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No-observed effect levels are associated with up-regulation of MGMT following MMS exposure

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ABSTRACT

The alkylating agents methyl methanesulphonate (MMS) and ethyl methanesulphonate (EMS) have non-linear dose–response curves, with a no-observed effect level (NOEL) and a lowest observed effect level (LOEL) for both gross chromosomal damage and mutagenicity. However, the biological mechanism responsible for the NOEL has yet to be identified. A strong candidate is DNA repair as it may be able to efficiently remove alkyl adducts at low doses resulting in a NOEL, but at higher doses fails to fully remove all lesions due to saturation of enzymatic activity resulting in a LOEL and subsequent linear increases in mutagenicity. We therefore assessed the transcriptional status of *N*-methylpurine-DNA glycoslase (MPG) and O^6-methylguanine DNA methyltransferase (MGMT), which represent the first line of defence following exposure to alkylating agents through the respective enzymatic removal of N7-alkylG and O^6-alkylG. The relative MPG and MGMT gene expression profiles were assessed by real-time RT-PCR following exposure to 0–2 μg/ml MMS for 1–24 h. MPG expression remained fairly steady, but in contrast significant up-regulation of MGMT was observed when cells were treated with 0.5 and 1.0 μg/ml MMS for 4 h (2.5- and 6.5-fold increases respectively). These doses lie within the NOEL for MMS mutagenicity (LOEL is 1.25 μg/ml), thus this boost in MGMT expression at low doses may be responsible for efficiently repairing O^6methylG lesions and creating the non-linear response for mutations. However, as the LOEL for MMS clastogenicity is 0.85 μg/ml, O^6-alkylG is unlikely to be responsible for the clastogenicity observed at these concentrations. Consequently, at low doses N7-methylG is possibly the predominant cause of MMS clastogenicity, while O^6-methylG is more likely to be responsible for MMS mutagenicity, with MGMT up-regulation playing a key role in removal of O^6-alkylG lesions before they are fixed as permanent point mutations, resulting in non-linear dose–responses for direct acting genotoxins.

1. Introduction

The shapes of dose–response curves provide important information for risk assessments to enable the safety evaluation of chemical agents. These curves may have a variety of shapes [1] that all have different implications as they are dependent upon multiple factors ranging from specific chemical characteristics (such as mode of action), to cellular barriers including DNA repair, metabolic activation or membranes that enclose and protect organelles [2]. However, for genotoxins that react directly with DNA the classical assumption (for carcinogen classification and regulatory purposes) has been that a linear relationship prevails with permanent genetic aberrations arising at any given level of exposure [3]. This linear model has largely prevailed because for most compounds the dose–response at low concentrations has not been considered, so as a precautionary measure linear extrapolations have been the default. We have recently challenged this theory and demonstrated that certain alkylating agents, a classical group of electrophilic DNA reactive genotoxins, have non-linear dose–responses with respect to both gross chromosomal damage and mutagenicity [4]. Both methyl methanesulphonate (MMS) and ethyl methanesulphonate (EMS) were shown to lack any significant biological consequence below a critical dose, resulting in a no-observed effect level (NOEL) and a lowest observed effect level (LOEL). MMS and EMS therefore demonstrated a LOEL for chromosomal damage at 0.85 and 1.4 μg/ml respectively, and for point mutations at 1.25 and 1.4 μg/ml. Identification of the biological mechanism that is responsible for the NOEL is necessary to jus-

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y and corroborate its existence [5], and is currently lacking for ese compounds.

Despite the methanesulphonates demonstrating a non-linear se–response, their counterpart nitrosoureas had linear shaped rves. This indicated that the governing factor was the alkyla- n target sites within the bases. The methanesulphonates are gh *s*-value alkylating agents that react with highly nucleophilic ntres, hence their primary targets are N7G, N3A and to a much sser extent O^6G. In contrast, the nitrosoureas have low *s*-values d so will target less nucleophilic centres, they are therefore ore potent alkylators of O^6G, O^2T and O^4T [2,6]. This suggests NA repair may be strongly involved in influencing the shape the respective dose–response curves as N7G, N3A and O^6G ve specific repair mechanisms associated with their removal, hile O^2T and O^4T are very poorly corrected [4]. Consequently, low methanesulphonate exposure levels, DNA repair is likely be primarily responsible for the efficient removal of the N7G, 3A and O^6G DNA adducts resulting in a NOEL, while failing fully remove all the damage at higher concentrations due to turation of enzymatic activity resulting in a LOEL and subse- ent dose dependent increases thereafter. With respect to the trosoureas, it is possible that at low exposure levels, the N7G, 3A and O^6G may be repaired, but those at the O^2T and O^4T posi- ns would persist, giving rise to the more linear dose–response served.

