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Brief Communication

The bacterial alkyltransferase-like (eATL) protein protects mammalian cells against methylating agent-induced toxicity

Karl-Heinz Tomaszowski[a], Dorthe Aasland[a], Geoffrey P. Margison[b,1], Emma Williams[b], Sarah I. Pinder[b,2], Mauro Modesti[c], Robert P. Fuchs[c], Bernd Kaina[a,*]

[a] Department of Toxicology, University Medical Center, Obere Zahlbacher Strasse 67, D-55131 Mainz, Germany
[b] Paterson Institute for Cancer Research, University of Manchester, Wilmslow Road, Manchester, M20 4BX Manchester, UK
[c] Centre de Recherche en Cancérologie de Marseille, CNRS-UMR7258, Inserm-U1068, Institut Paoli-Calmettes, Université Aix-Marseille, France

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ABSTRACT

In both pro- and eukaryotes, the mutagenic and toxic DNA adduct O^6-methylguanine (O^6MeG) is subject to repair by alkyltransferase proteins *via* methyl group transfer. In addition, in prokaryotes, there are proteins with sequence homology to alkyltransferases, collectively designated as alkyltransferase-like (ATL) proteins, which bind to O^6-alkylguanine adducts and mediate resistance to alkylating agents. Whether such proteins might enable similar protection in higher eukaryotes is unknown. Here we expressed the ATL protein of *Escherichia coli* (eATL) in mammalian cells and addressed the question whether it is able to protect them against the cytotoxic effects of alkylating agents. The Chinese hamster cell line CHO-9, the nucleotide excision repair (NER) deficient derivative 43-3B and the DNA mismatch repair (MMR) impaired derivative Tk22-C1 were transfected with eATL cloned in an expression plasmid and the sensitivity to N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) was determined in reproductive survival, DNA double-strand break (DSB) and apoptosis assays. The results indicate that eATL expression is tolerated in mammalian cells and confers protection against killing by MNNG in both wild-type and 43-3B cells, but not in the MMR-impaired cell line. The protection effect was dependent on the expression level of eATL and was completely ablated in cells co-expressing the human O^6-methylguanine-DNA methyltransferase (MGMT). eATL did not protect against cytotoxicity induced by the chloroethylating agent lomustine, suggesting that O^6-chloroethylguanine adducts are not target of eATL. To investigate the mechanism of protection, we determined O^6MeG levels in DNA after MNNG treatment and found that eATL did not cause removal of the adduct. However, eATL expression resulted in a significantly lower level of DSBs in MNNG-treated cells, and this was concomitant with attenuation of G2 blockage and a lower level of apoptosis. The results suggest that eATL confers protection against methylating agents by masking O^6MeG/thymine mispaired adducts, preventing them from becoming a substrate for mismatch repair-mediated DSB formation and cell death.

1. Introduction

DNA is known to contain various types of alkylation damage that are presumed to arise from exposure to exogenous chemical agents or endogenously as by-products of cellular metabolism [1]. One of the lesions found in DNA as a result of the reaction with methylating species is O^6-methylguanine (O^6MeG), a highly mutagenic and cytotoxic DNA lesion [2]. O^6MeG exerts its mutagenic effect through mispairing with thymine during DNA replication, leading after a further round of DNA replication to G:C to A:T transition mutations [3,4]. The cytotoxic effect of O^6MeG stems from the binding of the mismatch repair (MMR) protein MutSα, a dimer composed of MSH2 and MSH6, to O^6MeG:T mispairs. This initiates binding of other MMR proteins and the excision of the misincorporated thymine, but due to the mispairing properties of O^6MeG, thymine is reinserted opposite O^6MeG, resulting in futile cycles of MMR along with extended DNA single-strand gaps [5]. Replication of DNA containing these structures generates DNA double-strand breaks (DSBs), which trigger cell death by activating apoptosis pathways [6–8]. The more complex DNA

* Corresponding author. Tel.: +49 6131 17 9217; fax: +49 6131 17 8499.
E-mail address: kaina@uni-mainz.de (B. Kaina).
[1] Current address at: Centre for Occupational and Environmental Health, Faculty of Medical & Human Sciences, University of Manchester, Manchester M13 9PL, UK.
[2] Current address at: Genome Damage and Stability Centre, University of Sussex, Falmer, Brighton BN1 9QG, UK.

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adduct, O^6-chloroethylguanine (O^6ClEtG), which is generated by chemotherapeutic chloroethylnitrosoureas (CNUs) such as lomustine (CCNU), is also cytotoxic. However, induction of cell death following CNUs occurs in an MMR independent manner [9]. Thus, intramolecular rearrangement of the O^6ClEtG adduct leads ultimately to the formation of an N1-guanine-N3-cytosine interstrand crosslink (ICL) [10], which blocks replication, resulting in collapse of replication forks and the formation of DSBs [11].

