pRL-TK of 10:1. After transfection cells were incubated at 37°C in complete media, lysed and stored frozen at -20°C for the luciferase assay. The assays for firefly luciferase activity and Renilla luciferase activity were performed sequentially in one reaction tube according to manufacturer's instructions.

RESULTS AND DISCUSSION

Role of DNA Glycosylase in Sulfur Mustard Toxicity

The base excision repair (BER) pathways are present in diverse organisms from bacteria to mammalian cells [3]. In all organisms the first enzyme on the pathway, DNA glycosylase, determines the substrate specificity of the BER. In order to determine whether alkyladenine DNA glycosylase is able to process biologically relevant alkylation products in SM-exposed cells, we used *E. coli* cells that are either wild type or glycosylase-deficient, and measured their survival as a function of SM dose. Cells deficient in the nucleotide excision repair (NER) or deficient in both, NER and BER, were also included in the study. The results are shown in *figure 1*. As expected,

nucleotide excision repair protect sells against SM toxicity and cells lacking NER (uvrA$^-$ cells) are much more sensitive to SM then the wild type cells (*figure 1, left panel*). Surprisingly, however, cells lacking functional AlkA glycosylase (Alk$^-$, close symbols) survive better than cells that are wild type for glycosylase (open symbols). This sensitizing effect of AlkA glycosylase function occurs in cells regardless of their NER status; AlkA+ cells are more sensitive to SM than AlkA$^-$ cells whether or not they have functional nucleotide excision repair.

Another potential consequence of DNA damage, induction of mutations, was tested as the reversion of Arg$^-$ into the Arg+ phenotype in cells with different repair background. The results in *figure 1* (right panel) show that, in contrast to the NER that protects cells from accumulation of mutations, the presence of glycosylase function increases mutation frequency above that seen in the AlkA-deficient strain. Increased sensitivity of cells expressing glycosylase suggests that this enzyme does act on SM-induced lesions, but process them in a manner that increases toxicity. The sensitizing effect of AlkA glycosylase is specific for SM-exposed *E. coli* cells; AlkA glycosylase protects cells exposed to simple methylating agent, methyl methane sulfonate [8].

In order to examine whether the sensitization to SM by DNA glycosylase also occurs in mammalian cells we performed survival studies with wild type and DNA glycosylase (AAG) null mutants of mouse embryonic stem (ES) cells. The results in *figure 2* (left panel) demonstrate protective effect of glycosylase against the MMS toxicity. However, when cells are exposed to SM (*figure 2,* right panel), the presence of glycosylase function in wild type cells (WT, open symbols) has a similar effect as it has in bacterial cells, it increases sensitivity to SM; lack of AAG in null mutant cells (AAG-/-, close symbols) make cells more resistance to SM. Therefore, the results with ES cells indicate that the sensitizing effect of alkyladenine DNA glycosylase on SM toxicity is not unique for bacterial cells; sensitization occurs in mammalian cells as well. The results suggest that it may be possible to manipulate, diminish or inhibit glycosylase activity in order to both enhance survival and reduce mutagenic effects after SM exposure.

To determine the effects of mammalian DNA glycosylase on the repair of SM-induced DNA damage we applied the host cell reactivation assay to compare the levels of damaged plasmid reactivation in cells with and without DNA glycosylase. The repair of DNA damage restores or "reactivates" expression from the reporter gene and the difference in the levels of expression reflects the difference in DNA repair capacity between the two cell lines. *Figure 3* shows the results of experiments where undamaged or SM-damaged firefly luciferase reporter plasmid was transfected into the wild type (APNG+/+) or glycosylase deficient (APNG-/-) mouse embryonic fibroblasts. Cells were incubated at 37°C for 30 h after transfection, lysed and assayed for the levels of luciferase expression. The results show that in cells with functional DNA glycosylase (APNG+), reactivation of plasmid DNA exposed to higher SM doses is significantly lower than in cells deficient in DNA glycosylase (APNG-). These results are in agreement with the survival data demonstrating a sensitizing effect of DNA glycosylase and they suggest that at the higher level of alkylation DNA glycosylase interferes with restoration of the functional integrity of SM-damaged DNA.

Different DNA lesions and mechanisms could account for sensitization phenomenon. Although DNA monoadducts are natural substrates for glycosylase, it is possible that DNA crosslinks are responsible for sensitization. In order to address the question of whether monoadducts contribute to the increased lethal and mutagenic effects in cells with functional DNA glycosylase, we used monofunctional sulfur mustard, chloroethyl ethyl sulfide (CEES), which generates DNA monoadducts similar to those formed by SM, but does not form DNA crosslinks. Effects of CEES on bacterial cells with or without endogenous DNA glycosylase are compared in *figure 4*. Similar to the results with SM, the presence of a functional DNA glycosylase significantly increases both killing (*figure 4*, left panel) and mutation frequency (*figure 4*, right panel) in *E. coli* cells exposed to CEES. This sensitizing effect of glycosylase is not dependent on NER; DNA glycosylase increases mutagenesis in both NER-wild type and NER-deficient cells. The results strongly suggest that the sensitizing effect of DNA glycosylase on SM-exposed cells is, at least in part, due to the presence of mustard monoadducts in DNA.

The Role of Hypothermia in Protection against SM Toxicity

In contrast to heat shock, very little is known at the molecular level about the response of mammalian cells to cold. We have demonstrated [6] that hypothermia represents a p53-inducing condition and that lowering temperature to 28°C causes reversible cell cycle arrest in normal human fibroblasts. The effect of low temperature on cell cycle in damaged cells is likely to be dependent on a number of factors including cellular DNA repair status. Providing low temperature does not compromise protective pathways or intensify toxic ones, the delay in

progression is likely to be beneficial for survival by allowing more time for processing of damaged DNA before the onset of DNA synthesis and mitosis.

Since the activity of DNA glycosylase apparently contributes to SM toxicity when cells are incubated at 37°C after exposure, we asked what impact, if any, hypothermia has on this sensitization. To answer this question we measured survival of mouse embryonic stem cells, wild type or DNA glycosylase null mutants that were incubated at either 37°C or 28°C after the exposure to SM. The results in *figure 5* (left panel) clearly demonstrate a protective effect of post-exposure incubation at 28°C on cells with functional DNA glycosylase (WT). This increased survival may be the result of either increased activity of protective pathway(s) or weakened activity of toxic pathway(s). The results in *figure 5* (right panel) show the survival of glycosylase-deficient cells at two temperatures. Compared to the wild type, glycosylase-deficient cells survive better at both temperatures and the beneficial effect of low temperature is much less pronounced than in the wild type cells. This results strongly suggest that at least one component of protection from SM toxicity by hypothermia is due to the weakening of glycosylase-mediated sensitization at low temperature.

One possible mechanism of sensitization may be through the direct interaction of glycosylase with SM-damaged DNA in such a way that generates more toxic repair products. Such products can be formed either through the overwhelming AP site formation leading to the DNA strand brakes or through the lack of glycosylase dissociation from DNA that could interfere with subsequent repair steps. Alternatively, sensitization can be caused by the glycosylase interference with the repair of highly toxic DNA crosslinks, either directly or through the effect on cellular factors linking BER and NER pathways. One possible candidate is hHR23 protein that is, in complex with XPC protein, required for the damage recognition step in NER pathway [9]. It was recently found that hHR23 proteins also interact with human alkyladenine DNA glycosylase, the first enzyme in the BER pathway, and increases binding affinity of glycosylase for damaged DNA [10]. If indeed DNA glycosylase and XPC protein compete for hHR23, then glycosylase may weaken repair of crosslinks by depletion of hHR23 protein. Further studies on the mechanisms of sensitization by glycosylase and protection by hypothermia would help us to better determine conditions for efficient protection against SM toxicity.

REFERENCES

1. Matijasevic, Z., Precopio, M.L., Snyder, J.E., and Ludlum, D.B., Repair of sulfur mustard-induced DNA damage in mammalian cells measured by a host cell reactivation assay. Carcinogenesis, *22*, 661-664, 2001.
2. Matijasevic, Z., Stering, A., Niu, T.-Q., Austin-Ritchie, P., and Ludlum, D.B., Release of sulfur mustard-modified DNA bases by Escherichia coli 3-methyladenine DNA glycosylase II. Carcinogenesis, *17*, 2249-2252, 1996.
3. Friedberg, E.C., Walker, G.C., and Siede, W., DNA Repair and Mutagenesis. Washington, DC, ASM Press, 1995.
4. Engleward, B.P., Dreslin, A., Christensen, J., Huszar, D., Kurahara, C., and Samson, L., Repair-deficient 3-methyladenine DNA glycosylase homozygous mutant mouse cells have increased sensitivity to alkylation-induced chromosome damage and cell killing. EMBO J., *15*, 945-952, 1996.
5. Elder, H.R., Jansen, J.G., Weeks, R.J., Willington, M.A., Deans, B., Watson, A.J., Mynett, K.J., Bailey, J.A., Cooper, D.P., Rafferty, J.A., Heeran, M.C., Wijnhoven, S.W.P., van Zeeland, A.A., and Margison, G.P., Alkylpurine-DNA-N-Glycosylase knockout mice show increased susceptibility to induction of mutations by methyl methanesulfonate. Molec. Cell. Biol., *18*, 5828-2837, 1998.
6. Matijasevic, Z., Snyder, J.E., and Ludlum, D.B., Hypothermia causes a reversible, p53-mediated cell cycle arrest in cultured fibroblasts. Oncol. Res., *10*, 605-610, 1998.
7. McMahon, A.P., and Bradley, A., Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. Cell, *62*, 1073-1085, 1990.
8. Bonnano, K.C., Wyrzykowski, J., Chong, W., Matijasevic, Z. and Volkert M.R., Alkylation resistance of E. coli cells expressing different isoforms of human alkyladenine DNA glycosylase. hAAG, *in press.*

9. Sugasawa, K., Ng, J.M., Masutani, C., Maekawa, T., Uchida, A., van der Spek, P.J., Eker, A.P., Rademakers, S. Visser, C., Aboussekhra, A., Wood, R.D., Hanaoka, F., Bootsma, D., and Hoeijmakers, J.H., Two human homologs of Rad23 are functionally interchangeable in complex formation and stimulation of XPC repair activity. Molec. Cell. Biol., *17*, 6924-6931, 1997.
10. Miao, F., B.M., Dammann, R., Masutani, C., Hanaoka, F., Pfeifer, G., and O'Connor, T.R., 3-Methyladenine-DNA glycosylase (MPG protein) interacts with human RAD23 proteins. Journal Biol. Chem., *275*, 28433-28438, 2000.

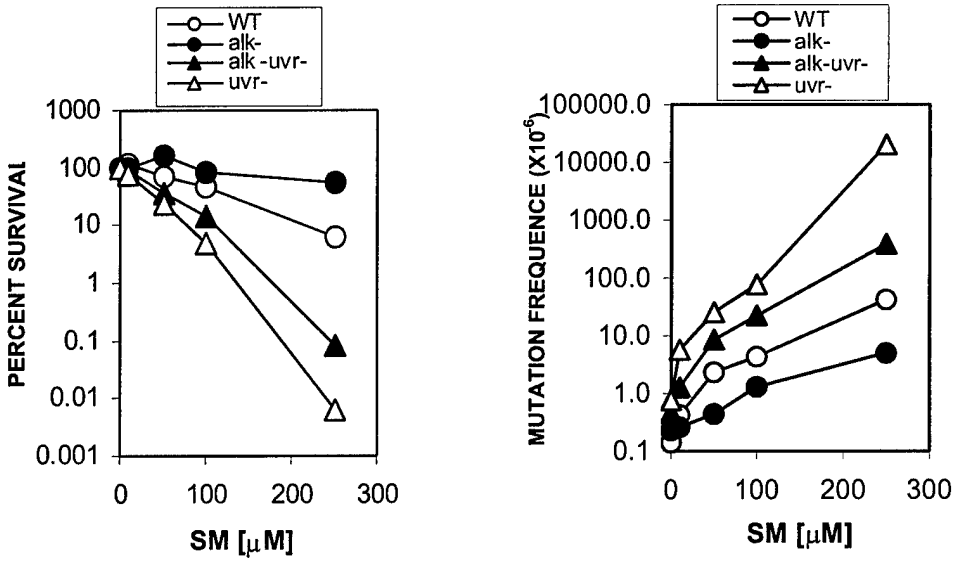


Figure 1. Effect of DNA repair on survival and mutation induction in *E. coli* cells exposed to sulfur mustard. Wild type (O), AlkA glycosylase-deficient (●), NER-deficient (△) or glycosylase and NER-deficient (▲) cells were exposed to SM for 30 min and plated for total cell number and for Arg+ revertants. Survival (left panel) was calculated as a percent of untreated control and mutation frequency (right panel) was calculated as a number of revertants among survivors.

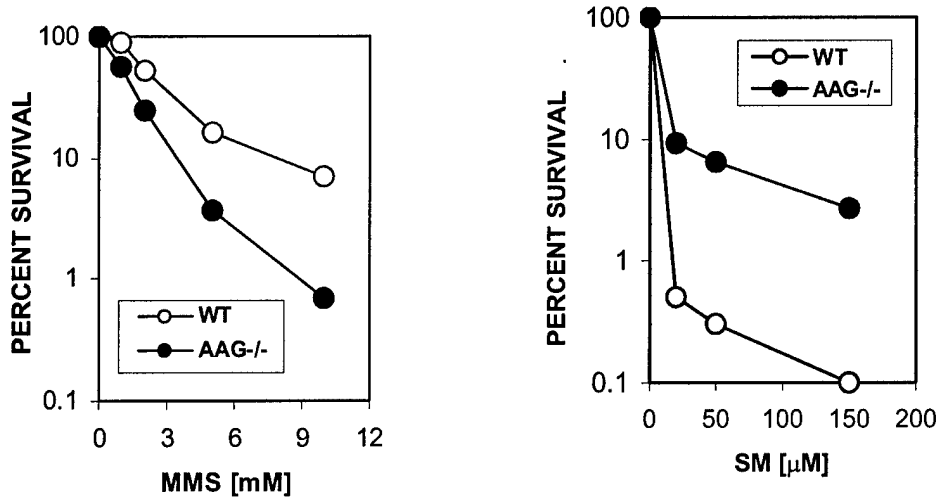


Figure 2. Effect of DNA glycosylase on survival of mouse embryonic stem cells exposed to MMS or SM. Mouse embryonic stem cells, wild type (WT, O) or DNA glycosylase null mutants (AAG-/-, ●) were exposed to MMS (left panel) or to SM (right panel) for 1h and incubated at 37°C for 2 days. Cell viability was determined by trypan blue exclusion assay. While presence of glycosylase protects cells from toxic effects of MMS, it increases their sensitivity to SM.

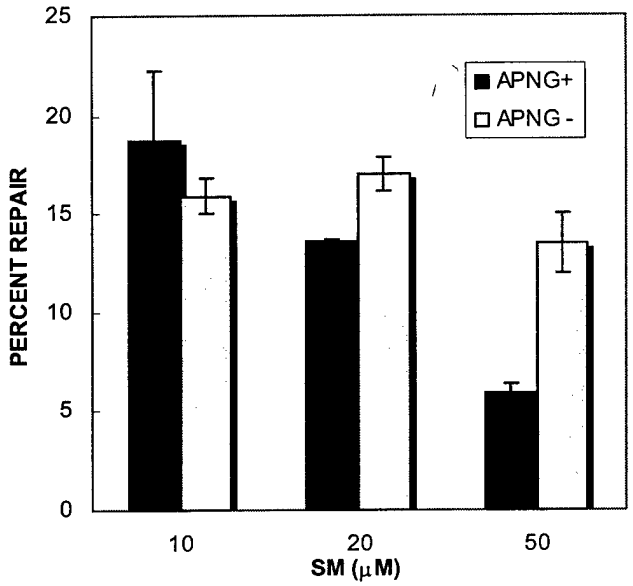


Figure 3. Effect of DNA glycosylase on luciferase expression from SM- damaged reporter plasmids.

Wild type cells (■) and APNG-deficient cells (□) were cotransfected with either the SM-damaged or undamaged firefly luciferase reporter plasmids and with undamaged Renilla luciferase reporter plasmids. Cells were incubated at 37°C and lysed. Firefly luciferase activity was normalized to correct for variations in transfection efficiency using Renilla luciferase as an internal standard. Firefly luciferase activity in cells transfected with damaged plasmid, expressed as a percent of activity in cells with undamaged plasmid, represents the extent of DNA repair for the given cell line.

