AD ________________

Award Number: DAMD17-00-C-0012

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

PRINCIPAL INVESTIGATOR: Zdenka Matijasevic, M.D., Ph.D.

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

REPORT DATE: April 2002

TYPE OF REPORT: Annual

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.

20021129 093

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2002	Annual (15 Mar 01 - 14 Mar 02)

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, M.D., Ph.D.

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

Report contains color.

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

13. ABSTRACT *(Maximum 200 Words)*

Our investigations of the mechanisms of sulfur mustard (SM) toxicity and conditions that can improve cell survival after exposure have previously demonstrated that cells with compromised nucleotide excision repair (NER) are very sensitive to SM. *In vitro* experiments implicated base excision repair (BER) as another pathway involved in the processing of SM-damaged DNA. We find that the DNA glycosylase that initiates BER, sensitizes cells to SM, probably due to formation of repair intermediates more toxic than the initial lesion. This represents a novel lead in the search for modulators of SM toxicity. Once we establish the mechanism of sensitization, we will pursue investigation directed toward the inhibitors of the glycosylase, and/or activators of alternative repair pathways. We have demonstrated that hypothermia can ameliorate the sensitizing effects of glycosylase activity. In addition, hypothermia also has a glycosylase-independent beneficial effect on cell survival. Our data indicate that p53-mediated cell cycle arrest induced by low temperature may account for the additional protection by hypothermia.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Sulfur Mustard, DNA modification, DNA repair, Hypothermia, Cell Cycle Arrest, p53 protein	77
	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

COVER 1
SF 298 2
INTRODUCTION 5
BODY 5
BACKGROUND 5
The Repair Mechanisms Involved in Processing of SM-induced DNA Lesions 5
The Role of Hypothermia in Protection Against SM Toxicity 6
MATERIALS AND METHODS 7
Bacterial Cells 7
Plasmids 7
Plasmid Propagation 8
Plasmid Purification 8
Bacterial Cell Survival and Mutation Frequency 9
Mammalian Cell Culture 9
Cell Cycle Analysis 9
Host Cell Reactivation Assay for DNA repair 10
Transient Saos-2 Transfection 10
Luciferase Assay 11
Immunofluorescent Staining 11
RESULT AND DISCUSSION 11
The Repair Mechanisms Involved in Processing of SM-induced DNA Lesions 11
Effect of DNA Glycosylase on Repair of SM-damaged DNA 11
Mustard Monoadducts are Responsible for Sensitizing Effect of DNA 13
Model for Sensitization to Sulfur Mustard by DNA Glycosylase 14

page

The Role of Hypothermia in Protection Against SM Toxicity 15

Role of p21^{WAF-1} in Cell Cycle Arrest by Hypothermia................ 15

Stable Transfection of Saos-2 Cells with p53........................... 15

Expression of p21 at Low Temperature.................................. 17

KEY RESEARCH ACCOMPLISHMENTS.. 18

REPORTABLE OUTCOMES 19

Publications Supported by this Contract 19

Personnel Receiving Contract Support 19

CONCLUSIONS 20

REFERENCES 20

APPENDICES 22

Abbreviations 22

Manuscripts 23

INTRODUCTION

Since DNA damage represents the initial event in sulfur mustard toxicity, we sought to first identify natural pathways involved in cellular responses to SM-induced DNA damage and in protection from its toxicity. We have identified 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 one involves the regulation of cell cycle progression.

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. Cells that have 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, cells lacking DNA glycosylase, an early enzyme of the BER pathway, are more resistant to SM (3). 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 (4) and some mammalian cell lines that have lost the ability to arrest in response to exposure are more sensitive to SM (Matijasevic, unpublished). These results suggest 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 (5) and protects cells from SM toxicity (6). A possible mechanism of protection based on the cell cycle arrest may be increased time for the repair of DNA lesions before the initiation of critical cellular events such as DNA replication and mitosis. Our recent data (3) suggested that the second, probably unrelated mechanism, diminished sensitization by DNA glycosylase at low temperature, also contributes to the protective effect of hypothermia.

BODY

BACKGROUND

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

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 NER-deficient cells compared to the cells with functional 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; we will address these questions in our future studies.

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 alkyl adenine DNA glycosylase (AAG), an early enzyme of the BER pathway, in protecting cells against cytotoxic effects of bifunctional alkylating agents (7,8). We have shown 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 (3). 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 (9).

In order to examine the effects of mammalian DNA glycosylase on the functional integrity of SM-damaged DNA we performed a host cell reactivation assay with SM-alkylated reporter plasmid transfected into mouse embryonic fibroblasts (MEF) that are either wild type or null mutants for DNA glycosylase. In agreement with survival data, the glycosylase-deficient MEFs show higher reactivation of damaged plasmid than the wild type cells.

Since the sensitizing effect of DNA glycosylase is manifested in the same cell systems in which glycosylase protects against toxicity of a simple monofunctional alkylating agent 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 monoadduct contributes substantially to glycosylase-dependent SM sensitization. We discuss possible mechanisms that may be responsible for sensitization.

The Role of Hypothermia in Protection Against SM Toxicity

Hypothermia decreases cytotoxicity of sulfur mustard (SM) for mammalian cells and the accumulating evidence suggests that this effect is an outcome of several pathways activated in cells in response to combined stress (3). 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 this point we are aware of at least two independent mechanisms that contribute to the protective effect of hypothermia. One of them is manifested through the diminished sensitizing effect of DNA glycosylase at low temperature. This mechanism apparently accounts for only a fraction of protection, since cells deficient in DNA glycosylase still survive better when incubated at low temperature after SM exposure.

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 (5). These two proteins have important roles in cells with damaged DNA; in response to DNA damage, p53 protein activates transcription of several genes including the cyclin-dependent kinase inhibitor $p21^{WAF-1}$, which is responsible for cell cycle arrest (10,11). In contrast to the DNA damage response, nothing is known about the mechanisms of accumulation and roles of p53 and p21 in response to hypothermia. Our finding that hypothermia induces a p53-dependent cell cycle arrest (5) suggests that a p53-mediated process is involved in cell protection at low temperatures. We have therefore initiated experiments to address the role and regulation of p21 in cell cycle arrest at low temperature. We now know that in addition to the tumor suppressor p53 protein, cell cycle arrest at low temperature also requires the functional kinase inhibitor $p21^{WAF-1}$ protein. The results also show that hypothermia activates transcription from the $p21^{WAF-1}$ promoter and suggests that two different regulatory pathways, p53-dependent and p53-independent, may be involved.

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 were obtained from Gibco BRL (Gaithersburg, MD). Fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibodies were from Caltag Laboratories, Burlingame, CA. Primary anti-p53 and secondary FITC (fluorescein isothiocyanate)-conjugated fluorescein antibodies were obtained from Oncogene Research Products, Boston, MA. Anti-[HA]-fluorescein antibodies were obtained from Boehringer Mannheim, Indianapolis, IN.

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: 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.

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 (10) (Figure1). 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

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.

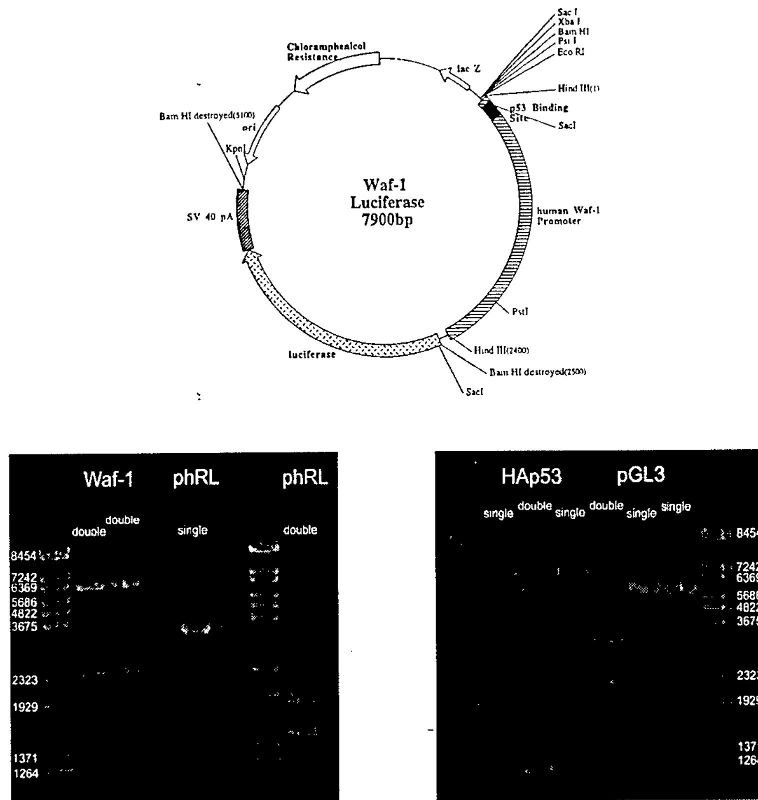


Figure 1. Map of p53-responsive Waf-1-*luc* reporter construct (10) and photographs of agarose gel electrophoresis performed on purified plasmids digested with appropriate restriction enzymes. The sizes (in base pairs) of λ ladder fragments are shown.

Plasmid Purification

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).

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 2-chloroethyl ethyl sulfide (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 alkyl adenine DNA glycosylase-null mutants (APNG-/-), both spontaneously transformed, were a gift from Dr. Rhoderick H. Elder from Paterson Institute for Cancer Research, Manchester, UK (8). Cells were grown in D-MEM/F-12 medium containing L-glutamine (GibcoBRL, Gaithersburg, MD, Cat. # 11320-033) supplemented with fetal bovine serum (10%), nonessential amino acids, nucleosides, penicillin and streptomycin. MEFs wild type for p53, p53 null mutants and p21 null mutants were gift from Stephen N. Jones, UMMS (12).

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 2x concentration of 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 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.* (13). 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 is damaged by SM 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. 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 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 2×10^4 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.

Transient Saos-2 Transfection

Saos-2 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 both the Waf-1-*luc* reporter plasmid and the phRL plasmid containing the *Renilla* luciferase gene. In addition, one set of cells was also transfected with HAp53 plasmid. In all transfections, 1.1 μg total DNA and 4μl transfection reagent (TransFast, Promega) in serum-free medium was used. Cells were incubated at either 37°C or 28°C for 72 or 120 h. Cells were lysed with 150 μl of Passive Lysis Buffer from the kit and stored frozen for the assay .

Luciferase Assay

Luciferase levels were determined using the Dual Luciferase Assay Kit (Promega). The assays for firefly luciferase activity and Renilla luciferase activity were performed sequentially in one reaction tube using 20 μl aliquots of cell lysates To measure firefly luminescence, cell lysates were added to 100 μl of Luciferase Assay Reagent II and the luminescent signal was 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 was added to the mixture to inhibit the firefly reaction and to provide a substrate for the *Renilla* luciferase. A second reading was obtained, and the ratio of firefly to *Renilla* luciferase was calculated.