The potential lethal effects of O^6-guanine alkylation damage have likely resulted in the evolution and conservation in pro- and eukaryotes of various strategies to eliminate the damage from DNA. One such mechanism, in all species except plants, involves the removal of the alkyl group by alkyltransferases (for human cells O^6-methylguanine-DNA methyltransferase (MGMT) [9]). These proteins bind to O^6-alkylguanine adducts in DNA and transfer the alkyl group to an internal cysteine residue in an autoinactivating stoichiometric process leading ultimately to proteasomal degradation [12]. In addition, in bacteria and yeast, there are proteins with some sequence homology to AGTs, collectively designated as alkyltransferase-like (ATL) proteins. Where examined, these proteins exhibit no alkyltransferase activity [13,14], but are able to bind to a wide variety of O^6-alkylguanine lesions [13,15] and confer resistance to the toxic and mutagenic effects of alkylating agents [16,17].

The protective effect of ATL proteins has been attributed to binding to the damaged DNA strand, which results in DNA bending and base flipping and the recruitment of nucleotide excision repair (NER) proteins that eliminate the O^6-alkylguanine adduct [18,19]. In *Schizosaccharomyces pombe*, the alkyltransferase-like protein 1 (Atl1) targets O^6MeG for global genome NER, whereas transcription-coupled NER participates in the repair of more complex adducts [20]. The ATL of *Escherichia coli*, initially described as ybaZ [21], was not only reported to initiate NER [17], but also to mask DNA damage and thus prevents the conversion of certain O^6-alkylguanines to toxic lesions by the MMR system [22]. This process was shown to reduce the transforming effect in *E. coli* of a plasmid containing O^6-hydroxyethyl-, O^6-1-hydroxypropyl- and O^6-2-hydroxypropylguanine, but not O^6MeG, albeit at the cost of a higher mutation frequency [22]. It was therefore proposed to be a lethality avoidance mechanism by damage tolerance.

ATL proteins, that is, O^6-alkylguanine sensing proteins that have no inherent catalytic activity but support the repair of O^6-alkylguanine adducts, have not so far been reported in mammalian cells. O^6MeG is a highly mutagenic and cytotoxic adduct, and, given that prokaryotes have evolved different strategies for removing this lesion from DNA, it is remarkable that mammalian cells appear to have only a single protein, MGMT, responsible for its repair. It might be speculated that mammalian cells either do not need a backup system for repairing O^6MeG (in which ATL is involved) or do not tolerate protein(s) with ATL function that probe the DNA for the presence of O^6-alkylguanines and support their repair. It was therefore of interest to investigate whether the expression of ATL in mammalian cells is tolerable and whether it impacts the effects of simple alkylating agents in cells that do not express MGMT. To examine the possible effects of ATL expression in mammalian cells, we introduced the ATL protein of *E. coli*, in the following referred to as eATL, into Chinese hamster cells and exposed them to alkylating agents. We show that expression of eATL is well tolerated, having no impact on cell proliferation and survival. It protects against cytotoxicity induced by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), but not lomustine (CCNU). Intriguingly, eATL expressing cells did not demonstrate increased capacity to eliminate O^6MeG from DNA. However, they showed a reduced number of DSBs, attenuated G2 arrest and apoptosis following MNNG indicating that protection against killing occurred by preventing the action of MMR on O^6MeG:T mispairs induced in replicating cells.

2. Materials and methods

2.1. Cell culture and drug treatment

The wild-type Chinese hamster cell line, CHO-9, the corresponding ERCC1 mutant 43-3B (NER-deficient) [23,24], the MMR-impaired derivative Tk22-C1 (designated originally as strain Tk22cos9/5-1/2-C1 [25]) and a CHO-9 derivative stably transfected with the human MGMT cDNA [26] were used in this study. All cell lines were cultured with Dulbecco's MEM/F-12 medium (1:1) containing 5% foetal calf serum (FCS) and penicillin (100 unit/ml) and streptomycin (100 μg/ml) in a humidified atmosphere in 7% CO_2 at 37 °C. MNNG (Sigma, Munich, Germany) was dissolved in dimethylsulfoxide (DMSO) and then diluted with sterile water to a final concentration of 10 mM. A solution of 10 mM lomustine [1-(2-chloroethyl)-3-cyclohexyl-L-nitrosourea; CCNU] (Sigma, Munich, Germany) was prepared by dissolving in ethanol. Aliquots of MNNG and CCNU were stored at −80 °C and −20 °C, respectively. For the treatment of exponentially growing cells MNNG or CCNU was added directly to the medium.

2.2. Plasmid constructions and transfection

The eATL pcDNA3.1 vector (containing a neomycin resistance cassette) was generated using the pcDNA3.1/V5 His Topo TA Expression Kit (Invitrogen, Darmstadt, Germany) according to the provided protocol. The eATL cassette was isolated by PCR using the vector pMBP-YbaZ [22] as template and eATL forward: 5′-GCCATGCGACTTCACTCGGGC-3′; reverse: 5′-TCAGTAGTTCCAGCGATAACG-3′ primers. The version of eATL pcDNA3.1 containing a hygromycin resistance cassette was obtained by excision of the eATL sequence with Hind III and Xho I from the eATL pcDNA3.1 (neomycin) vector and insertion into the Hind III-Xho I digested pcDNA3.1 (hygromycin) vector. The Effectene transfection kit (Qiagen, Hilden, Germany) was used for transient transfection of Tk22-C1 cells and to stably transfect CHO-9 and 43-3B cells with the eATL pcDNA.3.1 neomycin vector, as well as AT17-C3 cells with the eATL pcDNA.3.1 hygromycin vector. After transient transfection Tk22-C1 cells were allowed to regenerate before initiation of experiments. To obtained stably transfected clones cells were grown in media containing 1.5 mg/ml G418 (CHO-9, 43-3B) or 0.8 mg/ml hygromycin B and resistant clones were selected. Transfectants were routinely cultured in selective media, but the selective agents were omitted during the experiments.