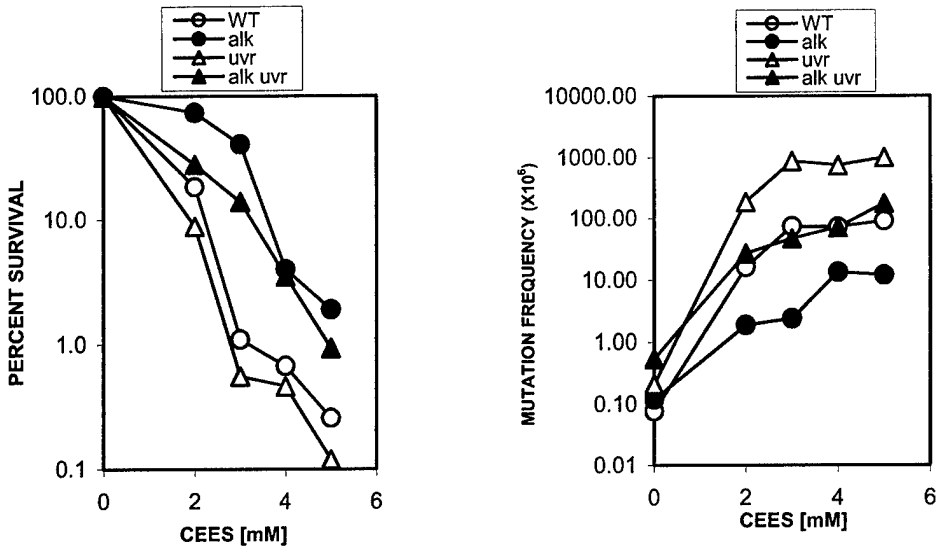


Figure 4. Effect of DNA repair on survival and mutation induction in *E. coli* cells exposed to 2-chloroethyl ethyl sulfide (CEES). Wild type (O), AlkA glycosylase-deficient (●), NER-deficient (△) or glycosylase and NER-deficient (▲) cells were exposed to CEES for 60 min and plated for total cell number and for Arg+ revertants. Survival (left panel) was calculated as a percentage of untreated control and mutation frequency (right panel) was calculated as the number of revertants among survivors.

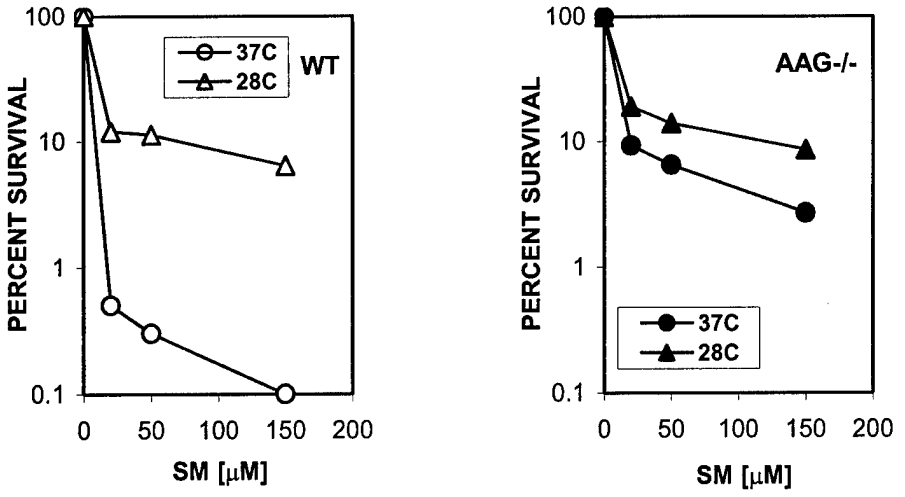


Figure 5. Hypothermia protects mammalian cells from SM toxicity by diminishing sensitizing effect of DNA glycosylase. Mouse embryonic stem cells, wild type (left panel, O and △) or DNA glycosylase null mutants (right panel, ● and ▲) were exposed to SM for 1h and then incubated at either 37°C (● and O) or 28°C (▲ and △) for two days. Cell viability was determined by trypan blue exclusion assay.

ELSEVIER

DNA Repair 1 (2002) 507–516

DNA REPAIR

www.elsevier.com/locate/dnarepair

Alkylation resistance of *E. coli* cells expressing different isoforms of human alkyladenine DNA glycosylase (hAAG)

Kenneth Bonanno [a,1], Jennifer Wyrzykowski [a], Wincha Chong [a,2], Zdenka Matijasevic [b], Michael R. Volkert [a,*]

[a] *Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA 01655, USA*
[b] *Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01655, USA*

Received 8 February 2002; accepted 3 April 2002

Abstract

The alkyladenine DNA glycosylase (AAG) has been cloned from mouse and humans. AAG knock out mouse cells are sensitized to a variety of alkylating and cross-linking agents suggesting AAG is active on a variety of substrates. In humans, two isoforms have been characterized that are generated by alternative splicing and contain either exon 1a or 1b (hAAG1 or hAAG2). In this study, we examine the ability of the both known isoforms of human AAG (hAAG) to contribute to survival of *Escherichia coli* from treatments with simple alkylating agents and cross-linking alkylating agents. Our results show that hAAG is effective at repairing methyl lesions when expressed in *E. coli*, but is unable to afford increased resistance to alkylating agents producing larger alkyl lesions such as ethyl lesions or lesions produced by the cross-linking alkylating agents *N,N'*-bis-chloroethyl-*N*-nitrosourea (BCNU), *N*-(2-chloroethyl)-*N*-nitrosourea (CNU) or mitomycin C. In the case of CNU, expression of hAAG causes increased sensitivity rather than resistance, suggesting deleterious effects of hAAG activity. We also demonstrate that there are no apparent differences between the two isoforms of hAAG when recovery from damage produced by all alkylating agents is tested.

Keywords: Alkylation resistance; *E. coli* cells; Human alkyladenine DNA glycosylase

1. Introduction

The human alkyladenine DNA glycosylase gene (*hAAG*) was identified by its ability to complement methyl methane sulfonate (MMS) sensitivity of bacterial mutants lacking their own AAG (AlkA and TagA) [1–3]. Studies with mouse embryonic stem (ES) cells indicate that mutation of the mouse AAG (*mAAG*) gene causes sensitivity not only to the methylating agents MMS and $MeOSO_2(CH_2)_{2'}$-lexitropsin, but also to the DNA cross-linking agents *N,N'*-bis-chloroethyl-*N*-nitrosourea (BCNU) and mitomycin C; no effects on UV sensitivity are detected [4,5]. In contrast, studies by others have shown that mouse embryonic fibroblast (MEF) cells carrying the AAG knockout mutation are sensitized to the lethal and mutagenic effects of high doses of MMS, but not to

* Corresponding author. Tel.: +1-508-856-2314; fax: +1-508-856-5920.
E-mail address: michael.volkert@umassmed.edu (M.R. Volkert).
[1] Present address: Millennium Pharmaceuticals, Cambridge, MA 02139, USA.
[2] Present address: Transitional Residency Program, University of Hawaii Medical School, Honolulu, HI 96813-2427, USA.

1568-7864/02/$ – see front matter
PII: S1568-7864(02)00051-4

BCNU [6]. The lack of any detectable alkyl base excision repair (BER) activity in AAG deficient mouse cells suggests that this is the only alkyl specific DNA glycosylase present in mammalian cells [5]. This conclusion is strengthened by the lack of any additional identifiable hAAG paralogues in the human genome [7].

The *hAAG* gene exists in two known isoforms that result from differential splicing, incorporating either exon 1a (hAAG1), or exon 1b (hAAG2) [8,9]. Both isoforms of hAAG appear to be expressed in all tissues examined and no differences in substrate specificity have been detected to date [9,10]. In order to further examine which lesions can be repaired by hAAG and to determine if any differences in substrate specificities can be detected when the two hAAG isoforms are compared, we tested their ability to protect repair deficient bacterial cells from lethality upon exposure to different alkylating agents.

Bacteria have two different AAGs, one encoded by the *tagA* gene, and a second encoded by the *alkA* gene. The AlkA glycosylase can excise not only chemically methylated purines and pyrimidines from DNA [11,12], but can also remove more complex lesions such as chloroethylated, ethylthioethylated bases as well as exocyclic DNA adducts generated by environmental and chemotherapeutic agents [13–17]. More complex alkyl lesions are also substrates for *uvrABC*-dependent nucleotide excision repair (NER). The NER pathway assists the BER pathway and, as the complexity of the lesion increases, probably replaces the *alkA* and *tagA* pathways as the primary mechanism of repair. It has also been demonstrated that the *alkA* encoded DNA glycosylase is unable to prevent formation of cross-links by interaction with intermediates in their formation [18].

2. Materials and methods

2.1. Chemicals

MMS, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) and mitomycin C were obtained from Sigma. *N*-(2-chloroethyl)-*N*-nitrosourea (CNU) and BCNU were obtained from the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD).

2.2. Construction of hAAG1 and hAAG2 expression plasmids

We constructed an IPTG inducible hAAG1 expression plasmid by transferring the EcoRI-HinDIII fragment which contains the hAAG1 coding sequence from pBU16 (obtained from Leona Samson, Massachusetts Institute of Technology), and cloning this fragment into the pTrc99a vector (Pharmacia). This plasmid allows expression of genes from the strong pTrc promoter and carries the *lacI*^{q} repressor gene to control expression. The EcoRI to HinDIII fragment carrying hAAG1 was first transferred from pBU16 to pTrc99a. Upstream pBU16 sequences were replaced by removing the EcoRI to BstEII fragment and replacing it by inserting two annealed oligonucleotides mv1 and mv2 (Table 1). These oligonucleotides are complementary to one another, producing single-stranded ends complementary to EcoR1 and the BstEII site present in hAAG1. They also contain a consensus ribosome binding site (AGGAGG) and the ATG start codon of the *hAAG1* gene appropriately positioned for optimal expression.

The plasmid encoding the histidine tagged form of hAAG1 was constructed by replacing the 3′ end of the cloned gene by digesting with CelII and HinDIII, then replacing the CelII to HinDIII fragment encoding the hAAG carboxyl terminus with the two self-complementary oligonucleotides mv3 and mv4 (Table 1). After annealing, these oligonucleotides produce unpaired ends compatible with CelII and HinDIII digested DNA. When inserted into the hAAG1 plasmid, they restore the last seven codons of the *hAAG* genes and insert six histidine codons upstream of the stop codon. All plasmid constructions were confirmed by DNA sequencing.

The difference between the hAAG1 and hAAG2 isoforms is due to alternative splicing resulting in the presence of either the 13 amino acid exon1a of hAAG1 or the 8 amino acid exon 1b of hAAG2 [8,10]. The hAAG1 clone was converted to hAAG2 in several steps. The first step replaced exon 1a with exon 1b using primer mv8 (Table 1) which contains an EcoR1 site near its 5′ end, followed by sequences encoding exon 1b and the first 24 bases of exon 2 which is common to both isoforms of hAAG. The second primer was mv6 (Table 1) which hybridizes downstream of the HinDIII sequence beyond the 3′ end of the hAAG

Table 1
Oligonucleotides used in hAAG plasmid constructions

Oligo	Sequence
mv1	AATTCTAAGGAGGTATCTAATG
mv2	GTGACCATTAGATACCTCCTTAG
mv3	TGAGCAGGACACACAGGCCCATCATCATCATCATCACTGA
mv4	AGCTTCAGTGATGATGATGATGATGGGCCTGTGTGTCCTGC
mv6	CTGTATCAGGCTGAAAATC
mv8	GCGCGAATTCTGATGCCCGCGCGCAGCGGGGCCCAGTTTTGCCGACGGATGGGGC
mv20	CATGGAATTCTAAGGAGGTATCTAATGCCCGCGCGCAGCGGGGCCCAGTTTTGC

coding sequence. This PCR fragment was cleaved with EcoRI and HinDIII and ligated into the pTrc99A vector to clone hAAG2, producing pMV514. Next, sequences upstream of the hAAG2 coding sequence were replaced using primers mv20 and mv6 (Table 1) and pMV514 as template (Table 1) in a PCR reaction to amplify hAAG2. Primer mv20 contains an EcoRI site near its 5′ end followed by sequences identical to those present upstream of hAAG1 and the first 30 bases of hAAG2. Cloning the EcoRI to HinDIII fragment from this PCR reaction into pTrc99A resulted in the production of a plasmid pMV536 that differs from the hAAG-$(his)_6$ expressing plasmid only in its exon 1 sequence. The upstream region and hAAG2 sequence was confirmed by DNA sequencing. The EcoRI to AfeII region of pMV536 was then purified and used to replace the EcoRI to AfeII region of pMV509 to produce the hAAG2 expressing plasmid pMV543. This same fragment was also used to replace the EcoRI to AfeII region of pMV513 to produce the his tagged hAAG2 expressing plasmid pMV545. The inducible hAAG1 and hAAG2 plasmids constructed by these methods proved to be stable and allow maintenance of the hAAG plasmids in *E. coli*.

2.3. Bacterial strains

All bacterial strains and plasmids used in this study are listed in Table 2.

2.4. Survival studies

Survival studies were performed as described previously [19]. Briefly, cells were grown to mid-log phase in standard LB medium, treated with IPTG (1 mM) for 90 min to induce hAAG expression, then treated with the alkylating agent at various concentrations for 30 min, then immediately diluted 1:100 in 4% $Na_2S_2O_3$ to stop exposure to alkylating agents. Cells were then diluted further and plated on LB plates.

Table 2
Bacterial strains

Strain	Relevant genotype	Plasmid	Source or reference
MV1161[a]	Wild type	none	[19]
MV1176	*uvrA6*	none	This study
MV4211	*alkA1 tagA1 ompT*	pMV513 (hAAG1-$(his)_6$)[b]	This study
MV4213	*alkA1 tagA1 ompT*	pMV536 (hAAG2-$(his)_6$)	This study
MV4224	*alkA1 tagA1*	pMV509 (hAAG1)	This study
MV4228	*alkA1 tagA1*	pTrc99a	This study
MV4232	*alkA1 tagA1*	pMV550 (hAAG2)	This study
MV4236	*alkA1 tagA1 uvrA6*	pTrc99a	This study
MV4237	*alkA1 tagA1 uvrA6*	pMV509 (hAAG1)	This study
MV4239	*alkA1 tagA1 uvrA6*	pMV550 (hAAG2)	This study

[a] Bacterial strains are derivatives of MV1161 which carries the following additional mutations: *thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1 rfa-550*.

[b] All plasmids are derivatives of pTrc99A (Pharmacia) and hAAG alleles are inserted into the EcoRI and HinDIII restriction sites of this vector.

Surviving colonies were counted after 24 h incubation at 37 °C. Presence or absence of the vector had no detectable effect on survival. All graphs represent the averages of two or more experiments and standard errors are included for all points on graphs.

2.5. Western blot analysis

Cells were grown as described for survival studies. After addition of IPTG (2 mM), cells were incubated for various times, adjusted to a constant cell concentration by adjusting OD_{600} to identical levels, centrifuged and resuspended in lysis buffer [20]. Samples were then boiled for 10 min and centrifuged, then identical volumes of samples were loaded onto a 12% SDS polyacrylamide gel. After electrophoretic separation, proteins were transferred to an immobilon P membrane (Millipore) using a blot transfer apparatus (Bio-rad). Blots were incubated in PBS containing 5% nonfat milk and 0.1% Tween-20, rinsed twice with PBS with 0.1% Tween-20 (PBST) and probed with anti-histidine antibody according to the manufacturers protocol (Qiagen). After 1 h incubation, the membrane was rinsed several times with PBST and treated with anti-mouse Ig horseradish peroxidase linked whole antibody from sheep (Amersham Life Science) for 1 h. After rinsing, the membrane was treated using an ECL+ Western blotting detection kit (Amersham-Pharmacia) for chemi-luminescence detection. Quantification was performed by densitometric scanning (Molecular Dynamics) ImageQuant (Molecular Dynamics) image analysis.