Immunofluorescent Staining

500 μl of Saos-2 cells were plated on sterile coverslips in 6-well plates at a density of $2\times10^4/cm^2$, and incubated at 37°C. Additional medium was added after cells had begun to adhere to the coverslip. After incubation periods of 76 or 96 h, cells were washed with phosphate buffered saline (PBS), fixed in 3.7% formaldehyde, washed again with PBS, fixed in 100% methanol, and rehydrated with PBS. They were then incubated with blocking buffer (1% bovine serum albumin and 0.5% Triton X-100 in PBS) to block nonspecific antibody binding sites. Samples were incubated with either anti-[HA]-fluorescein antibodies for 2 h, or primary anti-p53 antibodies for 1 h and then with secondary FITC-conjugated antibodies for 1 h. All samples were washed with PBS and incubated with Hoescht 33258 solution to stain the genomic DNA. They were washed again with PBS, and coverslips were mounted on glass slides with ProLong Antifade medium (from Molecular Probes, Eugene, OR). Slides were viewed using fluorescence microscopy. A yellow filter was employed to view the blue DNA, and a magenta filter to view the green fluorescein staining.

RESULTS AND DISCUSSION

Repair Mechanisms Involved in Processing of SM-induced DNA Lesions

Effect of DNA Glycosylase on Repair of SM-damaged DNA

In order to determine the effects of mammalian DNA glycosylase on the repair of SM-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 (Figure 2).

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-) 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 (3).

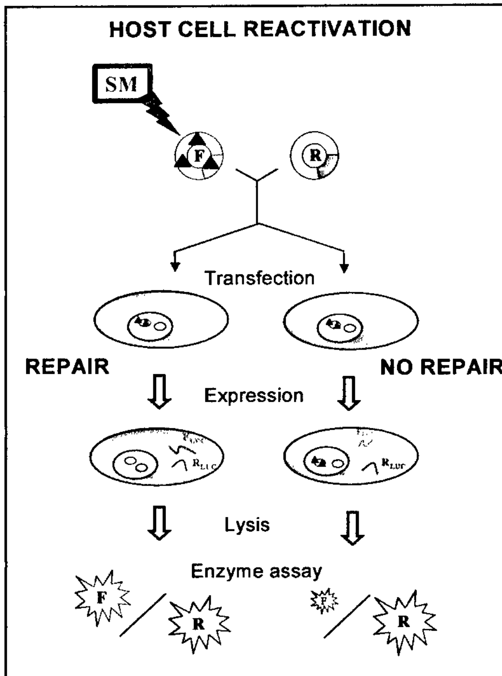


Figure 2. Host cell reactivation assay for monitoring DNA repair using firefly luciferase (F) expression plasmid damaged *in vitro* and transfected into the cells of different repair status. The level of firefly luciferase activity relative to Renilla (R) luciferase activity is a measure for the efficiency of DNA repair normalized for transfection efficiency.

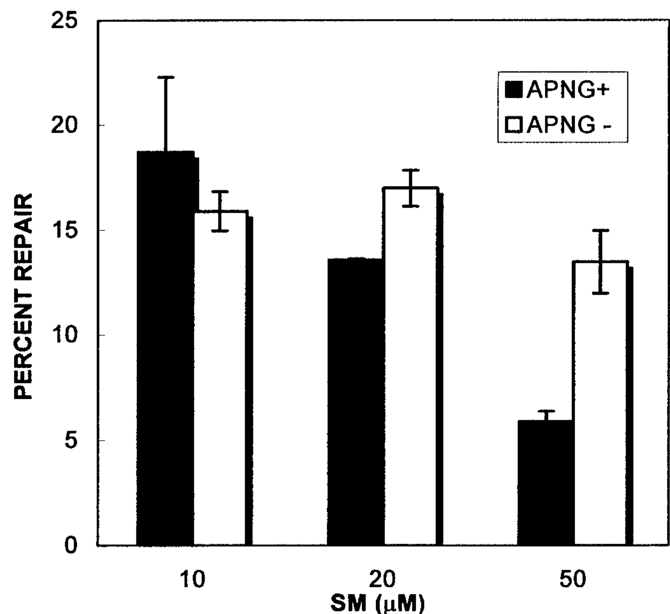


Figure 3. Effect of DNA glycosylase on luciferase expression from SM- damaged reporter plasmids. Wild type cells (■) and APNG-deficient cells (□) were cotransfected with either the SM-damaged or undamaged firefly luciferase reporter plasmids and with undamaged Renilla luciferase reporter plasmids. Cells 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.

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 4. 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.

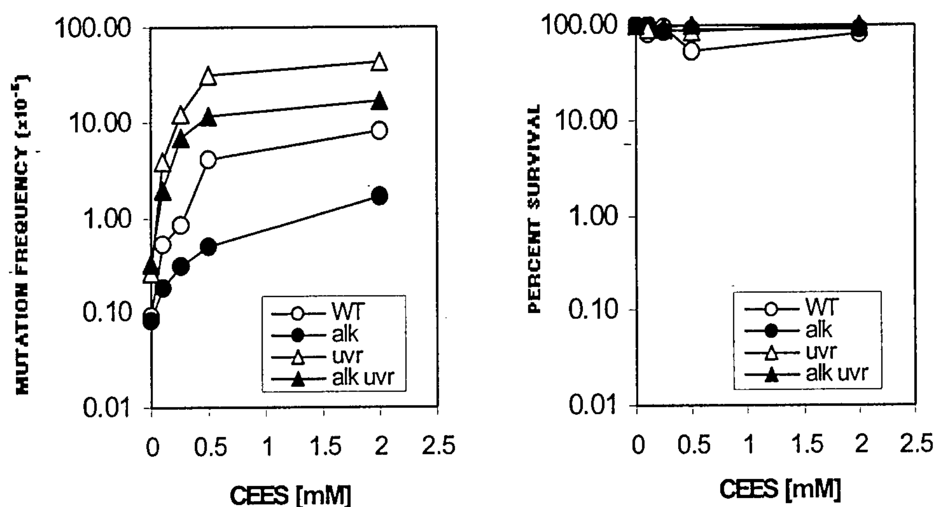


Figure 4. Effect of DNA repair on survival and mutation induction in repair deficient *E. coli* cells exposed to 2-chloroethyl ethyl sulfide (CEES). Wild type (O), AlkA glycosylase-deficient (●), NER-deficient (△) or glycosylase and NER-deficient (▲) cells were exposed to CEES for 60 min and plated for total cell number and for Arg+ revertants. 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. We plan to test the effects of DNA glycosylase on survival at higher, potentially cytotoxic, doses of CEES.

Model for Sensitization to Sulfur Mustard by DNA Glycosylase

Compromised repair of SM lesions in the presence of DNA glycosylase may be due to its interference with another, more efficient repair pathway such as the NER, or to the conversion of primary lesion into the more toxic ones by glycosylase. The interference with NER can be ruled out, at least for the bacterial cells, since both NER+ and NER- cells are sensitized to SM by the glycosylase. Apurinic (AP) sites formed by glycosylase activity are highly toxic and mutagenic if left unrepaired, however, they are also formed by glycosylase during the repair of MMS-modified bases. Sensitization may result from an aborted repair process where DNA glycosylase recognizes and binds to the damaged base in DNA but is unable to dissociate from it. One possible scenario would be the formation of a DNA-protein crosslink between the SM-alkylated base in DNA and one of the glycosylase residues contacting the alkylated base.

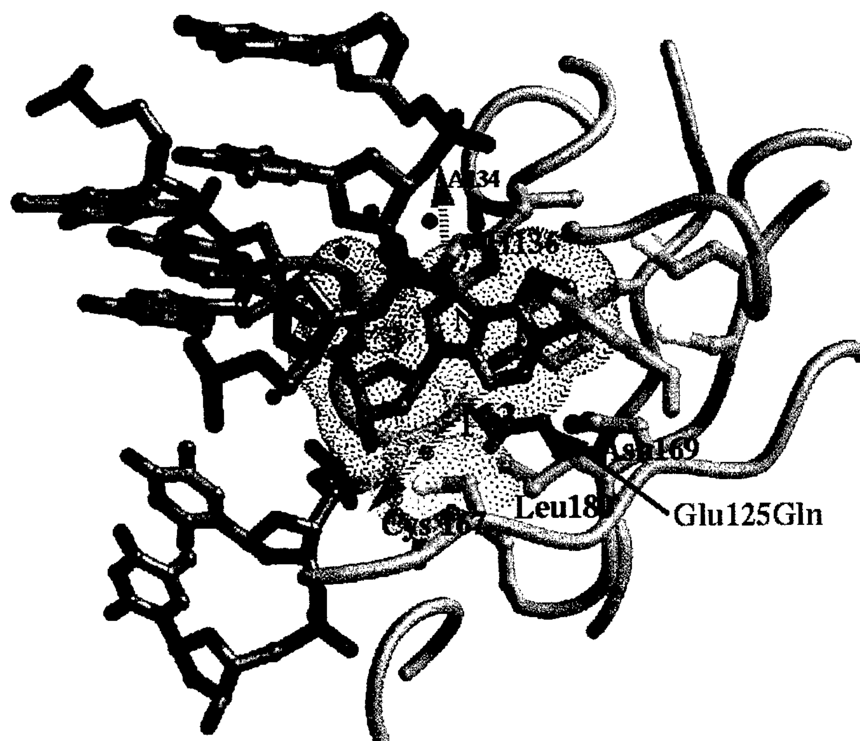


Figure 5. Structure of human alkyl adenine DNA glycosylase (hAAG) in complex with 1,N^6-ethenoadenine (εA)-DNA. Complex hAAG/εA-DNA as reported by Lau et al. (PNAS 97:13573 –13578, 2000) is shown here with partial electron density contours of flipped-out εA and the enzyme active site. Turquoise arrow pointing from N3 position of adenine indicates possible crosslinking between SM-modified adenine and Cys167 of the enzyme.

Crystal structure of human alkyl adenine DNA glycosylase is available and the structure of the enzyme in complex with DNA containing 1, N^6-ethenoadenine (εA) (14) is shown in figure 5. The mechanism by which most of the DNA glycosylases reach their target nucleotide in DNA is by flipping it out of the DNA double helix into the active site cleft of the enzyme. Considering broad specificity of hAAG for substrates, the binding site of the enzyme must be able to accommodate a structurally diverse group of damaged bases (15). Providing that the active site conformation with εA also applies to the complex with the SM-modified adenine, it could be predicted that the additional atoms in the N3 position of adenine, such as chloroethyltioethyl group, could reach down (indicated by the turquoise arrow in Figure 5) and form a crosslink with the Cys167 residue of the enzyme.

Formation of protein-DNA crosslinks may not only block further steps in the BER pathway but may also interfere with other DNA transactions such as transcription and replication.

The Role of Hypothermia in Protection Against SM Toxicity

Our results suggest that the protective effect of low temperature against SM toxicity may derive from at least two sets of different cellular processes taking place in exposed mammalian cells. In order to address the question of whether the hypothermia-induced protective mechanisms act in concert or separately, and whether we can increase the overall protective effect, we need to learn more about the individual mechanisms involved. One of the mechanisms is manifested through the diminished sensitizing effect of DNA glycosylase at low temperature (3) and learning more about the mechanisms of sensitization, as described above, would increase our understanding of the effects of hypothermia. We believe that the second mechanism is due to the activation of at least one branch of the p53 pathway, namely the one leading to cell cycle arrest in response to hypothermia.

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

The key factor in DNA damage-induced cell cycle arrest regulated by p53, is 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 (5), we examined cell cycle progression as BrdU incorporation in p21 null mutant mouse embryonic fibroblasts incubated at 28°C. The results in figure 6 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.