2.3. Polymerase chain reaction

Total RNA from cultured cells was isolated using the Nucleospin RNA II Kit (Macherey-Nagel, Düren, Germany). One microgram RNA was transcribed into cDNA using the Verso cDNA Kit (Thermo Scientific, St. Leon-Rot, Germany). PCR amplification was performed using Red-Taq Ready Mix (Sigma–Aldrich, Taufkirchen, Germany) and primers for eATL (forward: 5′-TCGCCACGGCACAATTTCGC-3′, reverse: 5′-AGTCGATTTGCCCGCTTCCCG-3′) and β-Actin (forward: 5′-GCTCTTTTCCAGCCTTCCTT-3′, reverse: 5′-GAGCCAGAGCAGTG ATCTCC-3′).

2.4. Antibody production

Anti-eATL polyclonal antibodies were obtained by immunisation of rabbits (Eurogentec) with recombinant MBP-eATL and purified by protein A affinity chromatography.

2.5. Preparation of cell extracts and western blotting

To achieve a probable accumulation of eATL protein in the nucleus (through binding of eATL to O^6MeG), cells were treated

with MNNG (10 μM MNNG, 1 h). AT17-C3 and their corresponding eATL clone underwent pre-treatment (1 h) with 10 μM O^6-benzylguanine for depletion of MGMT. Nuclear extracts were prepared and western blot analysis was performed as previously described [27]. Protein was visualised using the Odyssey system (LI-COR Biosciences). The following antibodies were used: anti-eATL (dilution 1:25); β-actin (dilution 1:4.000, Santa Cruz, Heidelberg, Germany) and donkey fluorophor-coupled secondary antibody (dilution 1:5.000, LI-COR Biosciences).

2.6. Colony formation assay

The colony formation assay was performed as previously described [28]. Briefly, exponential growing cells were seeded, allowed to attach for 6 h and treated with varying concentrations of alkylating agents. After incubation for 8 or 10 days, colonies were fixed, stained and counted.

2.7. Determination of apoptosis and necrosis

Induction of apoptosis or necrosis was determined by annexin V-FITC/propidium iodide (PI) staining and flow cytometry (FACSCanto, Becton Dickinson, Heidelberg, Germany) as previously described [27].

2.8. Quantification of O^6MeG

DNA was extracted from cell pellets and analysed for O^6MeG content by an MGMT competitive inactivation method as previously described [29].

2.9. Proliferation and cell cycle analysis

Cell proliferation status was assessed by flow cytometry to quantify the mean fluorescence intensity (MFI) of carboxyfluorescein diacetate succinimidyl ester (CFSE) as previously described [8]. Briefly, cells (1×10^6 cells/ml) were labelled in PBS containing 5 μM CFSE (Sigma, Munich, Germany) for 8 min at room temperature in the dark. Residual CFSE was quenched by adding 5 volumes FCS. Cells were washed twice with PBS and seeded in 60 mm cell culture dishes for 24 h. Following non-treatment or treatment with 10 μM MNNG for 1 h at 37 °C (defined as zero time, and 100% CFSE signal) and subsequent incubation for various times, cells were harvested and re-suspended in PBS with 16.7 μg/ml PI for 5 min at room temperature in the dark. Flow cytometry was performed using a FACSCanto (Becton Dickinson, Heidelberg, Germany). To analyse cell cycle progression, adherent cells were harvested and fixed in 70% ice cold ethanol for at least 30 min. After RNAase (30 μg/μl) digestion, DNA was stained with PI (16.7 μg/ml) in PBS. Samples were analysed by flow cytometry and the proportion of cells in each phase of the cell cycle were calculated using ModFitTM.

2.10. Fluorescence microscopy

Cells were seeded on cover slips, treated with MNNG (1 μM, 24 h) and prepared for γH2AX immunostaining. Cells were fixed in 4% formaldehyde-PBS and then ice-cold 100% methanol was added. For LSM, fixation occurred in methanol:acetone (v/v, 7:3) at −20 °C. Non-specific binding of antibodies was avoided by blocking with 5% BSA in 0.3% Triton X-PBS. Cells were incubated with the primary antibody (mouse anti-phospho-H2A.X, Millipore, 1:1000) at 4 °C overnight followed by incubation with secondary antibody (anti-mouse Alexa Fluor 488, Invitrogen, 1:500) at room temperature. Nuclei were counterstained with 100 nM DAPI (quantification of γH2AX) or 1 μM TO-Pro-3 (representative example) and slides were mounted in VECTASHIELD (Vector Laboratories). For quantification, foci were automatically scored by fluorescent microscopy using the Metafer Finder System v. 3.1 (MetaSystems). Representative examples for γH2AX staining were acquired by confocal microscopy (LSM 710; Carl Zeiss).