3. Results

3.1. Methylation resistance of E. coli cells expressing hAAG1 and hAAG2

Fig. 1 shows the effect of hAAG expression on resistance to methylation damage in repair deficient bacteria. *E. coli* strains lacking the ability to carry out BER of alkyl lesions due to inactivation of the *alkA* and *tagA* genes are very sensitive to treatments with the methylating agents MMS and MNNG. In contrast, inactivation of NER by mutation of the *uvrA* gene has little or no effect on survival (Fig. 1). Expression of either the hAAG1 or hAAG2 isoforms results in effective complementation of MMS and MNNG sensitivity indicating both hAAG isoforms are capable of repairing methyl lesions in DNA. Complementation of methylation sensitivity of *alkA tagA* double mutant bacteria by the human glycosylases also indicates that bacterial enzymes of BER downstream of the glycosylase, such as AP endonucleases, polymerase I and ligase, can effectively process repair intermediates produced by the human glycosylase. Moreover, the levels of these bacterial enzymatic activities are sufficiently high to successfully process intermediates generated by hAAG when it is expressed at high levels from the strong pTrc promoter and present on a high copy plasmid. The result that hAAG1 expression can actually increase MNNG resistance of the *alkA tagA* double mutant strain to a level higher than that of the wild type strain suggests that activities of the bacterial glycosylases are the rate limiting factor in repair of methyl lesions (Fig. 1B).

3.2. Levels of hAAG proteins in E. coli

Complementation by hAAG1 appears to be more efficient than that seen when hAAG2 is expressed (Fig. 1A, B). This could either indicate a difference in activities towards methyl damaged DNA, or differences in levels of protein in cells. To test this, histidine tagged forms of hAAG1 and hAAG2 suitable for immuno-detection were constructed; the 5′end of the gene was modified by adding six histidine codons between the last amino acid and the stop codon. The presence of the histidine tag has no apparent effect on the ability of either hAAG1 or hAAG2 to increase recovery from methylation damage, and the differences seen between the two histidine tagged isoforms are essentially identical to those seen when the non tagged forms of the protein are compared (Fig. 1). The histidine tags also do not alter the effects of hAAG proteins on recovery from any of the other DNA damaging agents used in this study (data not shown). The expression of the *hAAG*-$(his)_6$ genes was induced by IPTG treatment. At various times samples were removed, the cells lysed, and levels of hAAG protein measured by Western blot analysis using an anti-histidine antibody (Qiagen). Although both genes are driven from the same promoter and have identical DNA sequences between the promoter and ATG start codon, the expression of hAAG1 is considerably

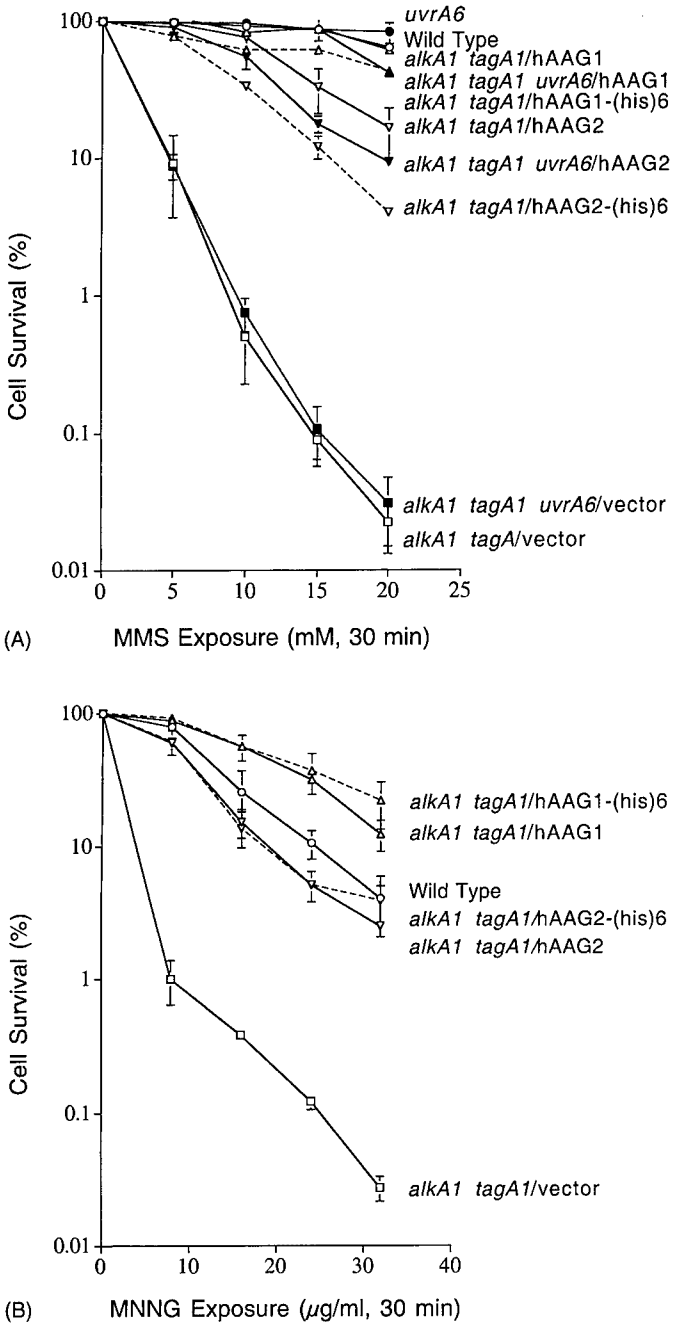


Fig. 1. Effect of hAAG expression on resistance of *E. coli* to methylating agents. Strains designations corresponding to the indicated genotypes are listed in Table 2: (○) wild type, (●) *uvrA*, (■) *alkA tagA uvrA*, (▲) *alkA tagA*/hAAG1, (▼) *alkA tagA uvrA*/hAAG2, (□) *alkA tagA*, (Δ–Δ) *alkA tagA*/hAAG1, (∇–∇) *alkA tagA*/hAAG2. Strains carrying histidine tagged forms of hAAG proteins are indicated as dotted lines, (Δ....Δ) *alkA tagA*/hAAG1-$(his)_6$, (∇....∇) *alkA tagA*/hAAG2-$(his)_6$. (A) MMS exposure. (B) MNNG exposure.

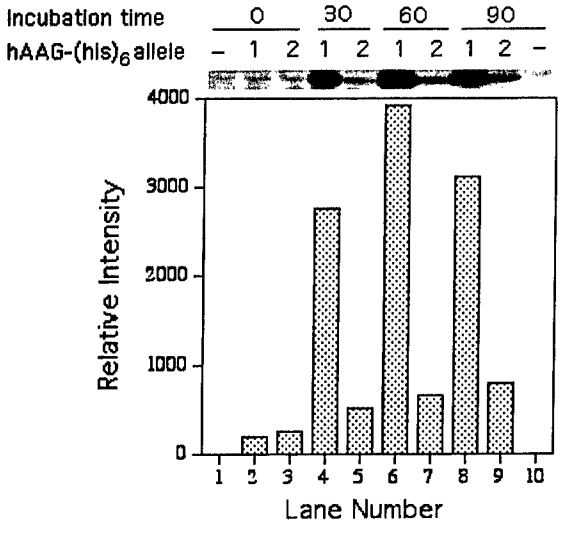


Fig. 2. Steady-state levels of hAAG1-(his)$_6$ and hAAG2-(his)$_6$ proteins. The 0 time samples were processed prior to addition of IPTG to induce hAAG expression from the pTrc99a promoter. All other samples indicate the amount of incubation time after IPTG addition. Relative intensities were determined by densitometic scanning (Molecular Dynamics) and quantitation by ImageQuant software (Molecular Dynamics) after subtracting the average of the intensity of the cross-reacting band present in the two vector control lanes.

higher than that of hAAG2 (Fig. 2). Intensities of the bands, measured by densitometry are shown in Fig. 2. The presence of a weak cross-reacting band migrating to the same position as hAAG is detectable in the vector control lanes. The average of the control intensities was subtracted to obtain the relative intensities of hAAG proteins. These measurements indicate that hAAG1 is present at a six-fold higher level at 60 min and a four-fold higher level at 90 min than hAAG2. The differences in steady-state levels of the two proteins could either be due to differences in the translation efficiency resulting from exon 1 DNA sequence differences, and/or the stability of the two proteins in *E. coli*. Although we can not rule out minor differences in activities of the two enzymes for the methyl substrates based on these results, it is likely that the higher steady-state level of hAAG1 accounts for most, if not all, of the higher level of complementation of the *alkA tagA E. coli* by hAAG1 versus hAAG2.

Both histidine tagged isoforms of hAAG migrate on SDS polyacrylamide gels to a position of approximately 37.5 kDa, consistent with the calculated molecular weights of 32,842 for hAAG1-(his)$_6$ and 32,160 for hAAG2-(his)$_6$. Amino terminal sequencing of hAAG1-(his)$_6$ protein partially purified from strain MV4211 by a nickel affinity column prior to electrophoresis confirms it is produced as a full length protein (Li, Wright, Matijasevic, Chong, Ludlum and Volkert, unpublished observation).

3.3. Effect of hAAG expression on survival from ENNG exposure

We next examined the ability of hAAG1 and hAAG2 to complement sensitivity of *E. coli* to agents that generate larger, more complex alkylation adducts in DNA. In bacteria, ENNG resistance requires both BER and NER pathways, since inactivation of either pathway increases ENNG sensitivity (Fig. 3A). The relative effect of inactivation of each of these bacterial DNA repair pathways on recovery from exposure to ENNG can be seen by comparing the ENNG survival of wild type, *uvrA, alkA tagA* and *alkA tagA uvrA* mutant strains (Fig. 3A). The results demonstrating the sensitization resulting from glycosylase deficiency are consist with the results of others [4,21]. Since both BER and NER contribute to ENNG resistance, we tested the ability of hAAG expression to enhance recovery from exposure to the ethylating agent ENNG in the *uvrA alkA tagA* triple mutant strain. As expected hAAG1 had no effect on alkylation resistance in ENNG treated cells (Fig. 3B) [4]. We find similar results when hAAG2 is tested for its ability to complement the *alkA tagA uvrA* triple mutant strain (Fig. 3B), indicating that neither hAAG isoform is capable of enhancing survival from exposure to ethylating agents, and neither isoform is able to effectively repair ethylated DNA. Thus it appears that the bacterial glycosylases, but not the two isoforms of human glycosylase, can repair ethyl lesions in *E. coli*.

3.4. Effect of hAAG expression on survival from treatment with cross-linking agents

AAG knock out mutants have been produced in mouse ES cells and have been shown to be sensitized to the cross-linking agents BCNU and mitomycin C [5]. We tested the ability of hAAG1 and hAAG2 to enhance recovery of repair deficient *E. coli* from

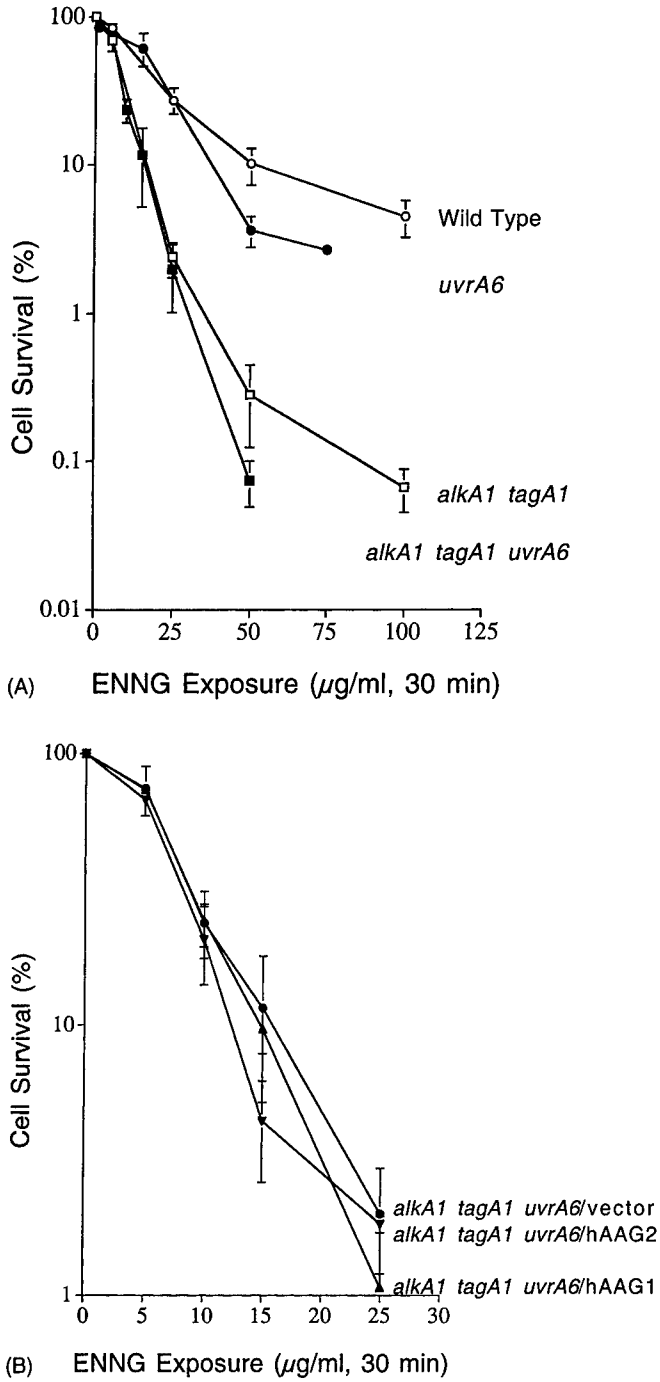
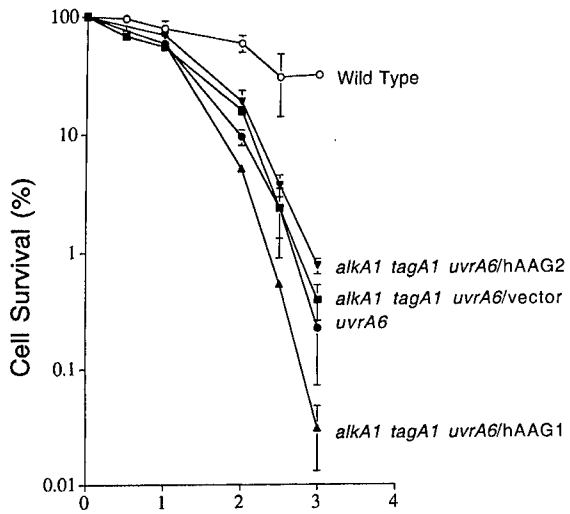


Fig. 3. Effects of hAAG expression on resistance of *E. coli* to ENNG. Symbols are the same as in Fig. 1. (A) Role of NER and BER in ENNG resistance as determined by effects of *uvrA6* and *alkA tag* genes on bacterial resistance to ENNG. (B) Effects of hAAG expression on ENNG survival of *uvrA6 alkA tagA* triple mutants of *E. coli*.

exposure to these two cross-linking agents and from exposure to another cross-linking agent, CNU. Neither bacterial, nor hAAG mediated BER, enhances survival to any of these agents (Fig. 4) indicating that the BER pathway is not effective in protecting *E. coli* from BCNU, mitomycin C, or CNU lethality regardless of whether the glycosylase initiating BER is of bacterial or human origin. A modest but consistent sensitization



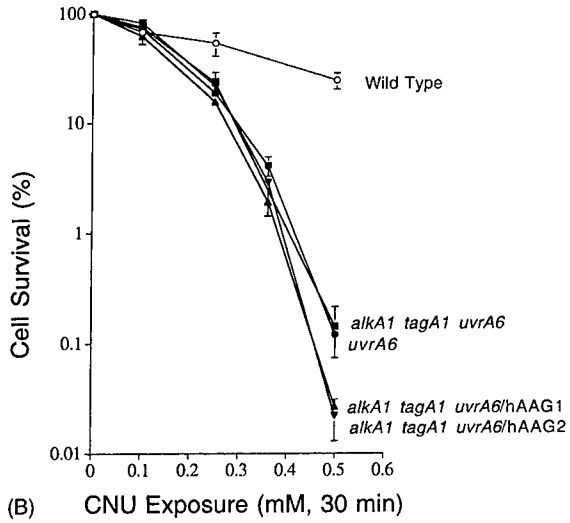


Fig. 4. Effects of hAAG expression on resistance to BCNU, CNU and mitomycin C. Symbols are the same as in Fig. 1. (A) BCNU exposure, (B) CNU exposure, (C) mitomycin C exposure.