Stable Transfection of Saos-2 Cells with p53

In response to DNA damage, $p21^{WAF-1}$ is one of the genes transcriptionally activated by p53. We have demonstrated that cellular levels of p21 protein are increased in response to hypothermia,

and that the cell cycle is arrested in a manner dependent on both p53 and p21. In order to address the question of how p21 expression is regulated at low temperature, we examined expression from the $p21^{WAF-1}$ promoter in human osteosarcoma Saos-2 cells at 37°C and 28°C. Saos-2 cells are p53-deficient; therefore, by introducing exogenous p53, we have generated an isogenic set of cells differing only in their p53 status, to study the requirements for p53 in cellular processes.

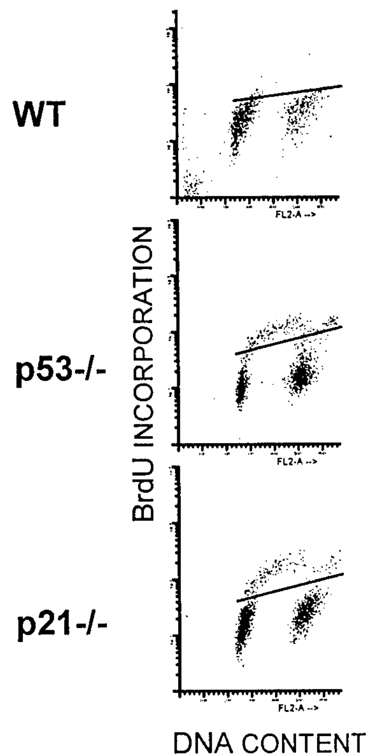


Figure 6. Effect of p53 and p21 status on cell cycle progression at low temperature. Mouse embryo fibroblasts were incubated at 28°C for 45h, labeled with BrdU for 2h and stained with anti-BrdU-FITC and propidium iodide for FACS analysis. Cell cycle arrest occurs in cells with wild type p53 but not in 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.

In order to generate Saos-2 cells stably transfected with p53, cells were plated on 100 mm tissue culture plates at a density of 2×10^4 cells/cm^2 and transfected with a mixture of p53 expression plasmid DNA (HAp53) and transfection reagent in serum-free medium. Cells were incubated at 37°C for 2.5 h when medium with serum was added. 24 h after transfection, cells were replated to provide more space for growth. Cells were grown for 1 week before the antibiotic Geneticin (G418) was added to select for clones that are resistant to Geneticin and therefore contain p53. Every third day, half of the medium was withdrawn and the equivalent amount with fresh G418 added. After an additional week of growth, trypsin was applied to the plate to loosen cells, and individual clones of stably transfected cells were extracted by pipet and replated into 24-well culture plates. Several stable transfectant clones were successfully generated.

In order to confirm the presence of exogenous p53 in transfected Saos-2 cells, cells were stained with p53-specific primary antibodies and FITC-conjugated secondary antibodies. Figure 7 shows the results of staining; transfected Saos-2 cells are shown in the upper row and nontransfected control cells in the lower row. The left panels show the green anti-p53

fluorescence, and right panels show the blue Hoechst-stained DNA in the same field of cells. The results show bright green staining indicative of the presence of p53 in the transfected cells, which is clearly above the background staining in the control cells.

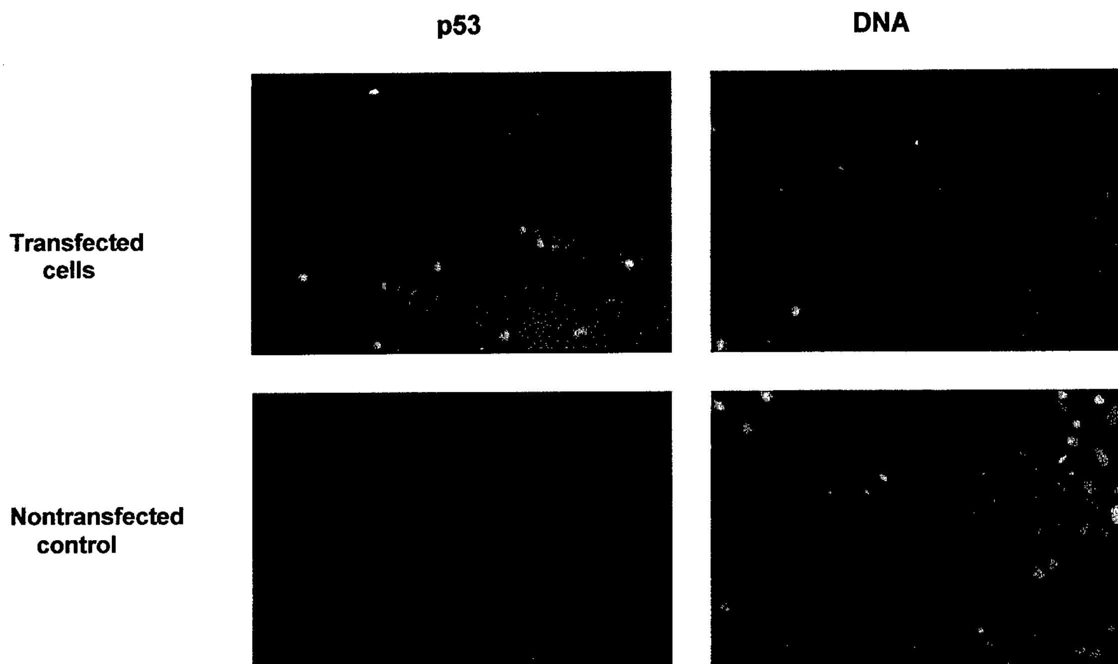
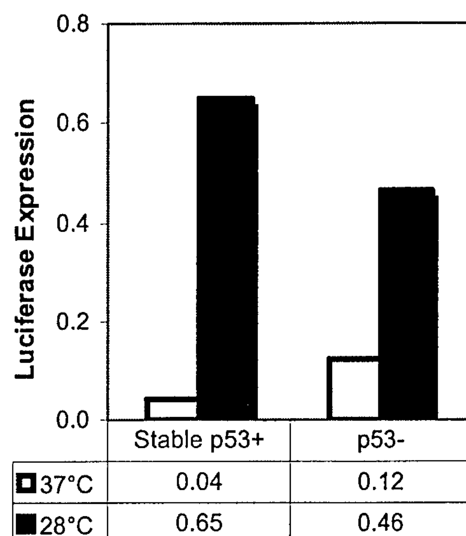


Figure 7. Immunostaining of Saos-2 cells transfected with p53-expressing plasmid. p53-transfected cells or nontransfected control cells were incubated with primary p53-specific antibodies, then with secondary FITC-conjugated antibodies (left panels) and Hoechst 33258 for the genomic DNA (right panels), and viewed under the fluorescent microscope.

Expression of p21 at Low Temperature

We have shown that hypothermia increases levels of p21 in normal human fibroblasts (5) and that cell cycle arrest at low temperature is p21-dependent (Figure 6). 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 or stably transfected with p53 were transiently transfected with p53-responsive $p21^{WAF-1}$- *luc* reporter plasmid containing the $p21^{WAF-1}$ promoter upstream of the gene for firefly luciferase (Figure 1). This plasmid allows for the determination of p21 expression by measuring the levels of luciferase in cell lysates. Figure 8 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.



	Stable p53+	p53-
37°C	0.04	0.12
28°C	0.65	0.46

Figure 8. 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 suggest that hypothermia activates expression of p21^{WAF-1} by two separate mechanisms, one that requires p53 and one that is p53-independent. p53-independent p21 expression was described for some tumor cell lines following serum stimulation (16) and for normal tissues as p53-independent compartmentalization (17). We will further investigate the hypothermia-induced regulatory pathways of p21 expression

KEY RESEARCH ACCOMPLISHMENTS

We have found that:

- human alkyladenine DNA glycosylase interferes with the restoration of sulfur mustard-damaged DNA function;
- the presence of mustard monoadducts in DNA is responsible for the sensitizing effect of DNA glycosylase;
- conditions such as hypothermia that can weaken glycosylase activity may protect cells against SM toxicity;
- the other mechanism of protection by hypothermia, cell cycle arrest, requires the presence of functional kinase inhibitor, p21 in cell;
- expression of p21^{WAF-1} is induced under the conditions of hypothermia;
- hypothermia activates expression of p21^{WAF-1} by two separate mechanisms, one that requires p53 and one that is p53-independent.

REPORTABLE OUTCOMES

Publications, Manuscripts and Presentations

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.

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)*

Li, Q., Wright, S.E., Matijasevic, Z., Chong, W., Ludlum, D.B., and Volkert, M.R. Isolation of a full length human alkyl purine glycosylase and its role in cellular resistance to chloroethylnitrosoureas. (*submitted*).

Matijasevic, Z. Selective protection of non-cancer cells by hypothermia. *(Completed, for submission).*

Powers, C.M., Santandrea, A.M. and Matijasevic, Z. DNA repair enzyme, alkyladenine DNA glycosylase, sensitizes cells to sulfur mustard (*in preparation*).

Matijasevic, Z., Snyder, J.E., and Ludlum, D.B. 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., 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.

Personnel Receiving Contract Support

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

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 the effect of DNA glycosylase, should provide protection against SM toxicity.

We have determined one type of DNA lesions involved in sensitization and identified one set of postexposure conditions, low temperature, that lessens sensitizing effects of glycosylase and increase 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.

So far we were the first to identify two proteins, p53 and p21, whose levels are increased at low temperature. Both proteins are required for the cell cycle arrest at low temperature and the expression of p21 occurs by both p53-dependent and p53-independent mechanism. 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. 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. Matijasevic, Z. Protection Against the Acute and Delayed Toxicities of Sulfur Mustard, Annual Report, 15 March 2000 – 14 March 2001, Contract No. DAMD17-00-C-0012, U.S. Army Medical Research and Developmental Command, 2001.

4. Matijasevic, Z., Stering, A., and Ludlum, D.B. 1996. Sulfur mustard-induced cell cycle arrest and the effect of hypothermia. Proc. Med. Defense Biosci. Rev., Vol. 2, 937-945.

5. 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.

6. Ludlum, D.B. Protection Against the Acute and Delayed Toxicities of Sulfur Mustard, Final Report, 15 Aug 96 – 01 Oct. 99, Contract No. DAMD17-96-C-6073, U.S. Army Medical Research and Developmental Command, 1999.

7. 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.

8. 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.

9. 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*).

10. 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.

11. 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.

12. 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.

13. 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.

14. Lau A.Y., Wyatt M.D., Glassner B.J, Samson L.D., and Ellenberger T. Molecular basis for discriminating between normal and damaged bases by the human alkyladenine glycosylase, AAG. PNAS *97*: 13573-13578, 2000.

15. Abner, C.,W., Lau, A.,Y., Ellenberger, T., and Bloom, L.,B. Base excision and DNA binding activities of human alkyladenine DNA glycosylase are sensitive to the base paired with a lesion. J. Biol. Chem. *276*: 13379-13387, 2001.

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

17. 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.

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

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*).

Li, Q., Wright, S.E., Matijasevic, Z., Chong, W., Ludlum, D.B., and Volkert, M.R. Isolation of a full length human alkyl purine glycosylase and its role in cellular resistance to chloroethylnitrosoureas. (*submitted*).