Cells have several DNA repair mechanisms, some of which are pable of correcting specific types of damage. With respect to kyl-DNA adducts, O^6-alkylG is primarily removed by the enzyme -methylguanine DNA methyltransferase (MGMT) in a single- ep reaction [7,8]. The enzyme transfers the alkyl group from the ducted base to an internal cysteine residue within the active ntre of the methyltransferase, resulting in its irreversible inac- vation, so one MGMT molecule will only remove one O^6G adduct . MGMT expression is induced in response to DNA damage (one gulatory pathway involves p53); a cells capacity to repair O^6G sions is therefore dependent upon the rate at which it can synthe- se MGMT [9]. MGMT is most efficient at removing methyl adducts, owever it is capable of removing larger adducts (e.g. ethyl, propyl, tyl adducts) although at a lower efficiency [8]. This enzyme is erefore very important in the protection of DNA against alkylat- g agents. However if MGMT fails to remove the alkyl group and e O^6-alkylG persists, it may stably mispair with thymine in the st cycle of replication, resulting in a GC to AT transition mutation the subsequent cycle [10,11]. In addition to point mutations, O^6- kylG is also associated with causing chromosomal damage as the ymine placed opposite this lesion is subject to mismatch repair. owever, this repair is often in vain as the DNA adduct is still present the parental strand, so the result is a single-stranded gap that in rn leads to double-strand breaks through stalled replication forks 2].

Alkyl adducts that arise at the N7G position are also thought be precursors for both chromosomal damage and point muta- ns as the modification destabilises the N-glycosidic bond and erefore they are prone to spontaneous depurination resulting in asic sites [7,13,14]. During replication these single-stranded gaps e unprotected and can stall replication forks resulting in double- randed breaks, but if they are detected prior to replication, they e subject to error prone repair leading to possible sequence aber- tions [10,15]. However, N7-alkylG adducts are not considered to as mutagenic as O^6-alkylG because the abasic sites are often rrectly repaired. Additionally, the N7-alkylG adducts are usually moved by enzymatic hydrolysis of the N-glycosylic bond, catal- ed by *N*-methylpurine-DNA glycoslase (MPG). This is the first step the initiation of repair of these lesions by the base excision repair ER) pathway [7,15,16].

In the present study, we have elected to investigate the mode of action that underlies the non-linear dose–response that the well known point mutagen and clastogen MMS demonstrates. We firstly demonstrated that alkyl adducts are indeed present following exposure to MMS doses within the NOEL by mass spectrometry, hence ruling out non-linear adduct formation. As MGMT and MPG represent the first line of defence against alkylation damage, we then assessed their gene expression patterns. MGMT was up-regulated suggesting its enhanced expression may have been responsible for removing the alkylation damage before it was fixed as measurable mutations, resulting in the NOEL. However, at concentrations above the LOEL for MMS, enhanced MGMT expression was not maintained, p53 activation was therefore also investigated to provide insights into its role in modulating MGMT transcriptional activity.

2. Materials and methods

2.1. Cell culture and treatment

The human lymphoblastoid B cell line AHH-1, was cultured in RPMI 1640 supplemented with 10% foetal horse serum and 1% L-glutamine (Gibco, Paisley, UK) at 37 °C, 5% CO_2. The cells were routinely sub-cultured to maintain a concentration of 1×10^5 cell/ml.

Cultures to be exposed to the alkylating agent were seeded at 1×10^5 cells/ml for 24 h and then treated with MMS (Sigma–Aldrich, Dorset, UK; CAS Registry Number 66-27-3) over a range of concentrations both above and below the previously identified LOEL for point mutations (1.25 μg/ml) [4]. The duration of exposure was 24 h for the mass spectrometry analysis, but 1, 2, 4, 6, or 24 h for all other assays. Following treatment cells were washed twice in PBS prior to DNA, RNA or protein extraction. For each time point and at every dose, the experiment was repeated in triplicate.

2.2. Mass spectrometry

DNA was isolated by a high salt extraction method (Stratagene, Amsterdam, The Netherlands) and each samples' concentration and purity was determined by spectrophotometery.