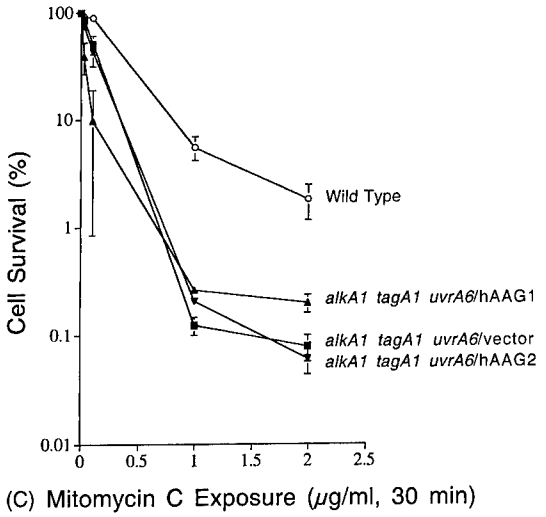


Fig. 4. (*Continued*).

to CNU was seen to result from hAAG expression, suggesting glycosylase activity may have deleterious effects when acting on CNU treated DNA. A similar sensitizing effect is also seen when hAAG1 expressing cells are tested by BCNU sensitivity (Fig. 4A). Thus, it appears that only the *uvrA* gene product is able to enhance recovery of *E. coli* from exposure to cross-linking agents, indicating these lesions are repaired primarily by NER mechanisms.

4. Discussion

We have produced several clones that allow expression of the two known isoforms of hAAG. Based on our results it appears that the two isoforms do not differ in their substrate specificity when expressed in bacteria and that the minor differences seen in the effectiveness of their ability to complement the sensitized bacteria is most likely due to differences in the steady-state levels of the hAAG isoforms. The result that both hAAG isoforms are very effective in their ability to complement the methylation sensitivity of *alkA tagA* double mutant strains indicates that the human proteins are functional when expressed in bacteria and that bacterial enzymes can efficiently process the repair intermediates generated by the human enzymes.

The result that hAAG expressing bacteria appear to be more sensitive to CNU treatment has several possible implications. This suggests that in bacteria, hAAG expression either interferes with other bacterial repair pathways resulting in decreased repair and increased lethality, or that hAAG processes primary lesions to a more lethal form. Either hypothesis requires hAAG to recognize and interact with DNA modified by CNU. A similar sensitization by glycosylase activity has been seen in *E. coli* expressing oxidative damage specific glycosylases upon exposure to ionizing radiation damage [22]. This sensitization appears to be due to the production of lethal repair intermediates by the Fpg, Nth and Nei glycosylases. It has been suggested that the primary ionizing radiation lesions may be more effectively repaired by other means, perhaps direct cleavage of the DNA backbone by an activity of AP endonucleases and that glycosylase activity interferes with this repair [23,24].

Engelward et al. [5], have shown that the AAG knockout mouse ES cells are sensitized to a variety of alkylating agents, including simple methylating as well as more complex alkylating DNA cross-linking agents. However, our results show that expression of functional hAAG does not increase the resistance of bacteria to alkylating agents other than those that methylate DNA. The result that AAG deficiency in mouse ES cells leads to sensitivity to mitomycin C and BCNU suggests that mAAG is able to repair these lesions. Several hypotheses can be proposed to explain this paradox. It is possible that the mammalian AAG enzymes require additional gene products not present in *E. coli* [25]. It is also possible that AAG enzymes are part of a repair complex and that assembly of this complex is required to repair alkyl lesions other than methyl lesions. By this model, the presence of the AAG protein may be required for repair activity in mammalian cells regardless of whether the actual repair is carried out by AAG itself, or some other component of the repair complex. Additional alternative possibilities are that the human form of the enzyme may differ from the mouse enzyme in its substrate specificity such that mouse, but not human, AAG is able to repair mitomycin C and BCNU lesions. It is also possible that the repair activity of the mammalian enzymes may require post translational modifications that do not occur in *E. coli*.

Acknowledgements

We thank Dr. David Ludlum for advice and support throughout this project. This work was supported by the US Army Medical Research and Materiel Command under contract Number DAMD17-00-C-0012 to ZM and by a grant from National Institutes of Health number GM56420 to MRV.

References

[1] D. Chakravarti, G.C. Ibeanu, K. Tano, S. Mitra, Cloning expression in *Escherichia coli* of a human cDNA encoding the DNA repair protein *N*-methylpurine-DNA glycosylase, J. Biol. Chem. 266 (1991) 15710–15715.

[2] T.R. O'Connor, J. Laval, Human cDNA expressing a functional DNA glycosylase excising 3-methyladenine and 7-methylguanine, Biochem. Biophys. Res. Commun. 176 (1991) 1170–1177.

[3] L. Samson, B. Derfler, M. Boosalis, K. Call, Cloning and characterization of a 3-methyladenine DNA glycosylase cDNA from human cells whose gene maps to chromosome 16, Proc. Natl. Acad. Sci. U.S.A. 83 (1991) 5607–5610.

[4] B.P. Engelward, M.S. Boosalis, B.J. Chen, Z. Deng, M.J. Siciliano, L.D. Samson, Cloning and characterization of a mouse 3-methyladenine/7-methyl-guanine/3-methylguanine DNA glycosylase cDNA whose gene maps to chromosome 11, Carcinogenesis 14 (1993) 175–181.

[5] B.P. Engelward, A. Dreslin, J. Christensen, D. Huszar, C. Kurahara, L. Samson, Repair-deficient 3-methyladenine DNA glycosylase homozygous mutant mouse cells have increased sensitivity to alkylation-induced chromosome damage and cell killing, EMBO J. 15 (1996) 945–952.

[6] R.H. Elder, J.G. Jansen, R.J. Weeks, M.A. Willington, B. Deans, A.J. Watson, K.J. Mynett, J.A. Bailey, D.P. Cooper, J.A. Rafferty, M.C. Heeran, S.W. Wijnhoven, A.A. van Zeeland, G.P. Margison, Alkylpurine-DNA-*N*-glycosylase knockout mice show increased susceptibility to induction of mutations by methyl methanesulfonate, Mol. Cell Biol. 18 (1998) 5828–5837.

[7] R.D. Wood, M. Mitchell, J. Sgouros, T. Lindahl, Human DNA repair genes, Science 291 (2001) 1284–1289.

[8] M.A. Vickers, P. Vyas, P.C. Harris, D.L. Simmons, D.R. Higgs, Structure of the human 3-methyladenine DNA glycosylase gene and localization close to the 16p telomere, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 3437–3441.

[9] T.R. O'Connor, Purification and characterization of human 3-methyladenine-DNA glycosylase, Nucl. Acids Res. 21 (1993) 5561–5569.

[10] A. Pendlebury, I.M. Frayling, M.F. Santibanez Koref, G.P. Margison, J.A. Rafferty, Evidence for the simultaneous expression of alternatively spliced alkylpurine *N*-glycosylase transcripts in human tissues and cells, Carcinogenesis 15 (1994) 2957–2960.

[11] P. Karran, T. Hjelmgren, T. Lindahl, Induction of a DNA glycosylase for *N*-methylated purines is part of the adaptive response to alkylating agents, Nature 296 (1982) 770–773.
[12] T.V. McCarthy, P. Karran, T. Lindahl, Inducible repair of *O*-alkylated DNA pyrimidines in *Escherichia coli*, EMBO J. 3 (1984) 545–550.
[13] Y. Habraken, C.A. Carter, M.C. Kirk, D.B. Ludlum, Release of 7-alkylguanines from *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea-modified DNA by 3-methyladenine DNA glycosylase II, Cancer Res. 51 (1991) 499–503.
[14] Z. Matijasevic, M. Sekiguchi, D.B. Ludlum, Release of *N*,2,3-ethenoguanine from chloroacetaldehyde-treated DNA by *Escherichia coli* 3-methyladenine DNA glycosylase II, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 9331–9334.
[15] Z. Matijasevic, A. Stering, T.Q. Niu, P. Austin-Ritchie, D.B. Ludlum, Release of sulfur mustard-modified DNA bases by *Escherichia coli* 3-methyladenine DNA glycosylase II, Carcinogenesis 17 (1996) 2249–2252.
[16] B. Hang, A. Chenna, S. Rao, B. Singer, 1,*N*-6-ethenoadenine and 3,*N*-4-ethenocytosine are excised by separate human DNA glycosylases, Carcinogenesis 17 (1996) 155–157.
[17] W.B. Mattes, C.S. Lee, J. Laval, T.R. O'Connor, Excision of DNA adducts of nitrogen mustards by bacterial and mammalian 3-methyladenine-DNA glycosylases, Carcinogenesis 17 (1996) 643–648.
[18] D.B. Ludlum, Q. Li, Z. Matijasevic, Role of base excision repair in protecting cells from the toxicity of chloroethylnitrosoureas, IARC Sci. Publ. 150 (1999) 271–277.
[19] M.R. Volkert, D.C. Nguyen, Induction of specific *Escherichia coli* genes by sublethal treatments with alkylating agents, Proc. Natl. Acad. Sci. U.S.A. 81 (1984) 4110–4114.
[20] A.J. Clark, M.R. Volkert, L.J. Margossian, A role for *recF* in repair of UV damage to DNA, in: Proceedings of the Cold Spring Harbor Symposium, Cold Spring Harbor, Quant. Biol. 43 (1979) 887–892.
[21] L. Thomas, C.H. Yang, D.A. Goldthwait, Two DNA glycosylases in *Escherichia coli* which release primarily 3-methyladenine, Biochemistry 21 (1982) 1162–1169.
[22] J.O. Blaisdell, S.S. Wallace, Abortive base-excision repair of radiation-induced clustered DNA lesions in *Escherichia coli*, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 7426–7430.
[23] B. Hang, A. Chenna, H. Fraenkel-Conrat, B. Singer, An unusual mechanism for the major human apurinic/apyrimidinic (AP) endonuclease involving 5′ cleavage of DNA containing a benzene-derived exocyclic adduct in the absence of an AP site, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 13737–13741.
[24] A.A. Ischenko, M.K. Saparbaev, Alternative nucleotide incision repair pathway for oxidative DNA damage, Nature 415 (2002) 183–187.
[25] P.E. Gallagher, T.P. Brent, Human 3-methyladenine-DNA glycosylase: demonstration of a stimulatory factor, Biochem. Biophys. Res. Commun. 101 (1981) 956–962.

Selective Protection of Non-cancer Cells by Hypothermia

ZDENKA MATIJASEVIC

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01655-0126, U.S.A.

Reprinted from
ANTICANCER RESEARCH *22:* 3267-3272 (2002)

ANTICANCER RESEARCH 22: 3267-3272 (2002)

Selective Protection of Non-cancer Cells by Hypothermia

ZDENKA MATIJASEVIC

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01655-0126, U.S.A.

Abstract. *Background: A serious limitation in cancer treatments is insufficient selectivity of drugs for cancer cells. We have previously demonstrated that, in contrast to p53-deficient cells, cells with wild-type p53 undergo a reversible cell cycle arrest when incubated at 28°C instead of 37°C. Since most of the human tumors are p53-deficient, it suggests that hypothermia may selectively protect normal cells from cytotoxic treatments that primarily target proliferating cells. Materials and Methods: We have examined the effect of hypothermia on the survival of wild-type and p53-deficient cells exposed to the anti-tumor drug 5-fluorouracil and compared BrdU incorporation at 28°C and 37°C of normal and tumor cells. Results: p53 wild-type fibroblasts, in contrast to p53-deficient cells, survive much higher doses of 5-fluorouracil when incubated at 28°C than at 37°C. Among tumor cells, the loss of the p53 function coincides with the inability to arrest cell cycle progression at low temperature and with increased sensitivity to prolonged hypothermia as a single modality. Conclusion: Hypothermia protects normal cells from cytotoxic treatments and may improve the therapeutic index of chemotherapy by mechanisms based on the differences in cell cycle regulation between normal and tumor cells.*

A major limitation of successful chemotherapy is the toxicity of anti-tumor drugs to normal, non-cancer cells. The sensitivity of normal cells during conventional, and particularly high-dose, systemic chemotherapy is manifested by a wide range of side-effects, some of them very severe. These side-effects are generally the result of toxicity to rapidly reproducing cells in tissue such as the gastrointestinal mucosa and bone marrow. Toxicity for normal hematopoietic stem cells during *ex vivo* chemo-purging of bone marrow for autologous transplantation results in delayed engraftment, increased post-transplantation transfusion requirements, increased risk of infection and bleeding (1, 2). Treatment under conditions that could either selectively increase toxicity for tumor cells or selectively reduce toxicity for non-tumor cells should lessen these side-effects, improve the therapeutic index of treatments and possibly decrease the risk of disease recurrence.

The selectivity of existing anti-cancer drugs for tumor cells is mainly based on the increased sensitivity to apoptosis of cells with de-regulated proliferation, rather than to the specific targeting of proliferative or anti-apoptotic processes in tumor cells (3-5). This selectivity is diminished in many human cancers by defects in the tumor-suppressor p53 pathway that compromise p53-dependent apoptotic death (6, 7). Loss or alteration of p53 function is frequently associated with advanced disease, resistance to conventional treatments, and poor prognosis (8-11). However, this very loss of p53 function may be an advantage through the concomitant impairment of cell cycle control in such cells. While normal cells have the ability to temporarily arrest their growth in response to certain environmental stimuli, p53-deficient cells may fail to do so. Consequently, lack of arrest may render such cells vulnerable to a number of classical anticancer treatments that are more effective against the proliferating than the arrested cells. With the current understanding of molecular mechanisms involved in the regulation of cell cycle progression, cell cycle-based protection of normal cells proposed many years ago by Pardee and James (12) became a more attractive strategy to increase the selectivity of chemotherapy (for review see Ref. 13). Such an approach requires identification of conditions that selectively and reversibly arrest cells with functional p53. The use of low doses of DNA damaging agents to selectively arrest p53 wild-type cells and protect them from microtubule-active drugs was suggested by Blagosklonny *et al.* (14). One problem associated with the use of DNA damaging agents may be the narrow window of doses that would induce selective arrest without causing permanent, lethal or mutagenic, DNA damage in non-cancer cells. A similar strategy exploiting the difference in growth factor dependence between normal and tumor cells (15) may provide reversible, less aggressive conditions for selective arrest.

We have recently discovered that normal human and

Correspondence to: Zdenka Matijasevic, Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, M4 01655-0126, U.S.A. Tel: 508-856-2459, Fax: 508-856-2700, e-mail: zdenka.matijasevic@umassmed.edu

Key Words: Hypothermia, cell cycle, p53, cancer treatment.

0250-7005/2002 $2.00+.40

mouse fibroblasts respond to the hypothermic conditions of 28°C by arresting cell cycle progression (16). This hypothermia-induced cell cycle arrest is reversible and characterized by the accumulation of tumor suppressor p53 protein in cells. Most importantly, this arrest is p53-dependent; cells deficient in p53 continue to progress through the cell cycle (16). Those results suggested that low temperatures might provide non-damaging conditions for selective arrest and protection of non-cancer cells against cytotoxic drugs. This hypothesis is supported by previous studies in mice showing that chlorpromazine-induced hypothermia has a protective effect against chemo- (17,18) as well as radio-toxicity (19). Here we show that p53 wild-type human and mouse fibroblasts incubated at 28°C indeed survive much higher doses of a commonly used antitumor drug, 5-fluorouracil (5-FU), than when incubated at 37°C. Cells that are p53-deficient, on the other hand, show no increase in survival at low temperature. We show that p53-deficient human lymphoma HL-60 and glioma SF126 cells do not arrest, while p53 wild-type MCF-7 breast cancer cells do arrest DNA synthesis at low temperature. Our results also suggest that, even in the absence of treatment with cytotoxic drugs, hypothermia as a single modality may decrease the survival of cell cycle-deregulated tumor cells. Therefore, hypothermia may improve the therapeutic index of chemotherapy by two independent mechanisms, both based on the differences in cell cycle regulation between normal and tumor cells. *Ex vivo* chemo-purging for autologous stem cell transplantation is a treatment that can most directly benefit from therapeutic intervention by hypothermia.