Isolation of a full length human alkyl adenine glycosylase and its role in cellular resistance to chloroethylnitrosoureas

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

[1]Department of Biochemistry & Molecular Pharmacology and [2]Department of Molecular Genetics & Microbiology, University of Massachusetts Medical School, Worcester, MA 01655-0126, USA.

*To whom correspondence should be addressed. Tel: 508 856-2314; FAX: 508 856-5920; Email: Michael.Volkert@umassmed.edu

Abstract

A full length, histidine-tagged human alkyl adenine glycosylase has been purified from the cloned human gene contained in a pTrc99A vector propogated in a *tag alkA* mutant *E. coli*. This human enzyme releases both m^3A and m^7G from methylated DNA but in contrast to previous studies with bacterial AlkA glycosylase, it does not release any adducts from [^3H]chloroethylnitrosourea-modified DNA. This finding suggests that the AAG-dependent resistance to the toxic effects of the chloroethylnitrosoureas previously reported in the literature may occur by a mechanism other than through direct glycosylase action.

Introduction

The chloroethylnitrosoureas (CNUs*) are antitumor agents whose cytotoxic action depends largely on the formation of a cross-link between guanine and cytosine in DNA [1]. Formation of this cross-link can be prevented by the DNA repair enzyme, O^6-alkylguanine-DNA alkyltransferase, with the result that high levels of alkyltransferase cause tumors to be resistant to treatment [2,3]. However, HPLC analysis of the DNA modifications in resistant cell lines treated with CNUs has shown that other adducts besides the GC cross-link are missing from resistant cells [4]. Previous studies have shown that many CNU-modified bases are *in vitro* substrates for the bacterial AlkA protein, 3-methyladenine DNA glycosylase II [5-7]. By analogy with the bacterial AlkA protein, we thought that mammalian alkyladenine-DNA glycosylase might be responsible for removing CNU adducts.

Investigations of the role of mammalian alkyladenine glycosylases (AAGs) in reducing the toxicity of CNU to cultured cells have not, however, shown a simple relationship between glycosylase content and cellular resistance. Matijasevic *et al* found higher levels of alkyladenine glycosylase in a glial cell line resistant to the chloroethylnitrosoureas than in a sensitive cell line [8],

***Abbreviations:** AAG, alkyladenine DNA glycosylase; CNU, *N*-(2-chloroethyl)-*N*-nitrosourea; hAAG, human alkyladenine DNA glycosylase; (hAAG-his_6), human alkyladenine DNA glycosylase with histidine tail; IPTG, isopropyl β-D-thiogalactopyranoside; m^3A, 3-methyladenine; m^7G, 7-methylguanine; MMS, methyl methanesulfonate; MNU, *N*-methyl nitrosourea;

however overexpression 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 cultured 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 base excision repair pathway; other enzymes are required to repair the abasic site left in DNA by AAG action. Accordingly, 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 obtained cloned human AAG from Leona Samson (Massachusetts Institute of Technology) and transferred it to a regulated bacterial expression vector. A carboxyl terminal histidine tag was attached to this glycosylase and it was tested for activity towards MNU and CNU-modified DNA. The human alkyladenine DNA glycosylase gene had been cloned independently by several research groups, but the separate isolates differ in their *N*-terminal amino acid sequences [12]. Since 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 on 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 that, either AAG requires an additional factor to be active *in vivo,* or this glycosylase provides protection by a mechanism other than its glycosylase action.

Materials and methods

Materials

N-(2-chloro-1,2-^{3}H-ethyl)-*N*-nitrosourea (^{3}H-CNU), specific activity 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 was purchased from Amersham. Unlabelled CNU was provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Optical markers of 3-methyladenine (m3A) and 7-methylguanine (m^{7}G) used in the HPLC separations were obtained from Cyclo Chemical (Los Angeles, CA). Other chemicals were reagent grade materials.

[^{3}H]MNU-modified and [^{3}H]CNU-modified DNA were prepared by alkylating calf thymus DNA with the radiolabelled MNU and CNU as described previously [5]. 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) while human glycosylase was assayed in its slightly different optimum buffer (50 mM HEPES, pH 7.5, containing 1mM NaEDTA, 5 mM mercaptoethanol, and 100 mM KCl).

Construction of plasmids expressing human glycosylase

Our constructs started with plasmid pBU16, a gift from Professor Leona Samson (Massachusetts Institute of Technology). Plasmid pBU16 carries a Bsu36I-XbaI fragment from pKT218 that encodes the human alkyl adenine DNA glycosylase gene (*hAAG*) [14] in the pSL301 vector (Invitrogen Corp., San Diego, CA). We subcloned the *hAAG*-bearing EcoRI-BstEII fragment from pBU16 into pTrc99A (Pharmacia Biotech) 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 EcoRI site. The presence of *lacI* on the plasmid results in tight repression of the promoter which can be induced by the addition of the *lac* inducer 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 EcoRI-BstEII fragment of pMV503 was removed by first digesting to completion with EcoRI, then partially digesting with BstEII to produce a 5152 bp fragment. This fragment was purified from a 0.8% agarose gel using the Gene Clean System (Bio101, Inc). Oligonucleotides MV1, (AATTCTAAGGAGGTATCTAATG), and MV2, (GTGACCATTAGATACCTCCTTAG), were first annealed to one another by heating and slow cooling, then ligated to the purified 5152 bp EcoRI-BstEII fragment of pMV503 to produce plasmid pMV509. Oligonucleotides MV1 and MV2 have four important features: (a) they are complementary to one another, (b) when annealed, they produce single-stranded DNA ends complementary to the EcoRI and BstEII sites of pMV503, (c) they reconstruct the ATG initiation codon of *hAAG*, which lies within the BstEII site, and (d) 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* pro-

moter of pTrc99A. Construction of pMV509 was tested by loss of the SalI restriction site that lies between EcoRI and BstEII of pMV503 and restoration of the EcoRI and BstEII sites. The hAAG sequence was confirmed independently.

To construct the histidine-tagged *hAAG* gene, pMV509 was digested to completion with CelII and HinDIII 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: (a) they are complementary to one another, (b) when annealed, they produce single stranded ends complementary to CelII and HinDIII, (c) they restore the last seven amino acid codons of the *hAAG* gene in the appropriate reading frame, and (d) 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 MluI restriction site that lies within the CelII-HinDIII fragment of pMV509 and restoration of the CelII and HinDIII sites, and DNA sequencing of the insert.

Effect of plasmids on sensitivity to MMS

Since 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) 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 [15]. Cultures were immediately diluted further and plated on LB ampicillin agar plates to assess cell survival. The results in Table 1 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 human glycosylase

The hAAG-(his)$_6$ enzyme was isolated from strain MV4126 (*alkA1tag-1*/pMV513). Cells were grown to a Klett reading of 70 (approximately 5 x 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 5h.

After incubation, cells (8 gms) 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 12,000g 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 x 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 mM to 500 mM imidazole in column buffer. Two ml 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 cpm (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 eluded with a 50 mM KH_2PO_4, pH 4.5 - acetonitrile buffer system at 1 ml/min. One min fractions were collected, counted in a Beckman liquid scintillation counter, and plotted versus fraction number as shown in Figure 3. Similarly, spontaneous release from a separate sample of [^{3}H]MNU-DNA substrate containing 136,000 cpm was determined after incubation for 1 h at 37C° in buffer (50 mM HEPES, pH 7.5, containing 1mM NaEDTA, 5 mM mercaptoethanol, and 100 mM KCl). Enzymatic release by 0.15 unit of bacterial or human enzyme was determined after a 1 h incubation at 37C° in the same buffer.

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 carboxyl terminus of the protein to facilitate its purification. This insert was positioned at the carboxyl terminus rather than at the amino 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 Methods section, 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 EcoRI site to control transcription. The presence of the wild type *hAAG* gene in the plasmids was tested by restriction digestion with BstEII, CelII, BglI, EcoRI, HinDIII and SacI followed by gel electrophoresis and confirmed by DNA sequencing.

The protective activity of pMV509 and pMV513 when these plasmids were propogated in *E. coli* cells deficient in glycosylase activity is shown in Table 1. 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 show a 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. 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 propogated in a glycosylase-deficient strain of *E. coli*, MV2157, that 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 [16]. 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 Methods section. A typical separation is shown in the upper panel of Figure 2. 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 kD 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 [17], who also reported purification of intact full length human glycosylase.

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, however, 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 [17].

With active full-length human glycosylase available, it was now possible to test the activity of hAAG towards chloroethylnitrosourea-modified DNA. Bacterial and human enzyme were incubated with ^{3}H-CNU-modified DNA for 10 min at 37°C and the cpms of adducted bases released into the supernatant were measured as shown in Figure 4. While the bacterial enzyme released CNU-adducted bases in an 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 alkyl adenine 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 alkyl adenine 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 adducted bases [5,6]. 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: 1) 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, 2) hAAG requires the presence of an additional cellular factor to act, 3) the activity of hAAG is affected by post-translational modification that differs in mammalian and *E. coli* host cells, or 4) 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, since the enzyme effectively protects against MMS exposure and methyl lesions are efficiently removed by the purified hAAG enzyme (Table 1 and figure 3).

Unless there are other minor but very toxic DNA adducts that we have not detected, possibilities 2-4 seem more likely. However, for possibility 2 to explain our results we would have to

conclude that some factor in *E coli* cells can 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 1 and Figure 3). Post-translational modification of the enzyme has not been reported, but cannot be ruled out. The final possibility, number 4, 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 choose among these possibilities.

Acknowledgments

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 U.S. 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**, 3102-5.

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**, 727-9.

3. Pegg, A.E., Dolan, M.E. and Moschel, R.C. (1995) Structure, function, and inhibition of O6-alkylguanine-DNA alkyltransferase. *Prog. Nucl. Acid. Res. Mol. Biol.*, **51**, 167-223.

4. 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**, 4489-92.

5. 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**, 1971-3.

6. 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.

7. Ludlum, D.B., Li, Q. and Matijasevic, Z. (1999) Role of base excision repair in protecting cells from the toxicity of chloroethylnitrosoureas. *IARC Sci Publ*, **150**, 271-7.

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**, 1568-70.

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**, 3965-73.

11. Elder, R.H., 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., van Zeeland, A.A. and Margison, G.P. (1998) Alkylpurine-DNA-N-glycosylase knockout

mice show increased susceptibility to induction of mutations by methyl methanesulfonate. *Mol Cell Biol*, **18**, 5828-37.

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 U S A*, **90**, 3437-41.

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**, 23690-7.

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. 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.

16. 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**, 5625-32.

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

Table I. Effect of Genotype on Cell Survival*

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%

* as percent of growth of unexposed cultures

Figure Legends

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.