Table 1
Cell lines, their repair status and population doubling times.

Cell line	Repair status	Doubling time (h)
CHO-9	Wild-type	11.6 ± 1.2
CHO eATL Cl. 3	eATL proficient	10.6 ± 1.2
CHO eATL Cl. 4	eATL proficient	13.0 ± 0.5
43-3B	ERCC1 mt (NER deficient)	11.7 ± 1.4
43-3B eATL Cl. 1	eATL proficient + NER deficient	12.7 ± 1.8
AT17-C3	MGMT proficient	10.5 ± 1.3
AT17 eATL Cl. 4	MGMT and eATL proficient	10.5 ± 1.4
Tk22-C1	MMR impaired (decline of MSH2)	13.8 ± 0.3
Tk22-C1 eATL	eATL proficient + MMR impaired	13.3 ± 0.4

2.11. Neutral comet assay

The procedure for detection of DSBs by neutral single-cell gel electrophoresis was performed as previously described [6]. Analysis of DNA migration occurred by means of an image analysis system (Komet 4.0.2; Optilas) and head/tail intensity of at least 50 cells per sample was determined.

3. Results and discussion

To determine the effect of eATL on the response of mammalian cells to alkylating agents, we transfected eATL into Chinese hamster cells and selected stably transfected clones. We used the wild-type CHO-9, its NER-defective mutant 43-3B (mutated in ERCC1) and the MGMT-overexpressing CHO-9 transfectant AT17-C3. RT-PCR of RNA isolated from G418 and hygromycin B resistant clones confirmed the expression of eATL (Fig. 1A) and western blotting demonstrated that the transfectants expressed detectable amounts of eATL (Fig. 1B). Two independent clones of CHO-9 showed different levels of expression of eATL on protein level, designated as Cl. 3 and Cl. 4 (Fig. 1B). Expression was stable during the course of the experiments. We also performed transient transfection experiments with the isogenic MMR-impaired cell line Tk22-C1. The expression of eATL mRNA (Fig. 1C) and protein (Fig. 1D) was confirmed 24 h after transfection. It remained still detectable 7 days thereafter (not shown). The eATL protein had no significant impact on the proliferation rate of the cells (Table 1).

In colony forming experiments, CHO-9 cells expressing eATL were clearly more resistant to MNNG than the parental line, and CHO-9 eATL Cl. 3 was more resistant than CHO-9 eATL Cl. 4 (Fig. 2A), which correlated with the eATL protein expression level (Fig. 1B). The CHO-9 derived mutant 43-3B did not differ significantly from the parent cell line in its sensitivity to MNNG (Fig. 2A and B), which was expected as ERCC1 is not involved in the repair of MNNG-induced DNA lesions [30]. Expression of eATL in 43-3B cells again resulted in enhanced survival rate, closely similar to those seen in CHO-9 eATL Cl. 3 (Fig. 2A and B), indicating that eATL provokes MNNG resistance independent of NER. CHO-9 cells do not express MGMT and expression of MGMT in the AT17-C3 cell line conferred a significant increase in resistance to MNNG *versus* CHO-9. Additional expression of eATL neither increased nor decreased MNNG resistance (Fig. 2C), indicating that eATL operates independently of MGMT in mammalian cells. We should note that eATL, due to its binding affinity to O^6MeG, reduces the capacity of MGMT for repairing the adduct, which was shown in assays *in vitro* [13]. The possible competition of eATL with MGMT in the context of the chromatin *in vivo* (in mammalian cells) remains to be elucidated.

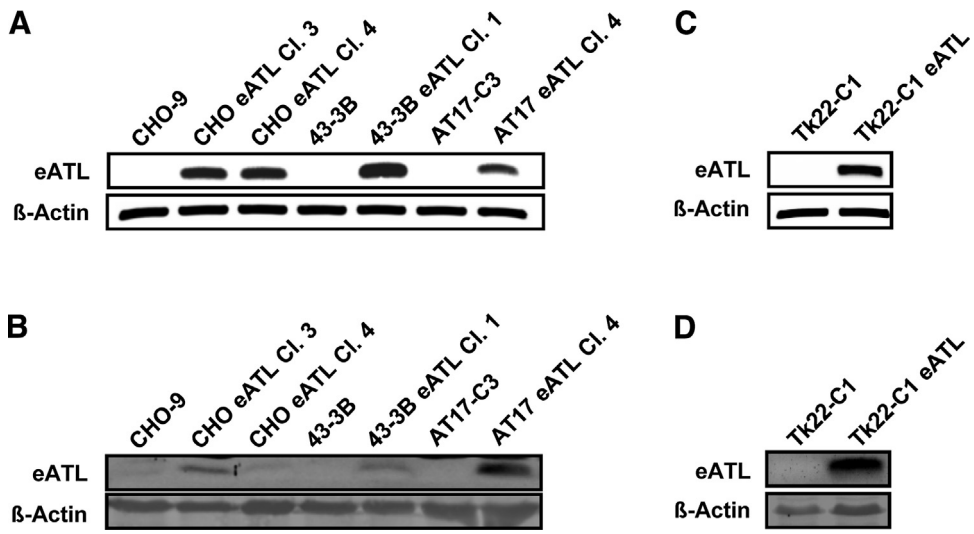

Fig. 1. Expression of eATL in stably and transiently transfected cell lines. CHO cells were transfected with eATL-expression vector and selected for G418 or hygromycin B resistance to obtained stable clones. Expression was confirmed by RT-PCR of mRNA (A and C) and western blot analysis of nuclear protein (B and D). For transient transfection, mRNA and protein level of eATL were analysed 24 h after transfection (C and D). β-Actin served as loading control.