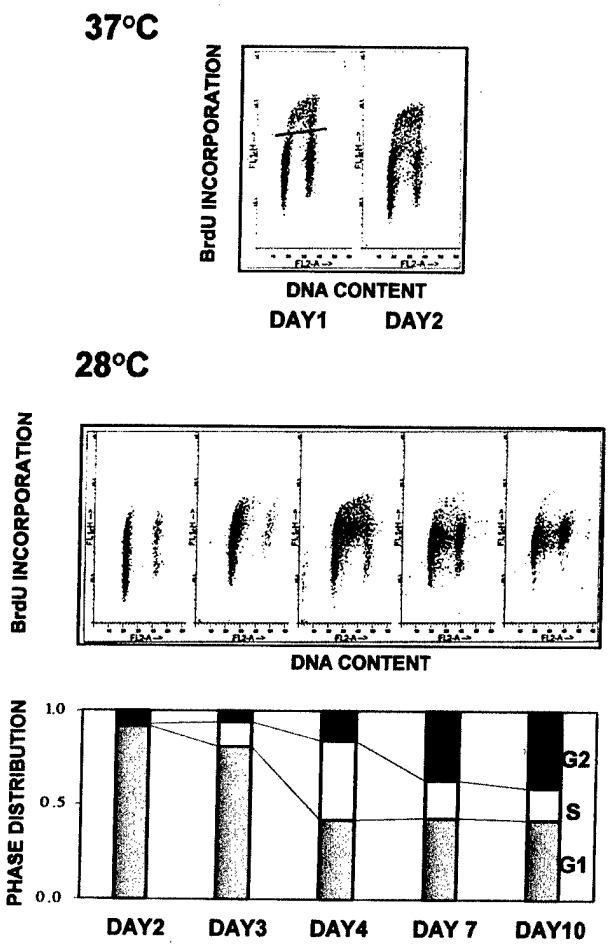


Figure 1. *Effect of hypothermia on cell cycle progression of normal human fibroblasts (NHF). The cells were incubated at 37°C (top panel) or 28°C (middle and bottom panel) and labeled with BrdU for 2 hours. After staining with anti-BrdU-FITC and propidium iodide, the cells were analyzed by flow cytometry. The lower temperature causes a delay in S-phase and an accumulation of cells in G2/M-phase. Bottom panel: fraction of cells in G1 (gray bars), S (open bars) or G2/M (black bars) at 28°C.*

Materials and Methods

Cell lines and culture conditions. The normal human diploid fibroblast cell line, AG01522, was obtained from the Aging Cell Repository, Coriell Institute for Medical Research (Camden, NJ, USA) at a population doubling level (PDL) of 15. Human fibroblasts were cultured as monolayers in Minimum Essential Medium (MEM) supplemented with nonessential amino acids (ICN Pharmaceuticals, Costa Mesa, CA, USA). Mouse embryonic fibroblast cells, wild-type for p53 and p53-null mutants, both at passage 2, were a gift from Stephen N. Jones, University of Massachusetts Medical School, USA (20). These cells were grown as monolayers in Dulbecco's modified Eagle medium (DMEM). Penicillin and streptomycin at concentrations of 100 IU/ml each and 15% heat-inactivated fetal bovine serum (FBS) were added to the media. HL-60 human promyelocytic leukemia cells with deletion in the p53 gene (21) were grown in suspension in RPMI 1640 medium. The human glioma cell line SF-126 with deletion in p53 (22) was a gift from William J. Bodell, Brain Tumor Research Center, University of California, San Francisco, USA. These cells were grown in Minimum Essential Medium (MEM) with 10% FBS and with penicillin and streptomycin. Human breast cancer MCF-7 cells, wild-type for p53 (23), were obtained from ATCC and were cultured in MEM with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate. The medium was supplemented with 0.01mg/ml bovine insulin and with 10% FBS. All media were from Gibco Invitrogen.

Viability determinations. Cells were plated at a density of 2×10^4 cells/cm^2 in 6-well tissue culture plates. Temperature experiments were performed with parallel sets of cells incubated at either 37°C or 28°C in humidified atmospheres with 5% or 4.6% CO_2, respectively. For the 5-fluorouracil (5-FU) treatments, 5-FU (Adrucil, SP Pharmaceuticals LLC, Albuquerque, NM, USA) was added to the media in the indicated concentrations on day one and day three after plating. On day five the cells were harvested by trypsinization and viability was determined by the trypan blue exclusion (TBE) assay. Survival was expressed as a percent of untreated control.

Cell cycle analysis. For cell cycle analysis, the cells were grown to confluency and plated at a density of 2×10^4 cells/cm^2 in T150 tissue culture flasks. The cells were incubated at either 37°C or 28°C for the required period of time. Replicative DNA synthesis was determined using 5-bromo-2'-deoxyuridine (BrdU) incorporation and a modified procedure described by Jones *et al.* (24). The cells were labeled in 13 μM BrdU for 2 hours, harvested by trypsinization, fixed with 70% ethanol and stored at -20°C until analysis. At least 1×10^6 cells were used for the

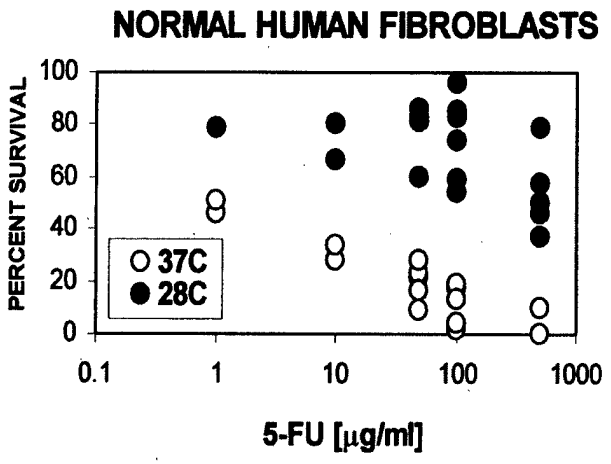


Figure 2. *Protective effect of hypothermia on normal human fibroblasts exposed to 5-fluorouracil. The cells were incubated at 37°C (○) or 28°C (●) with or without 5-fluorouracil and cell viability was determined five days after plating by TBE assay.*

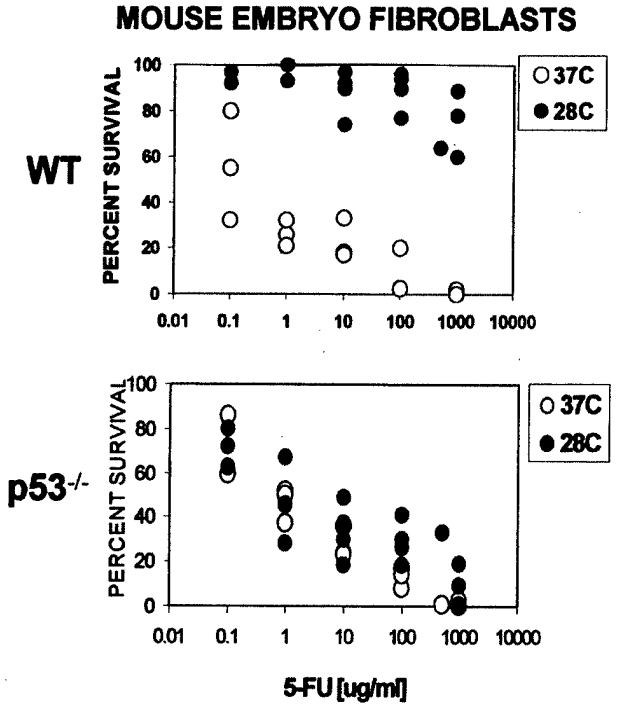


Figure 3. *Protective effect of hypothermia on p53-wild type mouse embryo fibroblasts (MEFs) exposed to 5-fluorouracil. MEF wild-type (upper panel) and p53-null mutant (lower panel) cells were incubated at either 37°C (○) or 28°C (●) with or without 5-fluorouracil. The cell viability was determined five days after plating by TBE assay.*

analysis. The samples were treated with 0.1 N HCl containing 0.5% Triton X-100 for 30 minutes at room temperature, boiled for 2 minutes and rapidly cooled to denature DNA. The cells were then washed twice with PermaCyte (BioErgonomics, White Bear Lake, MN, USA), incubated with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibodies for 30 minutes (Caltag Laboratories, Burlingame, CA, USA) and counterstained with propidium iodide for DNA content. Cell cycle analysis was performed using a Becton-Dickinson FACScan flow cytometer (Mountain View, CA, USA). At least 15,000 events were collected per sample. The percentage of cells in each phase of the cell cycle was determined using Modfit software (Verity Software House, Topsham, ME, USA). The BrdU-incorporating fraction was determined as a FITC-derived fluorescence above the control (BrdU-untreated cells) represented by the events above the horizontal line exemplified in Figure 1 (top panel, day one).

Results

Effect of hypothermia on BrdU incorporation in normal human fibroblasts. Normal mouse embryo fibroblasts (MEF) arrest DNA synthesis for at least 65 hours after plating when incubated at 28°C instead of 37°C (16). Figure 1 shows the results of DNA synthesis measured as BrdU incorporation in a population of normal human fibroblasts (NHF) incubated at 37°C (top panel) or 28°C (middle and bottom panels). Similar to the effects on mouse fibroblasts, there is a delay in DNA synthesis of at least two days in a population of human fibroblasts incubated at 28°C as compared to 37°C. After four days at 28°C, about 40% of cells are in S-phase (Figure 1, bottom panel). Longer incubation at low temperature results in the accumulation of cells in the G2/M-phase. Thus, lower temperature affects DNA synthesis and cell cycle progression of NHF in culture.

Effect of hypothermia on survival of 5-fluorouracil-exposed cells. The arrest or delay in cell cycle progression should affect the sensitivity of cells to certain classes of cytotoxic agents. We predicted that preventing DNA synthesis at low temperature might diminish cell sensitivity to S-phase-specific drugs such as 5-fluorouracil (5-FU). The results in Figure 2 demonstrate that low temperature indeed protects cells against 5-FU toxicity; normal human fibroblasts exposed to a wide range of 5-FU doses survive better if incubated at 28°C than at 37°C after exposure. Cell cycle arrest at low temperature appears to be p53-dependent; MEF cells that are null mutants for p53 fail to arrest at 28°C (16). If cell cycle arrest by hypothermia is indeed responsible for the increased resistance of normal cells to 5-FU, then one could expect a lack of such protection for non-arrested, p53 mutant cells. To test this hypothesis, we examined the effect of temperature on the survival of wild-type and p53-null mutant MEF cells exposed to 5-FU. As shown in Figure 3, hypothermia protects mouse cells with functional p53 from the cytotoxic effects of the drug (top panel). In contrast, this protective effect is not seen in p53-deficient cells (bottom panel).

Effect of p53 status on cell cycle progression at low temperature. The majority of human cancers have defects in the p53 pathway and consequently in regulation of cell cycle progression. It may be possible to take advantage of this

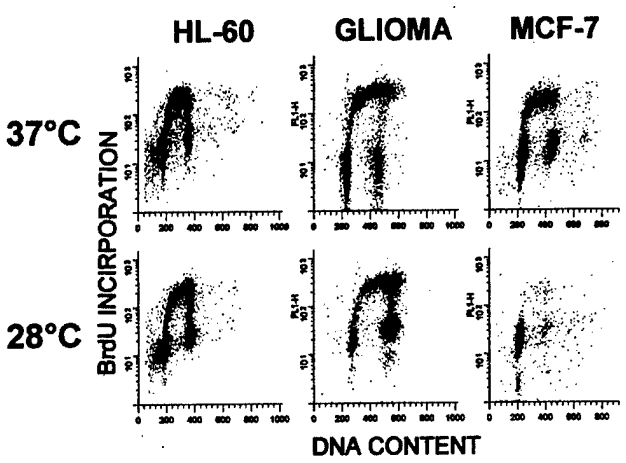


Figure 4. *Cell cycle progression of human tumor cell lines at 37°C and 28°C. The p53-deficient tumor cells, HL-60 and glioma SF126, and p53 wild-type breast cancer MCF-7 cells were incubated for 45 hours at 37°C or 28°C and labeled with BrdU for 2 hours. Ethanol-fixed cells were stained with anti-BrdU-FITC and propidium iodide for FACS analysis.*

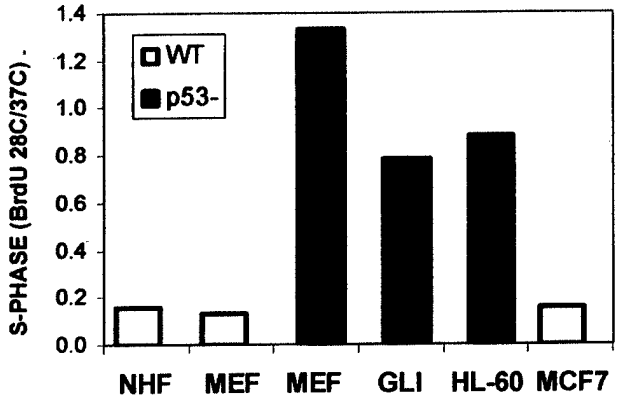


Figure 5. *Effect of p53 status on progression through S-phase under hypothermic conditions. The cells were incubated at 37°C or 28°C for 45 hours and labeled with BrdU for 2 hours. Ethanol-fixed cells were stained with anti-BrdU-FITC and propidium iodide and analyzed by flow cytometry. The results are presented as the ratio between the BrdU-positive cells at 28°C and at 37°C. p53 wild-type cells, open bars; p53-deficient cells, closed bars. NHF, normal human fibroblasts; MEF, mouse embryo fibroblasts; GLI, human glioma SF126 cells; HL-60, human lymphoma cells; MCF7, human breast cancer cells.*

difference between normal and tumor cells to increase the therapeutic index of anti-tumor treatments. To investigate this, we examined progression through S-phase at low temperature in several human tumor cell lines. In Figure 4, BrdU incorporation is shown for cells incubated for two days at either 37°C or 28°C. In two p53-deficient human tumor cell lines, leukemia HL-60 and glioma SF 126 cells, more than 30% of the population at both temperatures is in S-phase with active DNA synthesis. In contrast, in the population of breast cancer MCF-7 cells that have wild-type p53, only 4% of the cells at 28°C actively replicate DNA. There is also a small subpopulation of BrdU-negative MCF-7 cells with S-phase DNA content. Such S-phase-arrested cells are also seen in the population of normal human fibroblasts after several days at 28°C (see Figure 1). The effect of hypothermia on progression through the S-phase for cells with different p53 status is summarized in Figure 5. Clearly, p53-deficient cells do not arrest their DNA synthesis at 28°C.

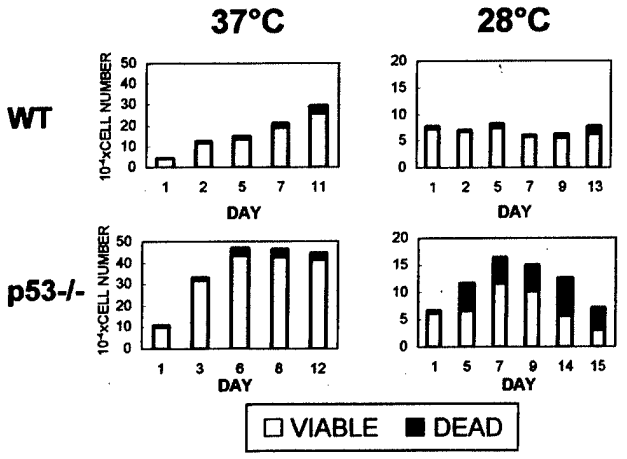


Figure 6. *Loss of p53 function increases the level of cell death under hypothermic conditions. Mouse embryo fibroblasts, wild-type or p53-null mutants, were incubated at 37°C or 28°C and viability was determined by the trypan blue exclusion assay. Dead cells, closed bars; viable cells, open bars.*

Effect of p53 status on survival under conditions of prolonged hypothermia. Since cell cycle arrest at low temperature requires functional p53, it raises the question of how the lack of p53 function, and concomitant lack of cell cycle arrest, affects cell growth and survival under the conditions of hypothermia as a single modality. To test this, p53 wild-type and p53-null mutant mouse embryo fibroblasts were incubated at 37°C and 28°C, and viability was determined by TBE assay. As shown in Figure 6, p53 wild-type cells maintain a constant viable cell number with very few dead cells in the population for the duration of the experiment. In contrast, survival of p53-deficient cells is compromised by prolonged periods of hypothermia. Although total cell number increases during the first several days at 28°C, the number of dead cells increases over time and, at later time-points, exceeds that of viable cells in the population. The results in Figure 7 suggest that at least some p53-deficient human tumor cells may be more sensitive to low temperature as a single modality than

normal cells. At 28°C the number of viable NHF and MCF-7 cells remains constant while the number of glioma and HL-60 cells declines. Thus, sensitivity to hypothermia appears to correlate with the lack of cell cycle arrest at low temperature; HL-60 and glioma cells that do not arrest at 28°C are more sensitive to prolonged hypothermia than normal human fibroblasts or p53 wild-type MCF-7 breast cancer cells that do arrest at 28°C.