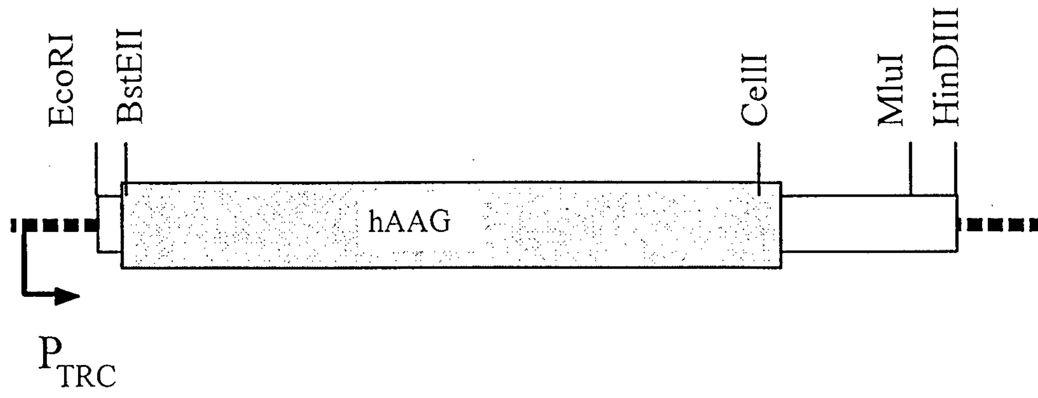
Fig. 2. Upper panel: elution profile of the hAAG-$(his)_6$ enzyme from a Ni-agarose column. Lower panel: gel separations of individual enzyme fractions 10-18. Molecular weight standards are in lanes marked S. *N* terminal analysis was performed on the 39 kD band from fraction 14.

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

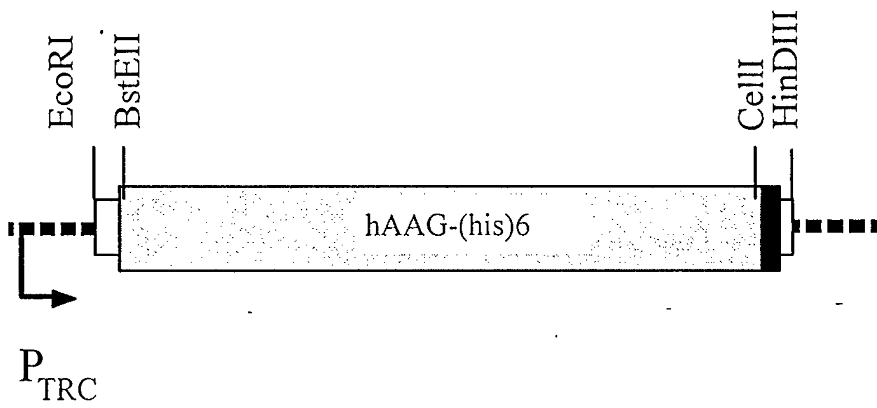
Fig. 4. Activity of bacterial (O) and his-tagged human enzyme (λ), towards ^{3}H-CNU-modified DNA. Incubation mixtures contained 27 μg of DNA with 2 x 10^4 cpm of ^{3}H-CNU adducts and 0.15 unit of bacterial or human glycosylase in 150 μl of buffer solution. Incubations were for 10 min at 37°.

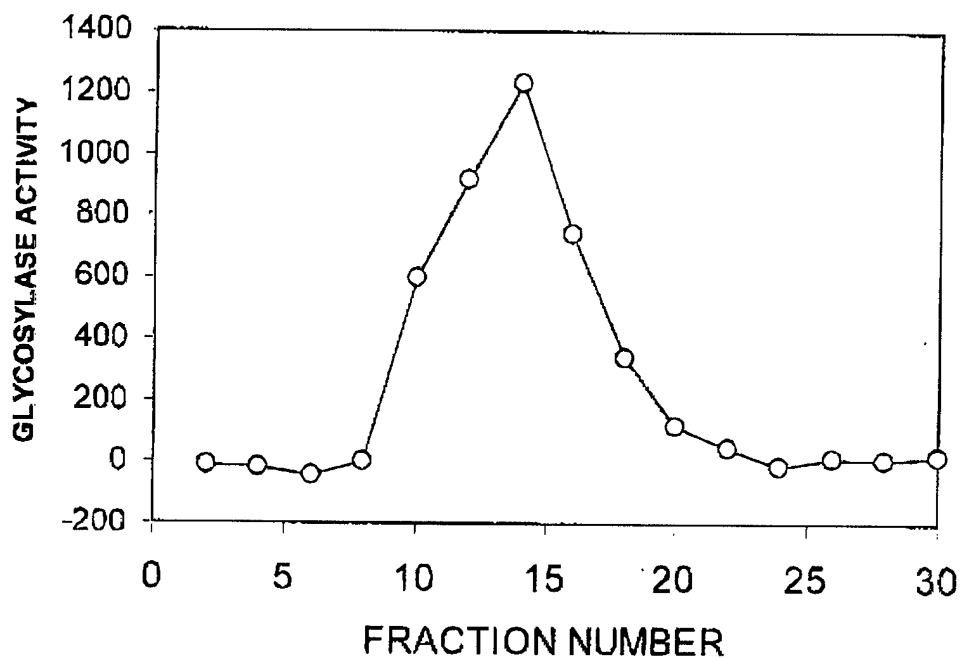
Figure 1

pMV509



pMV513





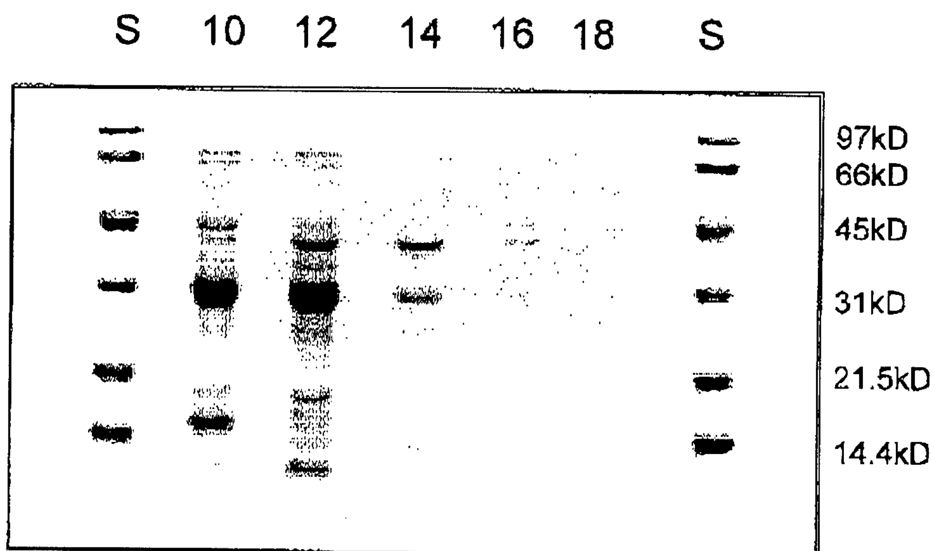


Fig. 2. Upper panel: elution profile of the hAAG-(his)$_6$ enzyme from a Ni-agarose column. Lower panel: gel separations of individual enzyme fractions 10-18. Molecular weight standards are in lanes marked S. *N* terminal analysis was performed on the 39 kD band from fraction 14.

Figure 3

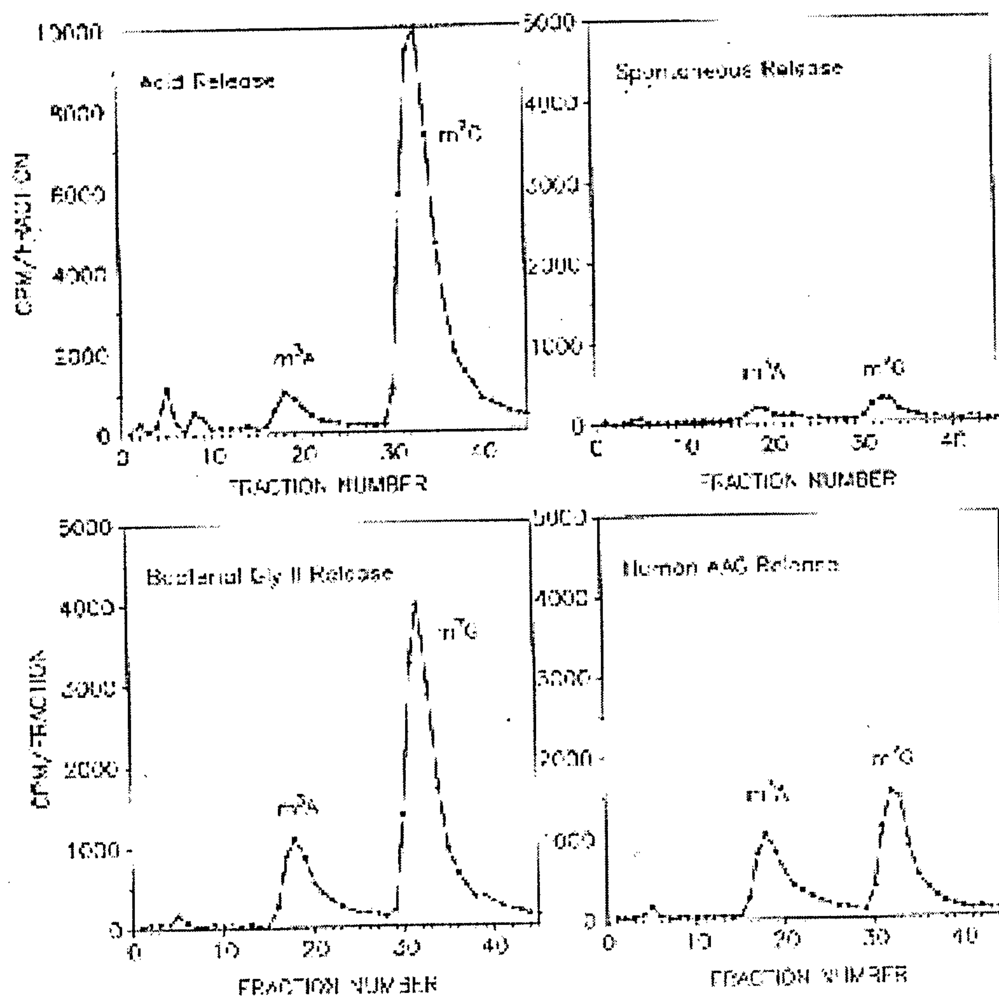
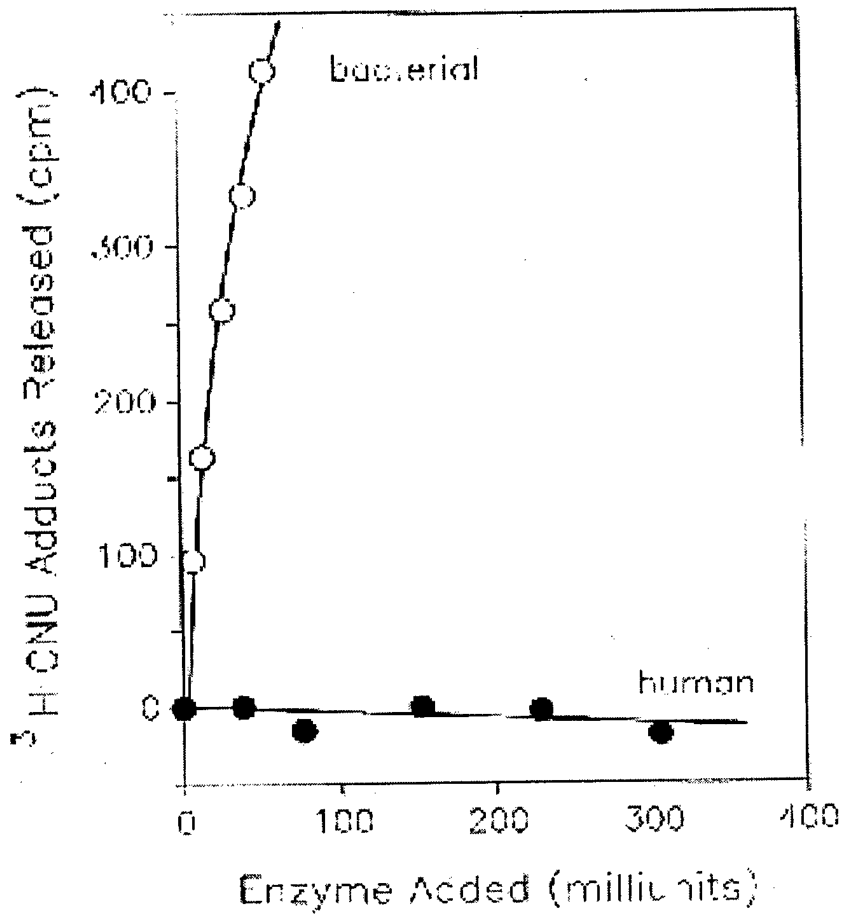