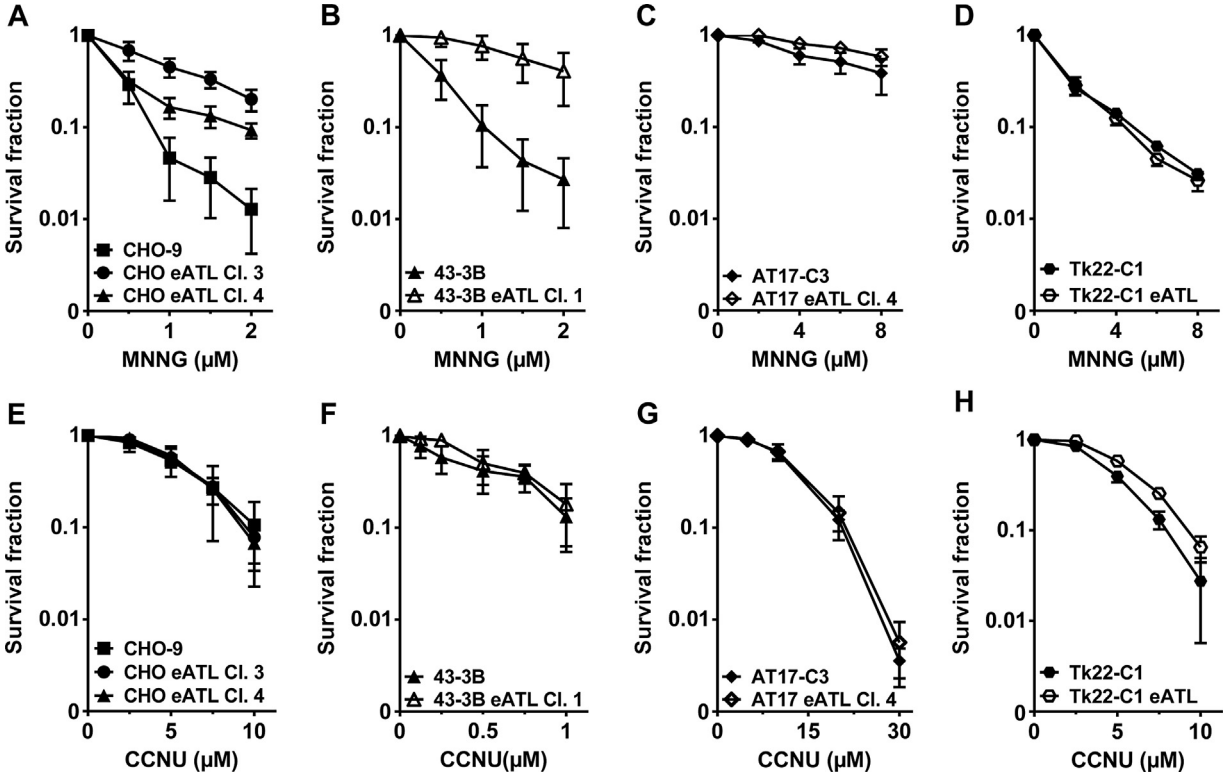

Fig. 2. Sensitivity of eATL non-expressing and expressing cell lines to MNNG and CCNU, determined in colony forming experiments. Cells were seeded and 6 h later exposed to various concentrations of MNNG (A-D) or CCNU (E-H). After 8 or 10 days colonies were fixed, stained and counted. Data are the mean of three independent experiments ± SD. The measure points for eATL transfectants compared to the parental cells shown in panel A and B are highly significant ($p < 0.05$).

Survival experiments were also performed with Tk22-C1 cells derived from CHO-9, which are characterised by downregulation of MSH2 expression (20% MSH2 protein compared to the wild-type) and increased MNNG resistance [25]. Expression of eATL in Tk22-C1 (Fig. 1C and D) did not change their response to MNNG (Fig. 2D), indicating that under MMR-impaired conditions eATL does not confer protection.