Discussion

We have previously demonstrated that normal, p53 wild-type cells respond to hypothermia at 28°C by reversibly arresting cell cycle progression, while p53-deficient cells fail to arrest (16). Here we investigated the effects of hypothermia as a function of p53 status on cell survival. The results demonstrate first, that hypothermia protects p53 wild-type cells from the cytotoxicity of anti-tumor drugs and second, that p53 wild-type cells tolerate prolonged periods of low temperature better than p53-deficient cells. This selective, p53-dependent protective effect of low temperature correlates with the ability to arrest/delay cell cycle progression and is probably due to the activation of two different cell cycle checkpoints by hypothermia. The delayed transition from G1 to S that occurs in normal fibroblasts at 28°C is very likely responsible for the increased resistance to S-phase specific cytotoxic drugs. Increased tolerance to prolonged hypothermia alone, on the other hand, could be due to activation of the G2/M checkpoint that is shown by an accumulation of G2/M cells at 28°C (see Figure 1). Progression into mitosis at low temperature is probably more detrimental for genomic stability than progression through S-phase. Low temperatures compromise tubulin binding to centrosomes (25) and cause microtubule depolymerization (26) that may trigger the G2/M cell cycle arrest. Further studies are needed in order to confirm a p53-dependent nature of hypothermia-induced G2/M arrest and the protective effect of this arrest on cell survival. Lack of G2/M arrest at low temperature may not only selectively sensitize cells to hypothermia alone, but also to M-phase-specific drugs and may further improve the therapeutic index of such treatments.

The relevance of protection by hypothermia that we have observed in cell culture is strongly supported by previous studies (17-19) demonstrating that chlorpromazine-induced hypothermia has a protective effect against radio- and chemotoxicity in animals. Selective arrest of non-cancer cells with proper cell cycle regulation in response to hypothermia, suggested by our results, offers one mechanistic explanation for the protection observed.

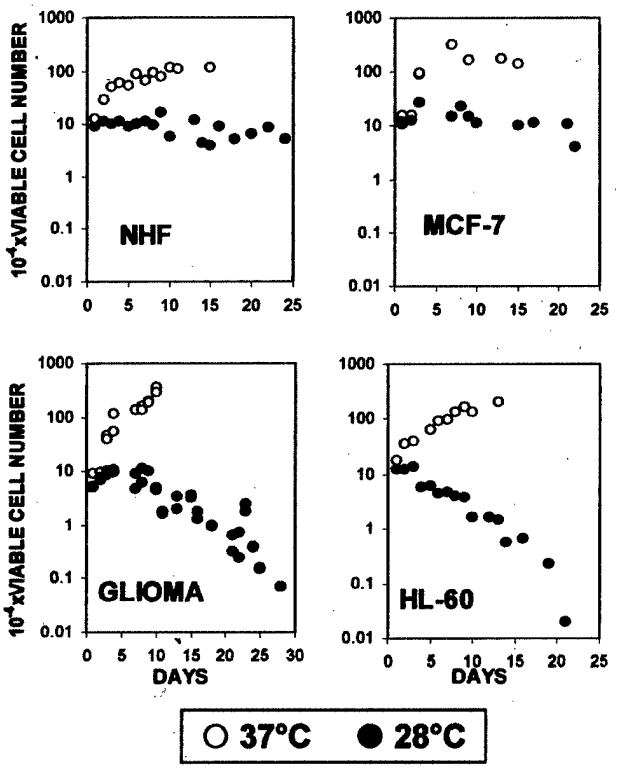


Figure 7. *Survival of normal human fibroblasts and human tumor cells under conditions of prolonged hypothermia. Normal human fibroblasts (NHF), p53 wild-type breast cancer MCF-7 cells, and p53-deficient tumor cells, HL-60 and glioma SF126 cells were incubated at either 37°C (○) or 28°C (●) and cell viability was determined by TBE assay at the indicated times.*

Acknowledgements

The author thanks Christine M. Powers and Alida M. Santandrea for their technical help, David B. Ludlum and Judith Luber-Narod for their helpful discussions and Debra Mullikin-Kilpatrick and Stephen N. Jones for critical reading of the manuscript. This work was supported by the U.S. Army Medical Research and Material Command under Contract DAMD17-00-C-0012.

References

1 Houtenbos I, Bracho F, Davenport V, Slack R, van de Ven C, Suen Y, Killen R, Shen V and Cairo MS: Autologous bone marrow transplantation for childhood acute lymphoblastic leukemia: a novel combined approach consisting of *ex vivo* marrow purging, modulation of multi-drug resistance, induction of autograft *vs.* leukemia effect, and post-transplant immuno- and chemotherapy (PTIC). Bone Marrow Transplant *27:* 145-153, 2001.

2 Martin TG and Linker CA: Autologous stem cell transplantation for acute lymphocytic leukemia in adults. Hematol Oncol Clin North Am *15:* 121-143, 2001.

3 Schmitt CA and Lowe SW: Apoptosis and therapy. J Pathol *187:* 127-137, 1999.

4 Evan G and Littlewood T: A matter of life and cell death. Science *281:* 1317-1322, 1998.

5 Evan GI and Vousden KH: Proliferation, cell cycle and apoptosis in cancer. Nature *411:* 342-348, 2001.

6 Gottlieb TM and Oren M: p53 and apoptosis. Semin Cancer Biol *8:* 359-368, 1998.

7 Weinstein JN, Myers TG, O'Connor PM, Friend SH, Fornace Jr AJ, Kohn KW, Fojo T, Bates SE, Rubinstein LV, Anderson NL, Buolamwini JK, van Osdol WW, Monks AP, Scudiero DA, Sausville EA, Zaharevitz DW, Bunow B, Viswanadhan VN, Johnson GS, Wittes RE and Paull KD: An information-intensive approach to the molecular pharmacology of cancer. Science *275:* 343-349, 1997.

8 Kirsch DG and Kastan MB: Tumor-suppressor p53: implications for tumor development and prognosis. J Clin Oncol *16:* 3158-3168, 1998.

9 Lowe SW, Bodis S, McClatchey A, Remington L, Ruley HE, Fisher DE, Housman DE and Jacks T: p53 status and the efficacy of cancer therapy *in vivo.* Science *266:* 807-810, 1994.

10 Righetti SC, Della Torre G, Pilotti S, Menard S, Ottone F, Colnaghi MI, Pierotti MA, Lavarino C, Cornarotti M, Oriana S, Bohm S, Bresciana GL, Spatti G and Zunino F: A comparative study of p53 gene mutations, protein accumulation, and response to cisplatin-based chemotherapy in advanced ovarian carcinoma. Cancer Res *56:* 689-693, 1996.

11 Cordone I, Masi S, Mauro FR, Soddu S, Morsilli O, Valentini T, Vegna ML, Guglielmi C, Mancini F, Giuliacci S, Sacchi A, Mandelli F and Foa R: p53 expression in B-cell chronic lymphocytic leukemia: a marker of disease progression and poor prognosis. Blood *91:* 4342-4349, 1998.

12 Pardee AB and James LJ: Selective killing of transformed baby hamster kidney (BHK) cells. Proc Natl Acad Sci USA *72:* 4994-4998, 1975.

13 Blagosklonny MV and Pardee AB: Exploiting cancer cell cycling for selective protection of normal cells. Cancer Res *61:* 4301-4305, 2001.

14 Blagosklonny MV, Robey R, Bates S and Fojo T: Pretreatment with DNA-damaging agents permits selective killing of checkpoint-deficient cells by microtubule-active drugs. J Clin Invest *105:* 533-539, 2000.

15 Blagosklonny MV, Bishop PC, Robey R, Fojo T and Bates SE: Loss of cell cycle control allows selective microtubule-active drug-induced Bcl-2 phosphorylation and cytotoxicity in autonomous cancer cells. Cancer Res *60:* 3425-3428, 2000.

16 Matijasevic Z, Snyder JE and Ludlum DB: Hypothermia causes a reversible, p53-mediated cell cycle arrest in cultured fibroblasts. Oncol Res *10:* 605-610, 1998.

17 Gailis L: Protection against doxorubicin toxicity in mice by chlorpromazine hypothermia. Res Commun Chem Pathol Pharmacol *53:* 319-30, 1986.

18 Hultborn R, Lundgren-Eriksson L, Ottosson-Lonn S, Ryd W and Weiss L: Chlorpromazine-induced hypothermia in tumour-bearing mice, acute cytotoxic drug lethality and long-term survival. Acta Oncol *29:* 941-4, 1990.

19 Lundgren-Eriksson L, Palm A, Ryd W, Warnhammar E and Hultborn R: Radio-and chemotoxicity in mice during hypothermia. Anticancer Res *21:* 3269-74, 2001.

20 Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery Jr CA, Butel JS and Bradley A: Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. Nature *356:* 215-221, 1992.

21 Wolf D and Rotter V: Major deletions in the gene encoding the p53 tumor antigen cause lack of p53 expression in HL-60 cells. Proc Natl Acad Sci USA *82:* 790-794, 1985.

22 Chen P, Iavarone A, Fick J, Edwards M, Prados M and Israel MA: Constitutional p53 mutations associated with brain tumors in young adults. Cancer Genet Cytogenet *82:* 106-115, 1995.

23 Zhan Q, Fan S, Bae I, Guillouf C, Liebermann DA, O'Connor PM and Fornace Jr AJ: Induction of bax by gentoxic stress in human cells correlates with normal p53 status and apoptosis. Oncogene *9:* 3743-3751, 1994.

24 Jones SN, Sands AT, Hancock AR, Vogel H, Donehower LA, Linke, SP, Wahl GM and Bradley A: The tumorigenic potential and cell growth characteristics of p53-deficient cells are equivalent in the presence or absence of Mdm2. Proc Natl Acad Sci USA *93:* 14106-14111, 1996.

25 Meda P, Chevrier V, Edde B and Job D: Demonstration and analysis of tubulin binding sites on centrosomes. Biochemistry *36:* 2550-2558, 1997.

26 Cassimeris LU, Wadsworth P and Salmon ED: Dynamics of microtubule depolymerization in monocytes. J Cell Biol *102:* 2023-2032, 1986.

Received June 10, 2002
Accepted July 19, 2002

Carcinogenesis vol.24 no.3 pp.589–593, 2003

The role of human alkyladenine glycosylase in cellular resistance to the chloroethylnitrosoureas

Qiong Li[1], Stephen E.Wright[1], Zdenka Matijasevic[1], Wincha Chong[2], David B.Ludlum[1] and Michael R.Volkert[2,3]

[1]Department of Biochemistry and Molecular Pharmacology and [2]Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA 01655, USA

[3]To whom correspondence should be addressed
Email: michael.volkert@umassmed.edu

To investigate the possible role of glycosylase action in causing tumor resistance, a full-length, histidine-tagged human alkyladenine glycosylase has been purified from the cloned human gene contained in a pTrc99A vector propagated in a *tag alkA* mutant *Escherichia coli*. This human enzyme releases both 3-methyladenine and 7-methylguanine from methylated DNA but in contrast to previous studies of the bacterial AlkA glycosylase, it does not release any adducts from [^{3}H]chloroethylnitrosourea-modified DNA. This finding suggests that the alkyladenine DNA glycosylase-dependent resistance to the toxic effects of the chloroethylnitrosoureas reported previously in the literature may occur by a mechanism other than through direct glycosylase action.

Introduction

The chloroethylnitrosoureas (CNUs) were introduced into clinical practice as antitumor agents in the 1970s. Although they have achieved an important place in the treatment of brain tumors and certain lymphomas, other tumors are usually resistant to their cytotoxic action. Because of the importance of the resistance phenomenon in the treatment of cancer generally, the mechanism of resistance to the CNUs has been investigated in detail. Cytotoxicity is caused by DNA modification, in particular the formation of a cross-link between guanine and cytosine (1). Formation of this cross-link can be prevented by the DNA repair enzyme, O^6-alkylguanine-DNA alkyltransferase (AGT); high cellular levels of AGT are a major cause of resistance (2,3). This mechanism of cytotoxicity and cellular resistance has now become a model for understanding the role of DNA repair in causing tumor resistance. Indeed, protocols designed to decrease cellular levels of AGT and thereby restore tumor sensitivity have reached clinical trial and have been reviewed recently (4).

This mechanism of tumor resistance is analogous to the adaptive response in *Escherichia coli* (5). In these bacteria, Ada removes alkyl groups attached to the O^6 position of guanine whereas a second enzyme, alkyladenine DNA glycosylase (AAG), releases 3- and 7-substituted purines from DNA. That a glycosylase might also be involved in repairing mammalian DNA and in causing tumor resistance was suggested by HPLC analysis of the DNA modifications in resistant tumor cells treated with a CNU (6). These studies showed that other DNA adducts besides the GC cross-link were absent from resistant cells in comparison with sensitive cells, indicating possible glycosylase action (6). Subsequent *in vitro* studies showed that many CNU-modified bases are substrates for the bacterial AlkA protein, 3-methyladenine DNA glycosylase II (7); further supporting the hypothesis that human AAG might be involved in the resistance of tumors to CNUs.

Investigations of the role of mammalian AAGs in reducing the toxicity of CNU to cultured cells have not, however, shown a simple relationship between glycosylase content and cellular resistance. Although a glial cell line resistant to the chloroethylnitrosoureas was found to have higher levels of alkyladenine glycosylase than a sensitive cell line (8), over-expression of human AAG in Chinese hamster ovary cells did not lead to increased resistance to CNUs (9). Comparison of CNU toxicity in wild-type and AAG knock out mouse embryonic stem cells showed a protective effect of AAG (10), but there was no evidence of protection when wild-type and AAG knock out mouse fibroblasts were compared (11).

In contrast to the enzyme alkyltransferase that completes DNA repair in a single step, the action of AAG is only the first step in the repair process; other enzymes are required to repair the abasic site left in DNA by AAG action. Thus, the varying results quoted above could depend on whether or not the glycosylase step is rate limiting in the repair process in these different cells.

As a first step in understanding these results, we have purified human AAG and tested its activity towards CNU-modified DNA. The human AAG gene has been cloned independently by several research groups, but the separate isolates differ in their N-terminal amino acid sequences (12). As these differences could affect substrate specificity, especially in view of the data of Roy *et al.* (13) showing that the N-terminal region of AAG is positioned close to the active site, we felt it was important to study the specificity of a full-length enzyme. The plasmid constructed for this study allows purification of full-length, histidine-tagged human AAG of the isoform described by Samson *et al.* (14). We report that the human enzyme differs from the bacterial enzyme in its activity towards CNU-modified DNA. Both human and bacterial glycosylases are able to remove modified bases from methylated DNA, but the human enzyme has no demonstrable activity towards CNU-modified DNA. As discussed below, this result could indicate either that hAAG requires an additional factor to be active on CNU lesions *in vivo* or that this protein provides cellular protection by a mechanism other than its glycosylase action.

Abbreviations: AAG, alkyladenine DNA glycosylase; AGT, O^6-alkylguanine-DNA alkyltransferase; CNU, *N*-(2-chloroethyl)-*N*-nitrosourea; hAAG, human alkyladenine DNA glycosylase; IPTG, isopropyl β-D-thiogalactopyranoside; m^3A, 3-methyladenine; m^7G, 7-methylguanine; MMS, methyl methanesulfonate; MNU, *N*-methyl-*N*-nitrosourea.

Materials and methods

N-(2-chloro-1,2-[^{3}H]-ethyl)-*N*-nitrosourea ([^{3}H]-CNU), sp. act. 1.3 Ci/mmol, was custom synthesized by Moravek Biochemicals (La Brea, CA). *N*-([^{3}H-methyl)-*N*-nitrosourea ([^{3}H]-MNU) with a specific activity of 17.0 Ci/mmol

Table 1. Bacterial strains

Strain name	Relevant genotype	Plasmid
MV1176	*uvrA6*	None
MV2157	*alkA1 tagA1*	None
MV4122	*alkA1 tagA1*	pMV509
MV4126	*alkA1 tagA1*	pMV513
MV4211	*alkA1 tagA1 ompT*	pMV513
MV4236	*alkA1 tagA1 uvrA6*	pTrc99a
MV4237	*alkA1 tagA1 uvrA6*	pMV509
MV4238	*alkA1 tagA1 uvrA6*	pMV513

was purchased from Amersham (Arlington Heights, IL). Unlabeled CNU was provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Optical markers of 3-methyladenine (m^3A) and 7-methylguanine (m^7G) used in the HPLC separations were obtained from Cyclo Chemical (Los Angeles, CA). Other chemicals were reagent grade materials.