Figure 4



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

Kenneth Bonanno[1,3], Jennifer Wyrzykowski[1], Wincha Chong[1,4], Zdenka Matijasevic[2], and Michael R. Volkert[1*]

[1]Department of Molecular Genetics and Microbiology

University of Massachusetts Medical School

Worcester, Massachusetts 01655

[2]Department of Biochemistry and Molecular Pharmacology

University of Massachusetts Medical School

Worcester, Massachusetts 01655

[3]Present Address: Millenium Pharmaceuticals

Cambridge, MA 02139

[4]Present Address: University of Hawaii Medical School

Transitional Residency Program

Honolulu, Hawaii 96813-2427

*Corresponding Author

Phone: 508-856-2314

Fax: 508-856-5920

Email: Michael.Volkert@umassmed.edu

The alkyladenine DNA glycosylase (AAG) has been cloned from mouse and humans. In humans two isoforms have been characterized that are generated by alternative splicing resulting and contain either exon 1a or 1b. AAG knock out mouse cells are sensitized to a variety of alkylating and crosslinking agents. In this study we examine the ability of the both known isoforms of human AAG to contribute to survival of *Escherichia coli* from treatments with simple alkylating agents and crosslinking alkylating agents. This study shows that human AAG provides a high degree of protection against methylation damage to DNA, but does not increase resistance to ethylating, or crosslinking agents when expressed in *E. coli*.

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 alkyladenine DNA glycosylases (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 BCNU [6]. The lack of any detectable alkyl base excision repair 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 alkyladenine DNA glycosylases, 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 substrate for the *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 crosslinks by interaction with intermediates in their formation [18].

In this report we show that human AAG 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 crosslinking alkylating agents BCNU, N-(2-chloroethyl)-N-nitrosourea (CNU) or mitomycin C. We also demonstrate that there are no apparent differences between the two isoforms of hAAG in the substrate specificities tested.

Materials and Methods

Chemicals. Methyl methane sulfonate (MMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG) and Mitomycin C were obtained from Sigma Chemical (St. Louis, Mo.). N-(2-chloroethyl)-N-nitrosourea (CNU) and N,N'-bis(2-chloroethyl)-N-nitrosourea (BCNU) were obtained from the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD).

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 6 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 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 hAAG1$(his)_6$ 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*.

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

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. Surviving colonies were counted after 24 hr incubation at 37°C. Presence or absence of the vector had no detectable effect on survival. All graphs represent the averages of 2 or more experiments and standard errors are included for all points on graphs.

Western Blot analysis. Cells were grown as described for survival studies. After addition of IPTG (2mM), 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 hr 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 hr. 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 Biosystems).

Results

Methylation resistance of E. coli cells expressing hAAG1 and hAAG2. Figure 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 base excision repair (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 (Figure 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 base excision repair 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 (Figure 1b).

Levels of hAAG proteins in *E. coli*. Complementation by hAAG1 appears to be more efficient than that seen when hAAG2 is expressed (Figure 1 a, 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 (Figure 1). The histidine tags also do not alter the effects of hAAG proteins on recovery from any of the other DNA damaging agents tested (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 higher than that of hAAG2 (Figure 2). Intensities of the bands, measured by densitometry are shown in Figure 2B. The presence of a weak cross-reactivating 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 6 fold higher level at 60 min and a 4-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).

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 is requires both BER and NER pathways, since inactivation of either pathway increases ENNG sensitivity (Figure 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 (Figure 3A) . These 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 (Figure 3B) [4]. We find similar results when hAAG2 is tested for its ability to complement the *alkA tagA uvrA* triple mutant strain (Figure 3B), indicating that neither hAAG isoform is capable of enhancing survival from exposure to ethylating agents, and is therefore not 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*.

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 exposure to these two crosslinking agents and from exposure to another crosslinking

agent, CNU. Neither bacterial, nor human AAG mediated BER, enhances survival to any of these agents (Figure 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 from bacterial or human origin. A modest but consistent sensitization to CNU was seen to result from hAAG expression, suggesting glycosylase activity may have deleterious effect when acting on CNU treated DNA. Thus, it appears that only the *uvrA* gene product is able to enhance recovery of *E. coli* from exposure to crosslinking agents, indicating these lesions are repaired primarily by NER mechanisms.

Discussion

We have produced several clones that allow expression of the two known isoforms of human alkyladenine DNA glycosylase. 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 [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 crosslinking agents. However, our results show that expression of functional human AAG 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 mouse AAG is able to repair these lesions. Several hypotheses can be proposed to explain this paradox. It is possible that the mammalian 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. It is also possible that the human form of the enzyme differs 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 and S. Mitra Cloning and 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 and 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 and 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. USA 83 (1991) 5607-5610.

[4] B.P. Engelward, M.S. Boosalis, B.J. Chen, Z. Deng, M.J. Siciliano and 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 and 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 and 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 and T. Lindahl Human DNA repair genes, Science 291 (2001) 1284-1289.

[8] M.A. Vickers, P. Vyas, P.C. Harris, D.L. Simmons and 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 and 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 and 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 and 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 and 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 and D.B. Ludlum Release of N2,3-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 and 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 and B. Singer 1,N6-ethenoadenine and 3,N4-ethenocytosine are excised by separate human DNA glycosylases, Carcinogenesis 17 (1996) 155-157.

[17] W.B. Mattes, C.S. Lee, J. Laval and 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 and 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 and D.C. Nguyen Induction of specific Escherichia coli genes by sublethal treatments with alkylating agents, Proc. Natl. Acad. Sci. USA 81 (1984) 4110-4114.

[20] A.J. Clark, M.R. Volkert and L.J. Margossian A role for recF in repair of UV damage to DNA, Cold Spring Harbor Symp. Quant. Biol. 43 (1979) 887-892.

[21] L. Thomas, C.H. Yang and D.A. Goldthwait Two DNA glycosylases in Escherichia coli which release primarily 3- methyladenine, Biochemistry 21 (1982) 1162-1169.

[22] J.O. Blaisdell and 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 and 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 and M.K. Saparbaev Alternative nucleotide incision repair pathway for oxidative DNA damage, Nature 415 (2002) 183-187.

Table 1 Oligonucleotides used in hAAG plasmid constructions.

Oligo	Sequence
mv1	AATTCTAAGGAGGTATCTAATG
mv2	GTGACCATTAGATACCTCCTTAG
mv3	TGAGCAGGACACACAGGCCCATCATCATCATCATCACTGA
mv4	AGCTTCAGTGATGATGATGATGATGGGCCTGTGTGTCCTGC
mv6	CTGTATCAGGCTGAAAATC
mv8	GCGCGAATTCTGATGCCCGCGCAGCGGGGCCCAGTTTTGCCGACGGATGGGGC
mv20	CATGGAATTCTAAGGAGGTATCTAATGCCCGCGCAGCGGGGCCCAGTTTTGC

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.

Figure Legends

Figure 1. Effect of human AAG expression on resistance of *E. coli* to methylating agents. Strains designations corresponding to the indicated genotypes are listed in Table 2. (O) 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 tag*A/hAAG1-$(his)_6$, (∇---∇) *alkA tagA*/hAAG2-$(his)_6$. A. MMS exposure. B. MNNG exposure.

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

Figure 3. Effects of human AAG expression on resistance of *E. coli* to ENNG. Symbols are the same as in Figure 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.*