A critical cytotoxic lesion induced in DNA by chloroethylating agents is *O*^{6}-chloroethylguanine (O^{6}ClEtG), which is also a substrate for MGMT. Therefore, we examined if eATL would have an impact on the cytotoxicity of this lesion. eATL expression had no effect on the sensitivity of either CHO-9 (Fig. 2E) nor 43-3B cells (Fig. 2F) to CCNU, suggesting that eATL has no impact on the repair of O^{6}ClEtG adducts. Of note, the NER-defective cell line 43-3B and the corresponding eATL clone showed much greater sensitivity to the chloroethylating agent than CHO-9 cells (note the much lower concentration of CCNU used for these cells) due to their inability to repair interstrand crosslinks (ICL), arising from O^{6}ClEtG adducts; ICL repair requires the involvement of functional NER [31]. In contrast, AT17-C3 cells (expressing MGMT) were clearly more resistant

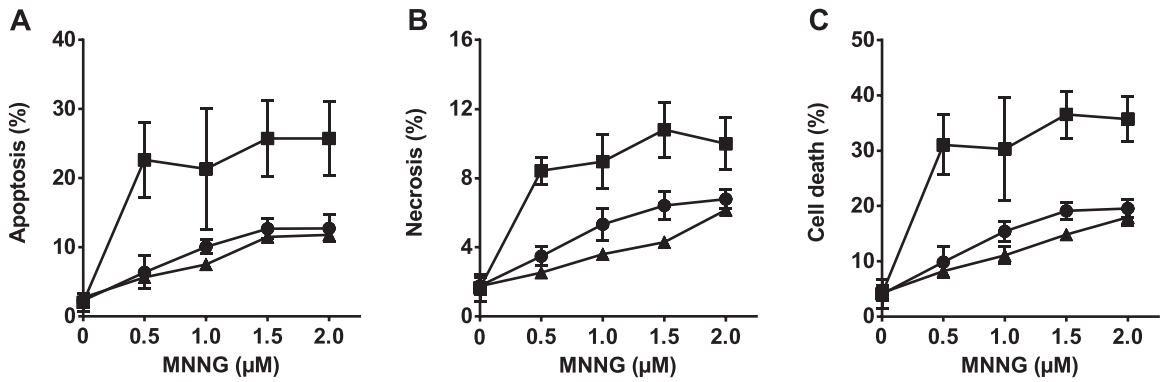

Fig. 3. Effect of eATL expression on apoptosis and necrosis after MNNG treatment. CHO-9 (■), CHO eATL Cl. 3 (●) and CHO eATL Cl. 4 (▲) were exposed to various concentrations of MNNG and annexin V/PI analyses were performed by flow cytometry 72 h after treatment. Data are the mean of three independent experiments ± SD.

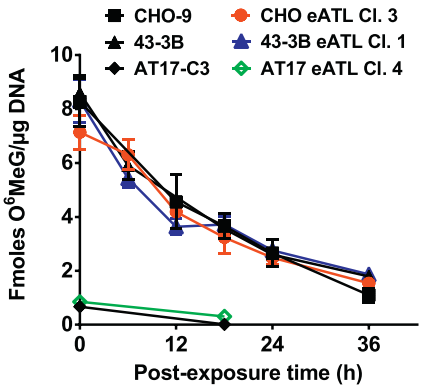

Fig. 4. Repair kinetics of O^6MeG. The cell lines indicated were treated with 10 μM MNNG for 1 h. Levels of O^6MeG in the DNA were determined immediately after treatment (0 h) and over a period of 36 h post-treatment.

to CCNU (Fig. 2G compare with Fig. 2E: note the change of scale of the X axis). The death of MGMT-expressing cells at high dose CCNU treatment is likely the result of N-chloroethylations, which are less toxic than O^6ClEtG [11]. Again, the expression of eATL in these cells had no impact on cell survival following CCNU treatment (Fig. 2G). The sensitivity of the MMR-impaired cell line Tk22-C1 to CCNU was comparable to CHO-9 cells, supporting the notion that cytotoxicity provoked by chloroethylating agents is MMR-independent. Expression of eATL in Tk22-C1 had no clear impact on their response to CCNU (Fig. 2H), suggesting, together with the results obtained for CHO-9 and 43-3B transfectants, that eATL does not influence the repair of O^6ClEtG.

Cell death following MNNG treatment in CHO-9 cells is the result of apoptosis triggered by O^6MeG [6], although some necrosis can also be induced [32]. The level of apoptosis and necrosis following increasing doses of MNNG in CHO-9 cells, CHO eATL Cl. 3 and CHO eATL Cl. 4 is shown in Fig. 3A and B, respectively. Fig. 3C shows the effect of increasing doses of MNNG on total cell death. The data shows that eATL-expressing cells are more resistant to MNNG-induced apoptosis and necrosis. This confirms the notion that eATL protects against O^6MeG, which is the major death triggering lesion in cells lacking MGMT [9].

In mammalian cell, the only known repair mechanism for O^6MeG is MGMT and, therefore, in cells lacking MGMT, O^6MeG is a persistent DNA lesion [33]. To examine the possibility that eATL enables the removal of O^6MeG from DNA, we measured the amount of O^6MeG in CHO-9 wild-type, 43-3B and AT17-C3 cells in the absence and presence of eATL immediately and up to 36 h after pulse treatment (1 h) with 10 μM MNNG. As shown in Fig. 4, the amount of O^6MeG was, as anticipated, significantly lower in