[^{3}H]MNU- and [^{3}H]CNU-modified DNA were prepared by alkylating calf thymus DNA with the radiolabeled MNU and CNU as described previously (15). Purification and assay of *E.coli* AlkA glycosylase and assay of human glycosylase also followed our published procedures (7) except that bacterial glycosylase was assayed in its optimum buffer (70 mM Tris, pH 7.6, containing 10 mM NaEDTA and 2 mM dithiothreitol) whereas human glycosylase was assayed in its slightly different optimum buffer (50 mM HEPES, pH 7.5, containing 1 mM NaEDTA, 5 mM mercaptoethanol and 100 mM KCl).

Bacterial strains

Bacterial strains used in this study and their relevant genotypes are listed in Table I. Plasmids are described in detail below.

Construction of plasmids expressing human glycosylase

Our constructs started with plasmid pBU16, a gift from Professor Leona Samson (Massachusetts Institute of Technology, MA). Plasmid pBU16 carries a *Bsu*36I–*Xba*I fragment from pKT218 that encodes the human alkyladenine DNA glycosylase gene (*hAAG*) (14) in the pSL301 vector (Invitrogen, San Diego, CA). We subcloned the *hAAG*-bearing *Eco*RI–*Bst*EII fragment from pBU16 into pTrc99A (Pharmacia Biotech, Piscataway, NJ) to produce plasmid pMV503. The pTrc99A vector carries the *lacI* gene of *E.coli* and has the synthetic *trc* promoter under *lac* operator control positioned upstream of the *Eco*RI site. The presence of *lacI* on the plasmid results in tight repression of the promoter that can be induced by the addition of the *lac* inducer isopropyl β-D-thiogalactopyranoside (IPTG), thus allowing IPTG-inducible expression of *hAAG*.

Plasmid pBU16 produces a fusion protein of *hAAG* and vector-encoded sequences 5′ to *hAAG*. To remove the pBU16 vector coding sequences from the 5′ end of the *hAAG* gene, the *Eco*RI–*Bst*EII fragment of pMV503 was removed by first digesting to completion with *Eco*RI, then partially digesting with *Bst*EII to produce a 5152 bp fragment. This fragment was purified from a 0.8% agarose gel using the Gene Clean System (Bio101, La Jolla, CA). Oligonucleotides MV1 (AATTCTAAGGAGGTATCTAATG) and MV2 (GTGACCATTAGATACCTCCTTAG) were first annealed to one another by heating and slow cooling, then ligated to the purified 5152 bp *Eco*RI–*Bst*EII fragment of pMV503 to produce plasmid pMV509. Oligonucleotides MV1 and MV2 have four important features: (i) they are complementary to one another; (ii) when annealed, they produce single-stranded DNA ends complementary to the *Eco*RI and *Bst*EII sites of pMV503; (iii) they reconstruct the ATG initiation codon of *hAAG* that lies within the *Bst*EII site; and (iv) they contain a consensus ribosome binding site, AGGAGG, appropriately positioned to allow optimal translation initiation from the downstream ATG initiation codon. The resulting plasmid, pMV509, carries the wild-type *hAAG* under the control of the IPTG-inducible *trc* promoter of pTrc99A. Construction of pMV509 was tested by loss of the *Sal*I restriction site that lies between *Eco*RI and *Bst*EII of pMV503 and restoration of the *Eco*RI and *Bst*EII sites. The hAAG sequence was confirmed independently.

To construct the histidine-tagged *hAAG* gene, pMV509 was digested to completion with *Cel*II and *Hin*DIII to remove the 3′ end of the gene and the resulting 5064 bp fragment was purified from a 0.8% agarose gel as described above to remove the 3′ end of the gene. Oligonucleotides MV3 (TGAGCAGGACACACAGGCCCATCATCATCATCATCACTGA) and MV4 (AGCTTCAGTGATGATGATGATGATGGGCCTGTGTGTCCTGC) were first annealed to one another as described above, then ligated to the 5064 bp fragment of pMV509 to produce plasmid pMV513. Oligonucleotides MV3 and MV4 have four important features: (i) they are complementary to one another; (ii) when annealed, they produce single-stranded ends complementary to *Cel*II and *Hin*DIII; (iii) they restore the last seven amino acid codons of the *hAAG* gene in the appropriate reading frame; and (iv) they add six histidine codons and one stop codon to the 3′ end of the *hAAG* gene. Construction of pMV513 was tested by loss of the *Mlu*I restriction site that lies within the *Cel*II–*Hin*DIII fragment of pMV509 and restoration of the *Cel*II and *Hin*DIII sites, and DNA sequencing of the insert.

Effect of plasmids expressing hAAG on sensitivity to methyl methanesulfonate

As the *hAAG* gene was initially cloned by functional complementation of an *E.coli* mutant strain lacking the *alkA* and *tag* DNA glycosylase genes (14), the function of the wild-type and histidine-tagged hAAG genes was tested by assessing the ability of pMV509 and pMV513 to complement the alkylation sensitivity of the *alkA1 tag-1 E.coli* double mutant. Strain MV2157 (*alkA1 tag-1*), and its isogenic, plasmid-bearing derivatives, MV4122 (MV2157/pMV509) and MV4126 (MV2157/pMV513), or their *uvrA6* derivatives, MV4236 (*alkA1 tag-1 uvrA6*/pTrc99 vector), MV4237 (*alkA1 tag-1 uvrA6*/pMV509), MV4238 (*alkA1 tag-1 uvrA6*/pMV513), were grown to early-log phase in LB ampicillin media at 37°C. Cultures were then divided into two aliquots, one of which was induced with IPTG (2 mM) and incubated at 37°C for an additional 90 min to allow induction. Cultures were then treated with methyl methanesulfonate (MMS) (20 mM) for 30 min at 37°C. After treatment, cells were diluted 1:100 in buffer containing sodium thiosulfate (4%) to inactivate MMS (16). Cultures were immediately diluted further and plated on LB ampicillin agar plates to assess cell survival. The results in Table II demonstrate that both plasmids are able to restore MMS resistance to MV2157 after IPTG treatment as expected if both the wild-type and histidine-tagged hAAG genes were inducible and active. Moreover, the level of resistance attained is similar, indicating that the addition of a histidine tag has not reduced activity of the hAAG enzyme.

Purification of glycosylases

Bacterial glycosylase II, the alkA protein, was isolated from *E.coli* MS23, which harbors the pYN1000 plasmid containing the *AlkA* gene (17). Lysed cells were treated with DEAE cellulose to remove DNA and the enzyme was purified through the phosphocellulose and DNA cellulose chromatography steps as described (17). The final product migrated as a single band with an apparent molecular weight of 39 000 Da as visualized by silver staining on a 12.5% polyacrylamide gel with 0.1% sodium dodecyl sulfate. This enzyme and the human glycosylase were assayed with [^{3}H]-MNU-DNA as described previously (18). One unit of enzyme activity was defined as the amount of enzyme that released 1 pmol of bases from [^{3}H]-MNU-DNA in 10 min at 37°C.

The hAAG-$(his)_6$ enzyme was isolated from strain MV4211 (*alkA1 tag-1 ompT*/pMV513). Cells were grown to a Klett reading of 70 (~5×10^8 cells/ml) in LB broth containing ampicillin (100 μg/ml), then induced by the addition of IPTG (2 mM). Fresh ampicillin (100 μg/ml) was also added at this time to insure plasmid maintenance, and cells were incubated for an additional 5 h.

After incubation, cells (8 g) were centrifuged, washed in saline buffer (10 mM Tris–Cl, pH 7.4, 1 mM EDTA, 100 mM NaCl) and lysed using a Kraft homogenizer followed by a French press. Crude cell extracts were then centrifuged at 12 000 *g* and the supernatant recovered. The supernatant was mixed with 24 ml of a 50% slurry of Ni–agarose (Qiagen) and, after stirring on ice for 1 h, was poured into a 1.6×24 cm column equilibrated with column buffer (5 mM Na_2HPO_4, pH 8.0, 300 mM NaCl, 10% glycerol). The column was washed with 200 ml of column buffer followed by 300 ml of 30 mM imidazole in column buffer, and finally eluted with a 100 ml gradient of 30–500 mM imidazole in column buffer. Two milliliter fractions were collected and assayed for glycosylase activity with [^{3}H]MNU-modified DNA. A sharp peak of activity appeared around fraction 14; fractions containing high activity were analyzed by gel electrophoresis as described in the Results section.

Substrate specificity of the human glycosylase

To obtain an HPLC profile of [^{3}H]MNU-modified DNA, a sample of the [^{3}H]MNU-DNA substrate containing 136 000 c.p.m. (49 μg) of [^{3}H]methyl adducts was depurinated in 0.1 N HCl for 18 h at 37°C. The hydrolysate was adjusted to pH 4.5 with 1 N NaOH and passed through a 2 ml A25 ion exchange column to remove oligonucleotides. Optical markers of m^3A and m^7G were added and an aliquot was separated on a C_{18} column eluted with a 50 mM KH_2PO_4, pH 4.5 acetonitrile buffer system at 1 ml/min. One minute fractions were collected, counted in a Beckman liquid scintillation counter, and plotted versus fraction number. Similarly, spontaneous release from a separate sample of [^{3}H]MNU-DNA substrate containing 136 000 c.p.m. was determined after incubation for 1 h at 37°C in buffer (50 mM HEPES, pH 7.5, containing 1 mM NaEDTA, 5 mM mercaptoethanol and 100 mM KCl). Enzymatic release from [^{3}H]MNU-DNA by 0.15 U of bacterial or human enzyme was determined after a 1 h incubation at 37°C in the same buffer. To investigate the activity of the bacterial and human enzymes for [^{3}H]CNU-DNA, 3×10^4 c.p.m. of [^{3}H]CNU-DNA were incubated with increasing

Table II. Effect of genotype on cell survival[a]

Genotype	MV1176	MV4236	MV4237	MV4238
alkA	+	–	–	–
tagA	+	–	–	–
uvrA	–	–	–	–
Plasmid	–	vector	pMV509 (hAAG)	pMV513 [(hAAG-(his)$_6$)]
Survival after 20 mM MMS	89%	0.04%	42.4%	46.5%
Survival after 0.5 mM CNU	0.12%	0.13%	0.023%	0.048%

[a]As percent of growth of unexposed cultures.

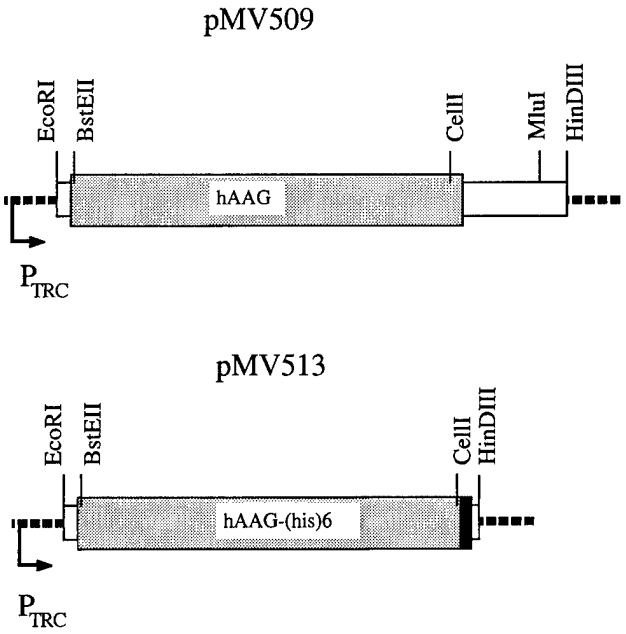


Fig. 1. Insert regions of the hAAG expression vector pMV509 (top) and the hAAG-(his)$_6$ expression vector pMV513 (bottom). Figures are drawn on the same scale. The P_{TRC} promoter is indicated by the rightward arrow labeled P_{TRC}, the hAAG coding sequences are indicated by the gray boxes, the histidine tag is indicated by the black box, and the ends of the pTrc vector sequences are indicated as heavy dotted lines.

amounts of the enzymes for 10 min at 37°C, conditions under which the bacterial enzyme release is 30% complete and spontaneous release is low (19).

Results

The design of plasmids pMV509 and pMV513 is shown in Figure 1. Both plasmids contain the full-length sequence for the human glycosylase gene; pMV513 also contains a (his)$_6$ insert at the C-terminus of the protein to facilitate its purification. This insert was positioned at the C-terminus rather than at the N-terminus because the amino end of the protein has been reported to be located near the active site (13), and a (his)$_6$ insert at this position might affect the specificity of the glycosylase. As described in the Materials and methods, the pTrc99A vector (Pharmacia Biotech) carries the *lacI* gene of *E.coli* and has the synthetic *trc* promoter under *lac* operator control positioned upstream of the *Eco*RI site to control transcription. The presence of the wild-type *hAAG* gene in the plasmids was tested by restriction digestion with *Bst*EII, *Cel*II, *Bgl*I, *Eco*RI, *Hin*DIII and *Sac*I followed by gel electrophoresis and confirmed by DNA sequencing.

The protective activity of pMV509 and pMV513 when these plasmids were propagated in *E.coli* cells deficient in glycosylase activity is shown in Table II. The doses of MMS and CNU used produced nearly identical levels of survival in the repair-deficient control strain MV4236. The base excision-proficient, nucleotide excision-deficient *E.coli* strain MV1176 shows an 89% survival after exposure to 20 mM MMS and a much lower, but appreciable, survival after exposure to 0.5 mM CNU. Survival after exposure to MMS and CNU in the repair-deficient control cells, *E.coli* MV4236 that lack glycosylase and excision repair capabilities but that harbor the unmodified vector, was lower. The presence of plasmids pMV509 and pMV513, carrying hAAG and hAAG-(his)$_6$, respectively, greatly increased survival after exposure to MMS in the repair-deficient strain, but did not seem to increase survival after exposure to CNU. In fact when hAAG is expressed in *E.coli*, it results in a small but reproducible decrease in survival, suggesting hAAG expression may either interfere with other DNA repair mechanisms active on CNU lesions, or convert CNU lesions to a more toxic form. This is similar to the glycosylase-mediated sensitivity to a variety of alkylators described by others [Matijasevic and Volkert, unpublished results; (20)]. Similar levels of MMS resistance and CNU sensitization were obtained regardless of the presence or absence of the histidine tag, thereby eliminating the possibility that the presence of the tag affects hAAG activity towards MMS or CNU lesions. Note that in this regard the glycosylase was originally identified by its ability to protect against the toxic effects of methylating and not chloroethylating agents.

In order to avoid contamination with bacterial glycosylases in purifying hAAG, plasmids containing the hAAG gene were propagated in a glycosylase-deficient strain of *E.coli*, MV2157, which lacks both the *tag* and *alkA* genes. Attempts to purify human glycosylase from pMV509-carrying cells using classical separation methods resulted in poor yields of the enzyme. Accordingly, further purification studies were performed using pMV513 that produces the hAAG enzyme with a (his)$_6$ tail.

An active glycosylase was obtained when human enzyme was isolated on a Ni–agarose column from *E.coli* MV4126, which harbors this plasmid. However, N-terminal amino acid analysis revealed that the enzyme had been truncated between two arginines near the amino acid end of the protein. This site of cleavage indicated that the enzyme that was responsible for this truncation was probably the OmpT protease found in the outer membrane of *E.coli* cells (21). Accordingly, the *OmpT* gene in MV4126 was disrupted by the introduction of an allele of *OmpT* containing an insertion element expressing kanamycin resistance. This host cell was designated MV4211.

The construct pMV513 that contains the human glycosylase was grown in these cells and purified through a Ni–agarose column step as described in the Materials and methods. A typical separation is shown in the upper panel of Figure 2.