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

Figure 1A

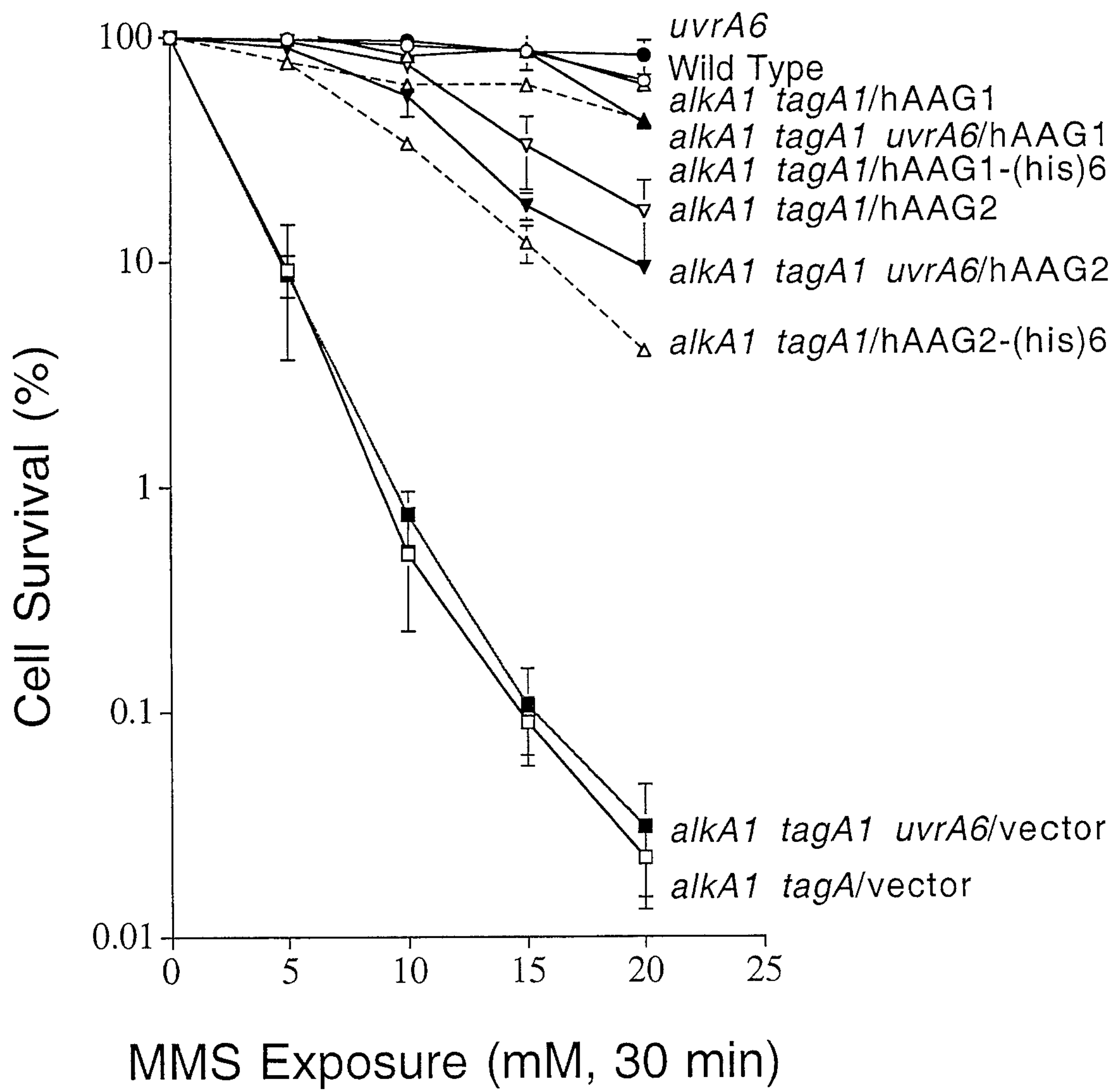
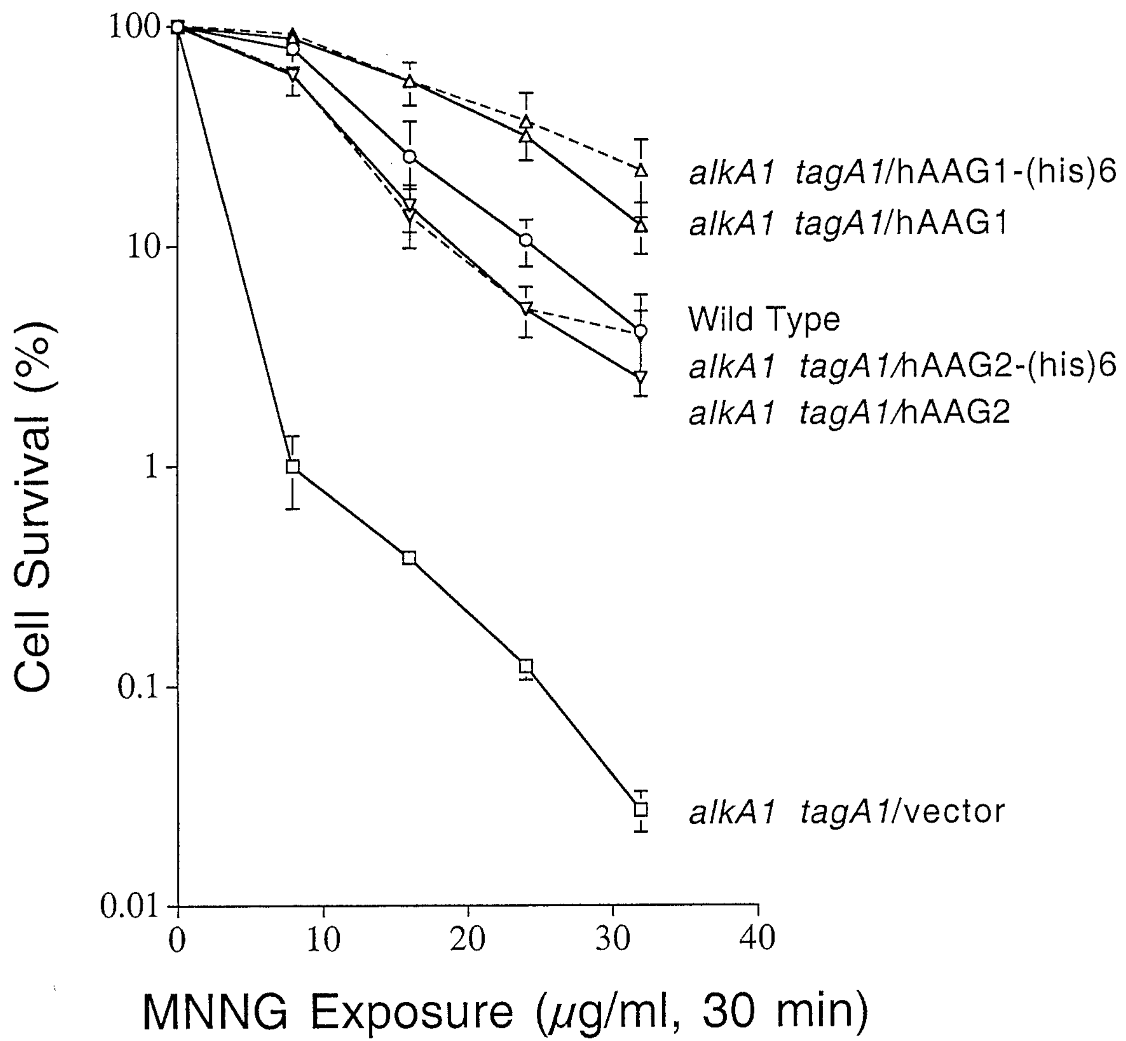
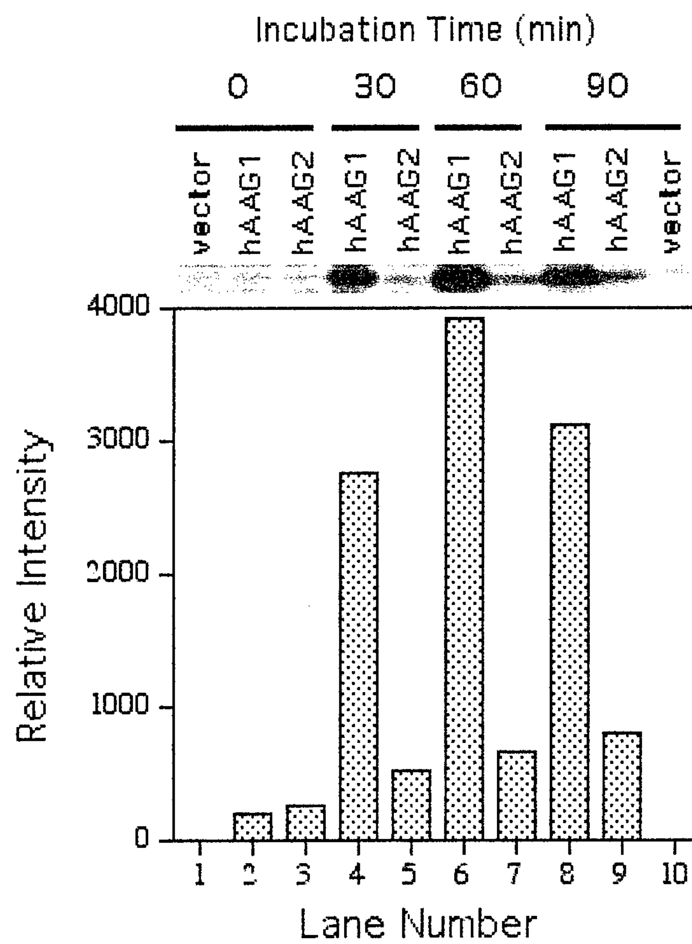


Figure 1B





Incubation Time (min)
0
30
60
90
vector
hAAG1
hAAG2
hAAG1
hAAG2
hAAG1
hAAG2
hAAG1
hAAG2
vector
Relative Intensity
4000
3000
2000
1000
0
1
2
3
4
5
6
7
8
9
10
Lane Number