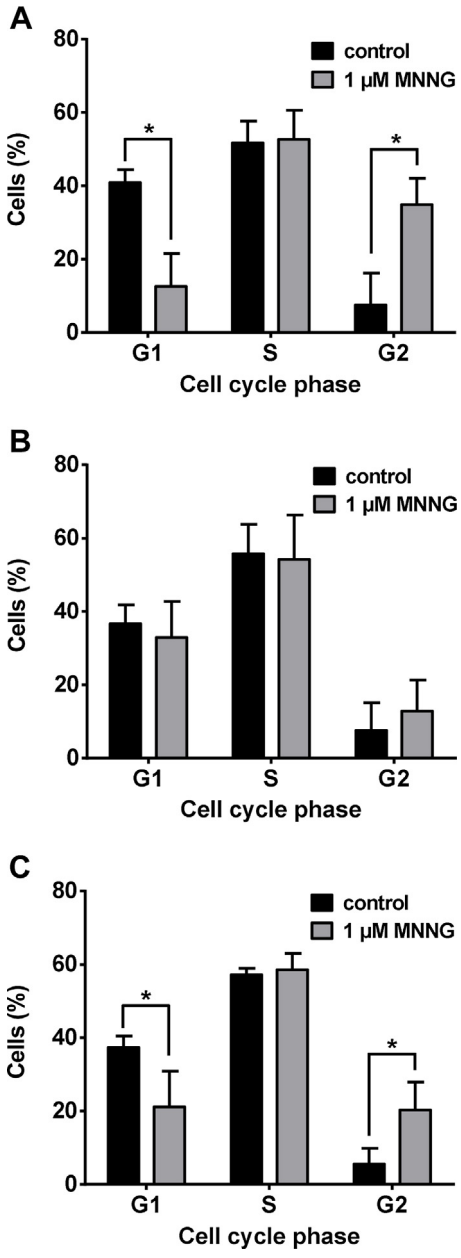

Fig. 5. Cell cycle progression following exposure to MNNG. (A) CHO-9 cells, (B) CHO eATL Cl. 3 and (C) CHO eATL Cl. 4 were treated with 1 μM MNNG and the proportion of cells in each cell cycle phase was quantified 24 h later by flow cytometry. Data are the mean of three independent experiments. *$p < 0.01$.

AT17-C3 cells (MGMT expressing) compared to the other isogenic lines and declined to undetectable levels when measured 18 h later. Most importantly, in all other cell lines lacking MGMT, the O^6MeG level was high immediately after treatment and declined at the same rates in parental cells and transfectants, as determined up to 36 h after treatment. This decline is the result of dilution of the DNA damage by cell proliferation, which was similar in all of the cell lines (data not shown). Overall, the data indicate that eATL has no impact on the removal of O^6MeG from DNA, despite the observation that it strongly impacts on the killing effects of this lesion.

The current model for the mechanism of cell killing by O^6MeG suggests that replication of DNA containing this lesion results in an O^6MeG:T mispair, which is recognised by MMR proteins. MMR processing of this mismatch conserves the O^6MeG adduct, but causes the persistence of long single-strand gaps in the DNA which, on the second round of replication, lead to collapse of replication forks, DSB formation and cell death [8]. eATL has been shown to bind to both O^6MeG:C base pairs and O^6MeG:T mismatches [22], so one possibility is that eATL binding to the mismatch blocks the access of MutSα, ultimately preventing the formation of DSB. To explore this hypothesis, we first analysed cell cycle progression following MNNG treatment, since O^6MeG processing by MMR results in CHO-9 cells in a G2 arrest in the 2nd post-treatment cell cycle [8]. As shown in Fig. 5A, treatment of CHO-9 cells caused a highly significant reduction of cells in G1 and an accumulation in G2 phase 24 h later. In contrast, eATL-expressing cells showed a reduced G2 block after MNNG treatment depending on their eATL expression level (Fig. 5B and C). Thus, treatment of CHO eATL Cl. 4 caused a moderate accumulation in G2 phase (20% compared to 35% in the wild-type), whereas in CHO eATL Cl. 3 cells there was no significant difference between untreated and MNNG-treated cells, demonstrating that eATL expression ablated the effects of MNNG on the cell cycle.

If eATL prevents O^6MeG:T mispairs from being processed by MMR proteins, which is required for DSB formation [6], we posited that DSBs will not be formed or formed at a lower level in the presence of eATL. To this end, we assessed the formation of γH2AX foci, a sensitive marker for DSBs [34], and also performed the neutral comet assay, which is an established method for DSB detection [35]. Representative photomicrographs of untreated and MNNG-treated CHO-9 and eATL-expressing cells are shown in Fig. 6A. Quantification showed a significantly lower number of γH2AX foci per cell (Fig. 6B) and a significantly lower fraction of cells with a high number of foci (>30) per cell in the MNNG resistant eATL-expressing population (Fig. 6C). Additionally, we observed in the neutral comet assay in eATL expressing cells a significantly lower tail intensity following MNNG, compared to CHO-9 (Fig. 6D). This supports the notion that eATL reduces the yield of DSBs in cells treated with MNNG.