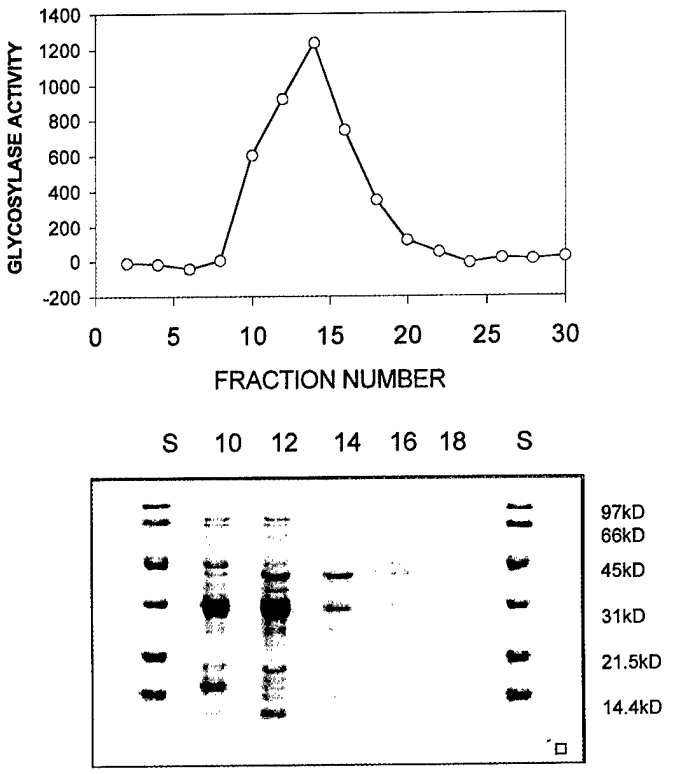


Fig. 2. (Top) Elution profile of the hAAG-(his)$_6$ enzyme from a Ni–agarose column. (Bottom) Gel separations of individual enzyme fractions 10–18. Molecular weight standards are in lanes marked S. N-terminal analysis was performed on the 39 kDa band from fraction 14.

Fractions containing enzyme activity were concentrated using 3K Microsep microconcentrators (Pall Filtron Corporation, Northboro, MA), and separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% gel as shown in the lower panel of Figure 2. Protein bands from the SDS–PAGE gel were transferred to a Millipore Immobilon-P^{SQ} transfer membrane (0.1 μm pore size) of polyvinylidene fluoride (PVDF) in preparation for N-terminal analysis. N-terminal sequencing by Edman degradation using an Applied Biosystems 494 Procise protein sequencer showed that the 39 kDa band from fraction 14 terminated in VTPALQMKKP in agreement with the sequence found by Samson *et al.* (14). The apparent molecular weight is also in agreement with the value obtained for this enzyme by O'Connor (22), who also reported purification of intact full-length human glycosylase. The contaminating protein at 31 kDa was identified by N-terminal analysis as the histidine-rich *E.coli* protein, SlyD.

The activity of this glycosylase towards [^{3}H]MNU-modified DNA substrate is shown in Figure 3. It is clear from this figure that hAAG releases both m^{3}A and m^{7}G from [^{3}H]MNU-DNA; although the human enzyme shows similar activity towards m^{3}A, it shows somewhat lower activity towards m^{7}G than the bacterial glycosylase. This result also agrees with O'Connor's data on purified human glycosylase (22).

With active full-length human glycosylase available, it was now possible to test the activity of hAAG towards chloroethylnitrosourea-modified DNA. Bacterial and human enzymes were incubated with [^{3}H]-CNU-modified DNA for 10 min at 37°C and the c.p.m. of adducted bases released into the supernatant were measured as shown in Figure 4. While the bacterial enzyme released CNU-adducted bases in an

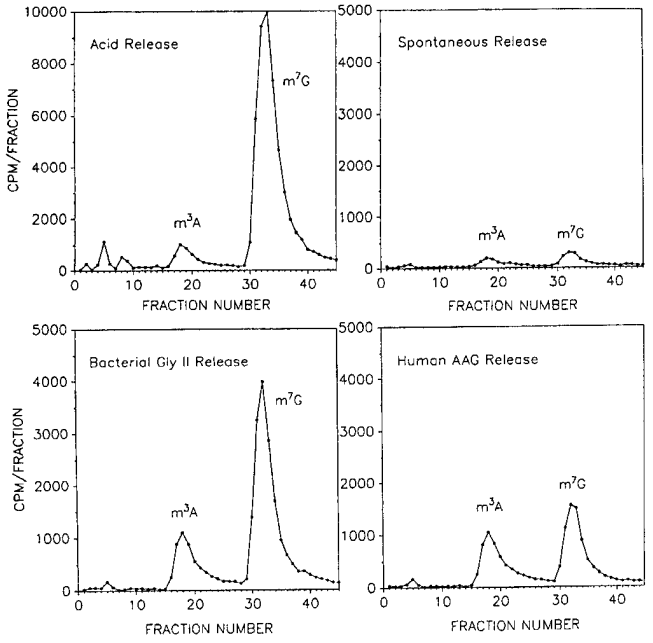


Fig. 3. HPLC profiles of bases released from methylated DNA by acid treatment, by spontaneous release, by 0.15 U of bacterial enzyme, and by 0.15 U of the hAAG-(his)$_6$ enzyme. Incubation mixtures contained 49 μg of DNA with 1.36×10^5 c.p.m. of [^{3}H]-MNU adducts in 500 μl of buffer solution. Optical markers are shown for peaks corresponding to m^{3}A and m^{7}G.

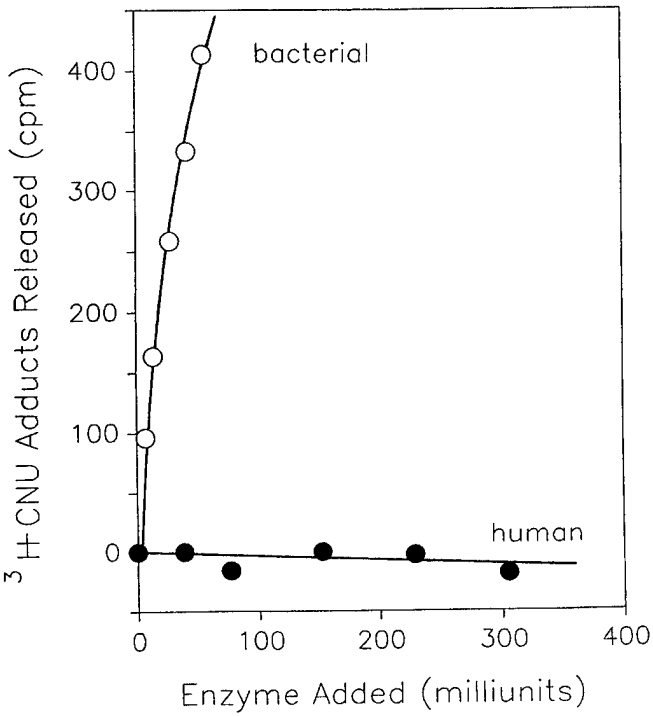


Fig. 4. Activity of bacterial (open circle) and histidine-tagged human enzyme (shaded circle), towards ^{3}H-CNU-modified DNA. Incubation mixtures contained 27 μg of DNA with 2×10^4 c.p.m. of [^{3}H]-CNU adducts and the indicated number of units of bacterial or human glycosylase in 150 μl of buffer solution. Incubations were for 10 min at 37°C.

enzyme-dependent way, there was absolutely no evidence for their release by the human enzyme even when much higher levels of enzyme were added to the incubation mixture. Thus, the hAAG has no *in vitro* activity towards [^{3}H]-CNU-modified DNA under conditions where the bacterial enzyme is highly

active suggesting that other factors must be involved in the cellular activity of hAAG as discussed below.

Discussion

After the construction of pMV513 and the development of the *E.coli* host cells MV4211, human alkyladenine glycosylase was successfully isolated from a Ni–agarose column as described above. This has made it possible to study the specificity of a full-length human alkyladenine glycosylase on CNU-modified DNA *in vitro*. In view of our earlier results showing that bacterial 3-methyl-adenine DNA glycosylase II releases a wide range of CNU-modified bases (7), we were indeed surprised at the lack of activity of the human enzyme towards CNU-modified DNA.

We had thought that hAAG might protect against the toxic effects of CNU in a manner similar to the bacterial glycosylase, which releases CNU-adducted bases (15,23). Although the data of Allan *et al.* (10) show that the presence of mouse AAG decreases the sensitivity of mouse cells to chloroethylnitrosoureas and the data of Matijasevic *et al.* suggest that hAAG may play a role in the resistance of human tumors to CNU (8) human AAG does not seem to release chloroethylnitrosourea-modified bases from CNU-modified DNA *in vitro*. Possible explanations for this could be that: (i) hAAG releases an extremely toxic base that is present in such small amounts that its release is not apparent in our experiments with [^{3}H]-CNU-modified DNA; (ii) hAAG requires the presence of an additional cellular factor to act on CNU-modified DNA; (iii) the activity of hAAG is affected by post-translational modification that differs in mammalian and *E.coli* host cells; or (iv) the hAAG protein serves some additional function besides base recognition and release in the cellular environment. The possibility of additional factors, post-translational modification or additional functions of hAAG must be specific for its role in repair of CNU lesions and do not apply to its interactions with methyl lesions, as the enzyme effectively protects against MMS exposure and methyl lesions are efficiently removed by the purified hAAG enzyme (Table II and Figure 3).

Unless there are other minor but very toxic DNA adducts that we have not detected, possibilities (ii)–(iv) seem more likely. However, for possibility (ii) to explain our results we would have to conclude that some factor in *E.coli* cells can selectively activate, or some factor from mammalian cells is not needed to activate the human enzyme towards repair of methyl lesions, because the human gene was identified by complementation of glycosylase-negative *E.coli* cells (14) and it is clearly active against this type of DNA damage (Table II and Figure 3). Post-translational modification of the enzyme has not been reported, but cannot be ruled out. The final possibility, number (iv), is intriguing but again there is no evidence for such a function. Because of the importance of the resistance problem in treating human tumors, further experimentation is needed to discriminate between these possibilities.

Acknowledgements

The authors would like to express their thanks to Professor Leona Samson for supplying the cloned human glycosylase and Kenneth Bonanno for technical assistance. Supported by NIH grant CA44499, by the Massachusetts Division of the American Cancer Society, and by the US Army Medical Research and Materiel Command under Contracts DAMD17-96-C-6073 and DAMD17-00-C-0012. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the views of the supporting organizations.

References

1. Tong,W.P., Kirk,M.C. and Ludlum,D.B. (1982) Formation of the cross-link 1-[N3-deoxycytidyl],2-[N1-deoxyguanosinyl]ethane in DNA treated with N,N′-bis (2-chloroethyl)-N-nitrosourea. *Cancer Res.*, **42**, 3102–3105.
2. Erickson,L.C., Laurent,G., Sharkey,N.A. and Kohn,K.W. (1980) DNA cross-linking and monoadduct repair in nitrosourea-treated human tumour cells. *Nature*, **288**, 727–729.
3. Pegg,A.E., Dolan,M.E. and Moschel,R.C. (1995) Structure, function and inhibition of O6-alkylguanine-DNA alkyltransferase. *Prog. Nucleic Acid Res. Mol. Biol.*, **51**, 167–223.
4. Gerson,S.L. (2002) Clinical relevance of MGMT in the treatment of cancer. *J. Clin. Oncol.*, **20**, 2388–2399.
5. Samson,L. (1992) The suicidal DNA repair methyltransferases of microbes. *Mol. Microbiol.*, **6**, 825–831.
6. Bodell,W.J., Tokuda,K. and Ludlum,D.B. (1988) Differences in DNA alkylation products formed in sensitive and resistant human glioma cells treated with N-(2-chloroethyl)-N-nitrosourea. *Cancer Res.*, **48**, 4489–4492.
7. Ludlum,D.B., Li,Q. and Matijasevic,Z. (1999) *Role of Base Excision Repair in Protecting Cells from the Toxicity of Chloroethylnitrosoureas*. IARC Scientific Publications, IARC, Lyon, Vol. 150, pp. 271–277.
8. Matijasevic,Z., Bodell,W.J. and Ludlum,D.B. (1991) 3-Methyladenine DNA glycosylase activity in a glial cell line sensitive to the haloethylnitrosoureas in comparison with a resistant cell line. *Cancer Res.*, **51**, 1568–1570.
9. Bramson,J., O'Connor,T. and Panasci,L. (1995) Effect of alkyl-N-purine DNA glycosylase overexpression on cellular resistance to bifunctional alkylating agents. *Biochem. Pharmacol.*, **50**, 39–44.
10. Allan,J.M., Engelward,B.P., Dreslin,A.J., Wyatt,M.D., Tomasz,M. and Samson,L.D. (1998) Mammalian 3-methyladenine DNA glycosylase protects against the toxicity and clastogenicity of certain chemotherapeutic DNA cross-linking agents. *Cancer Res.*, **58**, 3965–3973.
11. Elder,R.H., Jansen,J.G., Weeks,R.J. *et al.* (1998) Alkylpurine-DNA-N-glycosylase knockout mice show increased susceptibility to induction of mutations by methyl methanesulfonate. *Mol. Cell. Biol.*, **18**, 5828–5837.
12. Vickers,M.A., Vyas,P., Harris,P.C., Simmons,D.L. and Higgs,D.R. (1993) Structure of the human 3-methyladenine DNA glycosylase gene and localization close to the 16p telomere. *Proc. Natl Acad. Sci. USA*, **90**, 3437–3441.
13. Roy,R., Kumar,A., Lee,J.C. and Mitra,S. (1996) The domains of mammalian base excision repair enzyme N-methylpurine-DNA glycosylase. Interaction, conformational change and role in DNA binding and damage recognition. *J. Biol. Chem.*, **271**, 23690–23697.
14. Samson,L., Derfler,B., Boosalis,M. and Call,K. (1991) Cloning and characterization of a 3-methyladenine DNA glycosylase cDNA from human cells whose gene maps to chromosome 16. *Proc. Natl Acad. Sci. USA*, **83**, 5607–5610.
15. Habraken,Y., Carter,C.A., Sekiguchi,M. and Ludlum,D.B. (1991) Release of N2,3-ethanoguanine from haloethylnitrosourea-treated DNA by *Escherichia coli* 3-methyladenine DNA glycosylase II. *Carcinogenesis*, **12**, 1971–1973.
16. Volkert,M.R. and Nguyen,D.C. (1984) Induction of specific *Escherichia coli* genes by sublethal treatments with alkylating agents. *Proc. Natl Acad. Sci. USA*, **81**, 4110–4114.
17. Nakabeppu,Y., Miyata,T., Kondo,H., Iwanaga,S. and Sekiguchi,M. (1984) Structure and expression of the alkA gene of *Escherichia coli* involved in adaptive response to alkylating agents. *J. Biol. Chem.*, **259**, 13730–13736.
18. Habraken,Y., Carter,C.A., Kirk,M.C., Riordan,J.M. and Ludlum,D.B. (1990) Formation of N2,3-ethanoguanine in DNA after *in vitro* treatment with the therapeutic agent, N-(2-chloroethyl)-N′-cyclohexyl-N-nitrosourea. *Carcinogenesis*, **11**, 223–228.
19. Carter,C.A., Habraken,Y. and Ludlum,D.B. (1988) Release of 7-alkylguanines from haloethylnitrosourea-treated DNA by *E.coli* 3-methyladenine-DNA glycosylase II. *Biochem. Biophys. Res. Commun.*, **155**, 1261–1265.
20. Roth,R.B. and Samson,L.D. (2002) 3-Methyladenine DNA glycosylase-deficient Aag null mice display unexpected bone marrow alkylation resistance. *Cancer Res.*, **62**, 656–660.
21. Sugimura,K. and Nishihara,T. (1988) Purification, characterization and primary structure of *Escherichia coli* protease VII with specificity for paired basic residues: identity of protease VII and OmpT. *J. Bacteriol.*, **170**, 5625–5632.
22. O'Connor,T.R. (1993) Purification and characterization of human 3-methyladenine-DNA glycosylase. *Nucleic Acids Res.*, **21**, 5561–5569.
23. Habraken,Y., Carter,C.A., Kirk,M.C. and Ludlum,D.B. (1991) Release of 7-alkylguanines from N-(2-chloroethyl)-N′-cyclohexyl-N-nitrosourea-modified DNA by 3-methyladenine DNA glycosylase II. *Cancer Res.*, **51**, 499–503

Received March 22, 2002; revised December 2, 2002; accepted December 16, 2002