Figure 3A

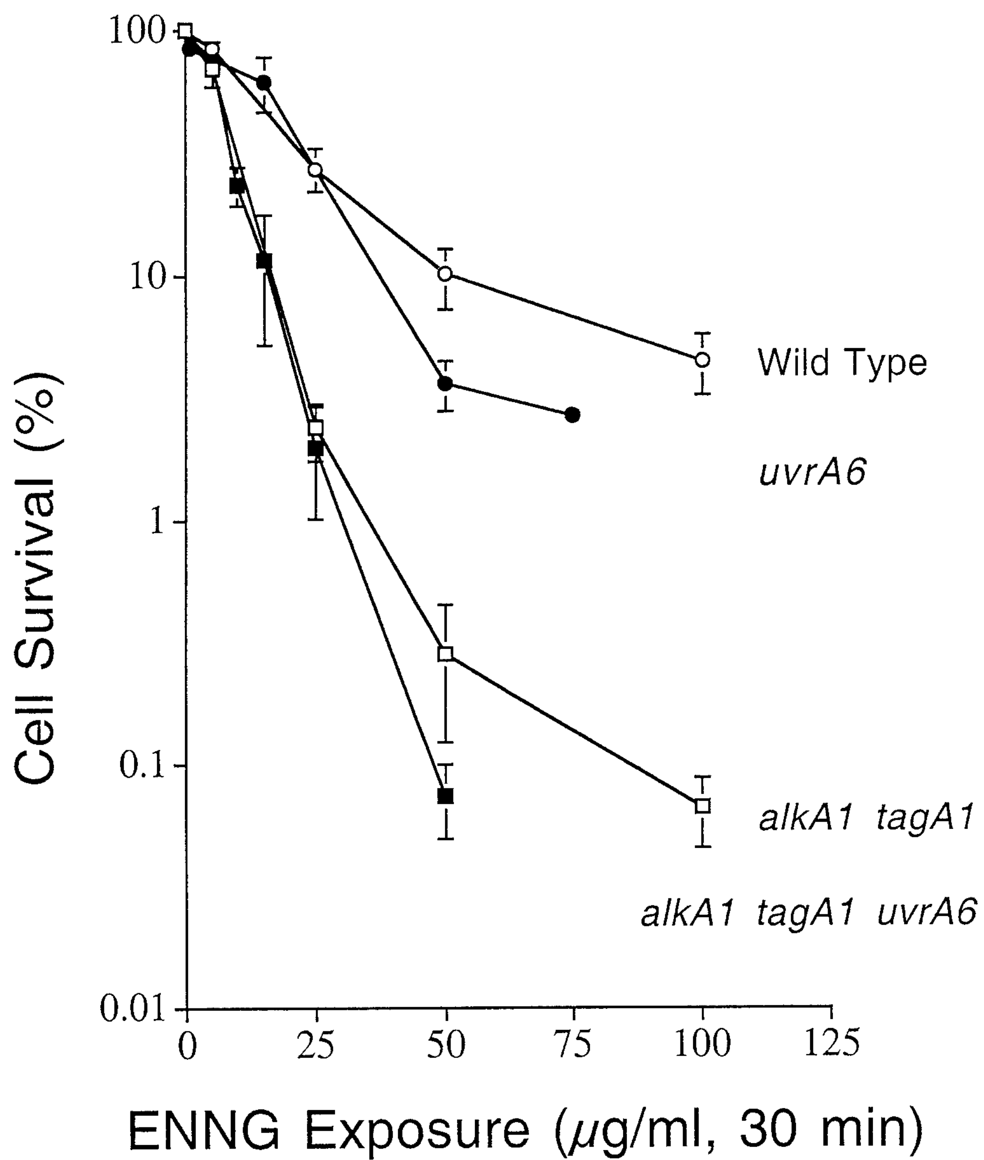


Figure 3B

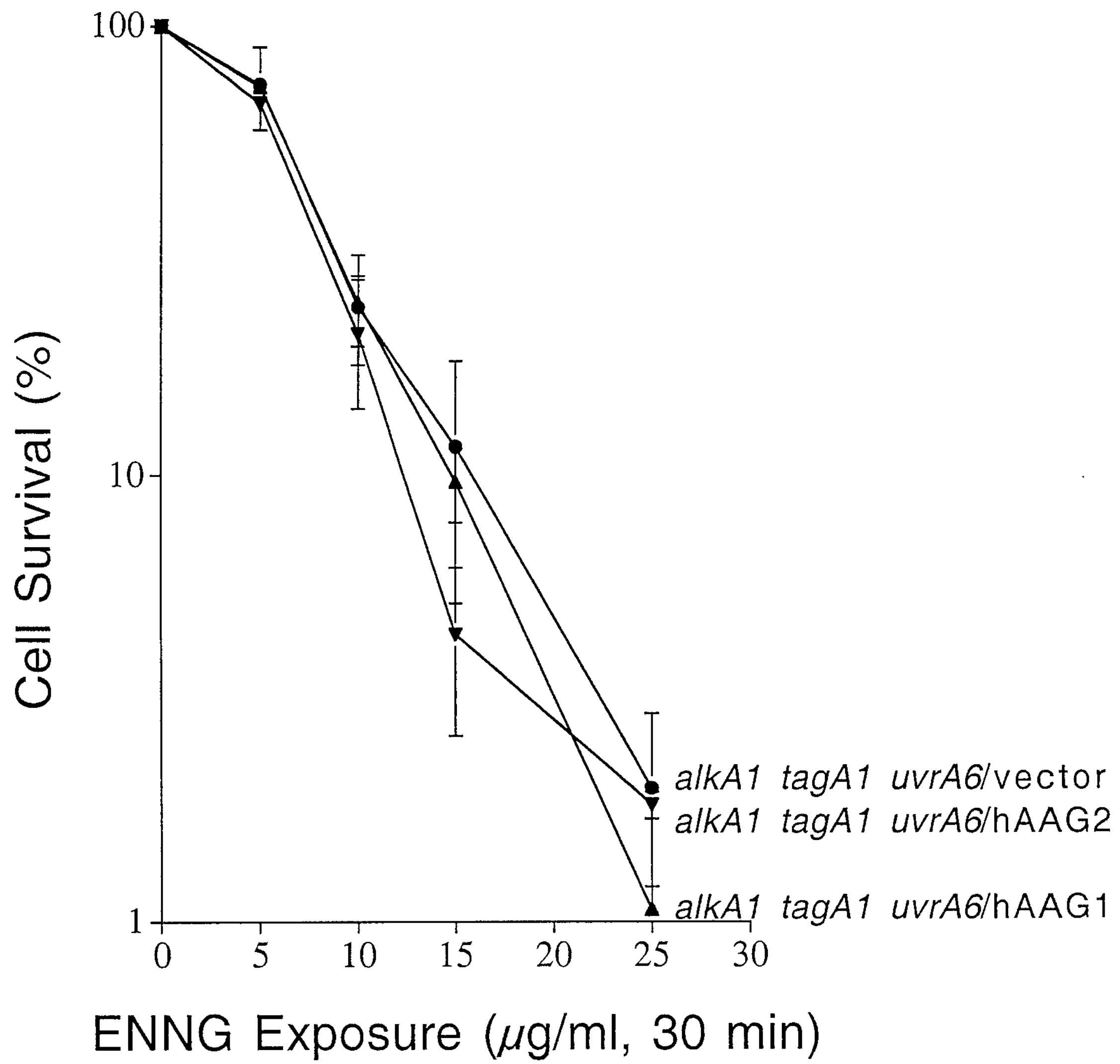


Figure 4A

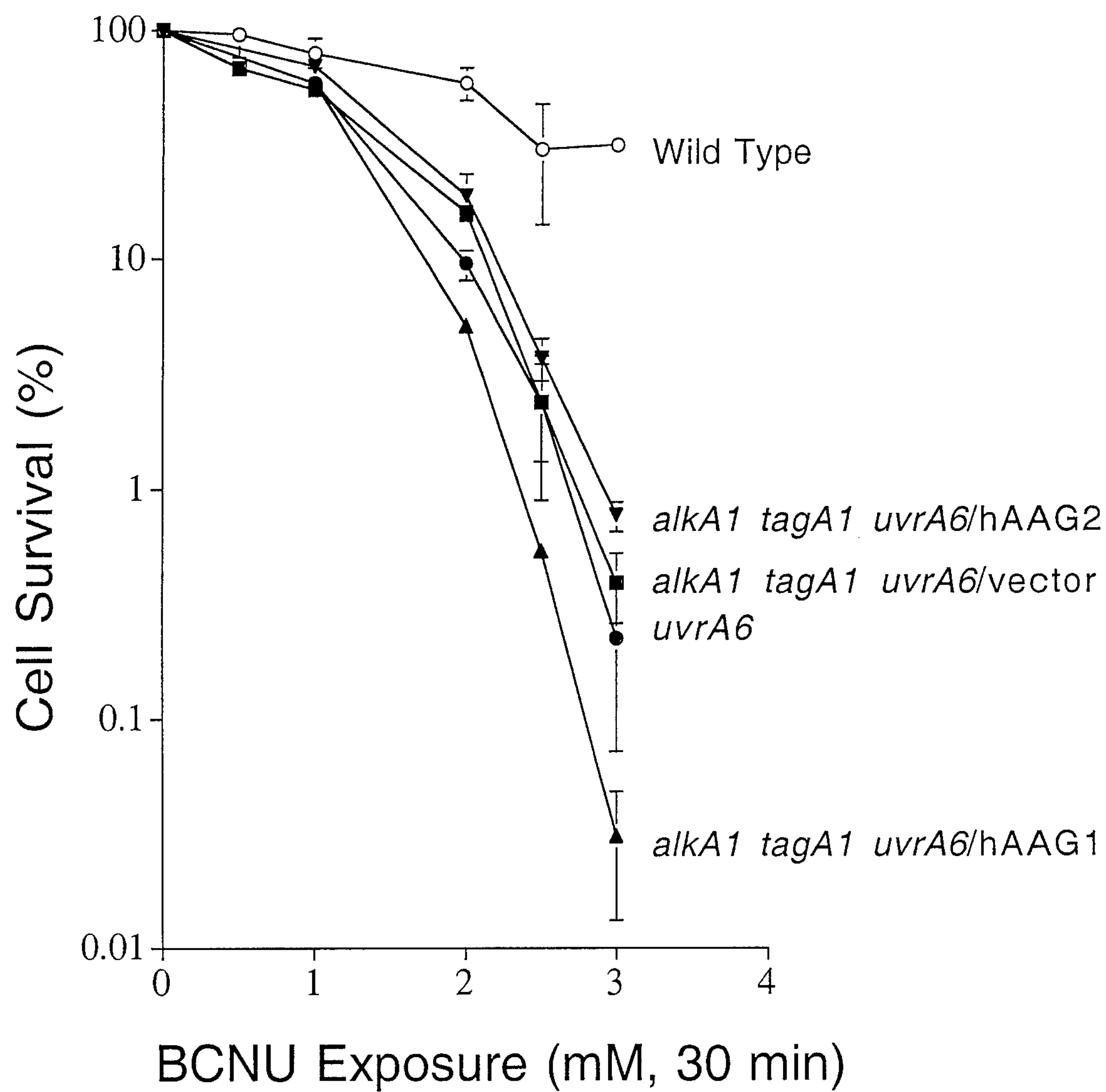


Figure 4 B

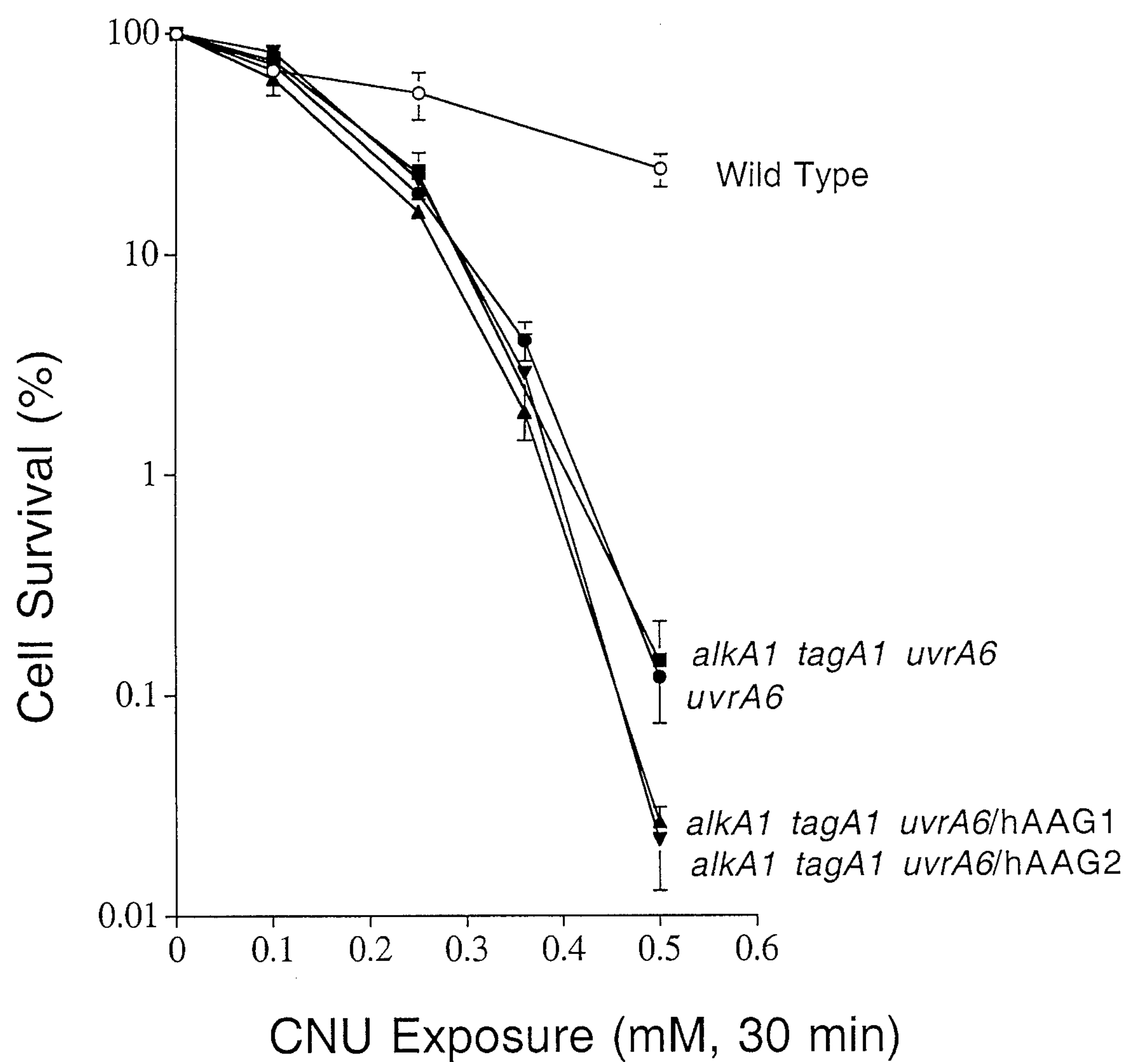


Figure 4C