In summary, we demonstrate that mammalian cells tolerate the expression of eATL and that eATL confers protection against the killing effect of the *O*^{6}-methylating agent MNNG. While eATL expression did not affect the persistence of O^6MeG in MGMT lacking cells treated with MNNG, we show that the level of apoptosis and necrosis and the frequency of DSBs triggered by O^6MeG were significantly reduced in the presence of eATL. These observations are consistent with the hypothesis that eATL masks the O^6MeG:T

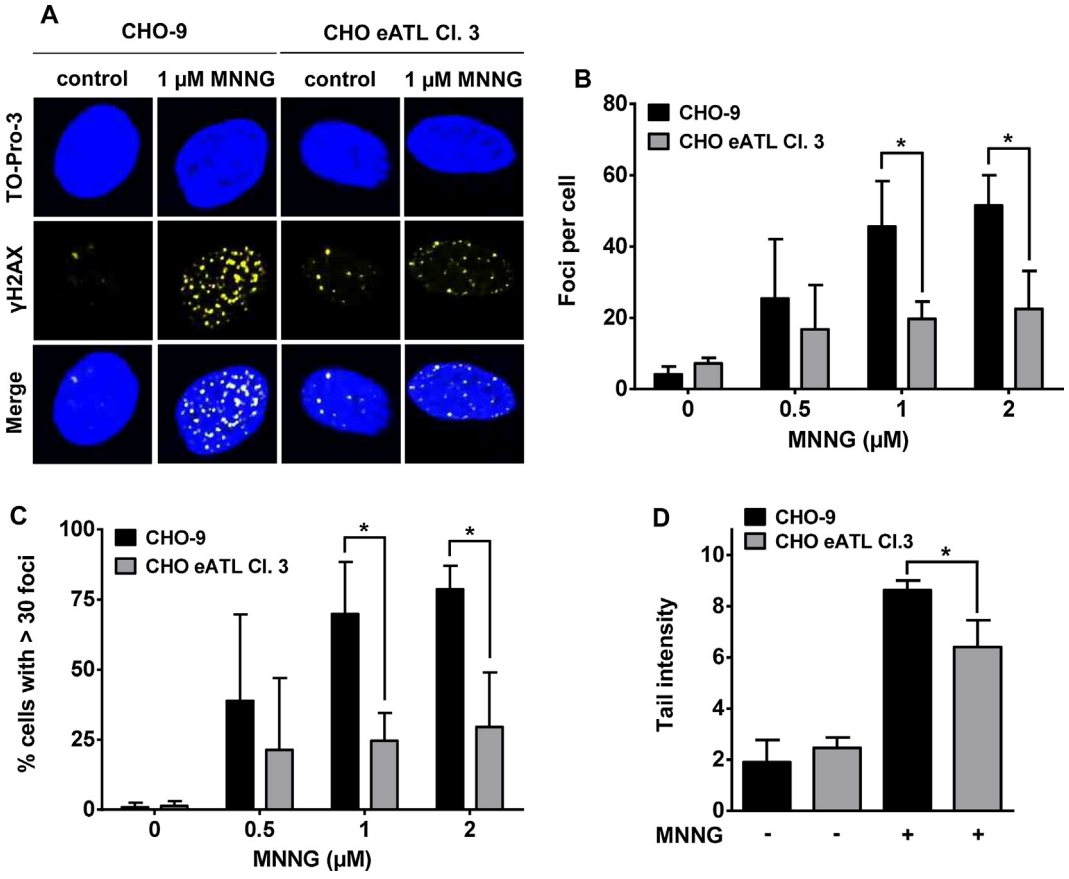

Fig. 6. Formation of MNNG-induced DNA double-strand breaks. (A) Examples of γH2AX foci (yellow) in CHO-9 and CHO eATL Cl. 3 cells 24 h after treatment with 1 μM MNNG. Nuclei (blue) were stained with TO-Pro-3 and pictures were taken from LSM. (B) γH2AX foci were quantified by immunofluorescence microscopy as described in methods and shown as number of foci per cell or (C) percentage of cells showing more than 30 foci. Data are the mean of three independent experiments ± SD in which at least 200 nuclei were counted per dose. $^*p < 0.05$. (D) Level of DSBs measured by the neutral comet assay in non-treated and 1 μM MNNG treated cells, determined 24 h after treatment. The level of DNA breakage was expressed by the relative tail intensity. $^*p < 0.05$.

mispairs and therefore prevents them from being converted by futile MMR cycles and replication into cytotoxic DSBs. The finding that in MMR impaired cells eATL was ineffective, as it did not further enhance the resistance to MNNG, supports this notion. In experiments with *E. coli* the eATL (YbaZ) protein was shown to bind to O^6MeG/T and also to larger O^6alkylguanine adducts. However, it supported only at low efficiency the rescue of a plasmid containing the O^6MeG adduct in a cell transformation assay [22]. Here we measured survival of mammalian cells harbouring O^6MeG adducts in their genomic DNA in the presence and absence of eATL, and it is reasonable to posit that the DNA damage is processed and signals death in a way that is not identic to *E. coli*. Therefore the data reported here do not contradict findings obtained with bacteria. Given that a functional homologue of ATL that might act as a backup protection mechanism against O^6MeG has not so far been reported in mammalian cells, it is reasonable to speculate that cell death triggered by O^6MeG, which was left unrepaired by MGMT, is a process that serves to remove O^6MeG harbouring cells from the population.

Conflict of interest

No conflict of interest.

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