Three hundred micrograms of each DNA sample were depurinated at 100 °C for 7 min [17], allowed to cool and passed through a 30,000 MWCO Microcon centrifugal filter (Millipore, Durham, UK). Following freeze-drying, samples were re-suspended in 10 μl water. N7-methylguanine and N7-ethylguanine standards (NCI Chemical Carcinogen Reference Standard Repository, Missouri, USA) were prepared at concentrations ranging from 10–10,000 fM per injection. The N7-methylguanine was used to generate a standard curve, while the ethylguanine was used as an external standard in the quantitative assay to normalise the data from different experiments, thereby providing a robust and accurate quantitative assay. The samples and standards (3 μl) were injected onto a C18 PepMap100 column (LC Packings, Surrey, UK) of length 25 cm, I.D. 300 μm, using an Ultimate capillary LC (LC Packings, Surrey, UK) system connected online with a Micromass Q-TOF Ultimate API Mass spectrometer (Waters, UK). The LC was used with an isocratic solvent flow of 82% A (H_2O with 0.1% CHOOH) and 18% B (MeOH with 0.1% CHOOH) maintained at 4 μl/min. Acquisition of mass spectra was initiated by a signal sent by the LC system after injection. The mass spectrometry performed two single reaction monitoring (SRM) acquisitions simultaneously: 1. Tandem mass spectrum (MS/MS) of the protonated methylguanine (m/z 166) followed by the selective detection of the major product ion at m/z 149 and 2. MS/MS of the protonated ethylguanine ion m/z 180 and detection of the product ion at m/z 152. The operation settings were: capillary 3.00 kV; cone 35; source temperature 110 °C; desolvation temperature 120 °C; cone gas 200 l/min; desolvation gas 250 l/min; collision gas 16. Methanol blanks were run after each DNA sample to clean the column and avoid carry over.

2.3. Gene expression analysis

Total RNA was extracted utilising the RNeasy Mini Kit (Qiagen, Sussex, UK), according to the manufacturers' instructions. Residual DNA was removed by DNase 1 treatment (DNA-free™, Ambion Ltd., Cambridgeshire, UK), then the RNA concentration, purity and quality was assessed by spectrophotometry.

Real-time reverse transcription-PCR (RT-PCR) was used to quantify the expression of MGMT, MPG and p21. One microgram RNA from each sample was reverse transcribed into cDNA using oligo(dT) primers and the RETROscript kit™ (Ambion Ltd., Cambridgeshire, UK). The initial denaturation step was omitted, but otherwise manufacturers' instructions were followed. Real-time RT-PCR was then performed on an iCycler iQ Thermal Cycler (Bio-Rad, Hertfordshire, UK) as previously described [18]. Appropriate primer sets for the test genes were designed to span an intron and validated to ensure they all had the same efficiency as

the β-actin internal control set: MGMT forward 5′-CTATCGAAGAGTTCCCCGTGCC-3′ and reverse 5′-GCTGCTAATTGCTGGTAAGAAATCAC-3′; p21 forward 5′-GACTCT-CAGGGTCGAAAACG-3′ and reverse 5′-GGATTAGGGCTTCCTCTTGG-3′; MPG forward 5′-CTTCTGCATGAACATCTCCAGC-3′ and reverse 5′-AGGGTGCTGCGAAGCTGACGC-3′.

The resultant threshold cycle (C_T) data was analysed by the $2^{-\Delta\Delta C_T}$ method and normalised to the β-actin housekeeping gene to determine the relative gene expression changes in treated samples as compared to the control untreated samples [19]. *N*-fold differences observed were only considered significant changes in gene expression if <0.5 or >1.5 as previously defined [18]. Students paired *T*-test was used to determine if up- or down-regulations proved to be significant changes in expression as compared to the control samples.

2.4. Western blotting

Treated cell pellets were re-suspended in lysis buffer (50 mM Tris–HCL pH 7.4, 1 mM EDTA, 1% IGEPAL, 0.25% sodium deoxycholate) with the protease inhibitors 1 mM AEBSF, 1 μg/ml leupeptin, and phosphatase inhibitors 1 mM sodium orthovanadate, 1 mM sodium fluoride. One volume of Laemmli buffer (250 mM Tris pH 6.8, 10% glycerol, 4% SDS, 2% β-mercaptoethanol, 0.006% bromophenol blue) was added to the lysate. It was then boiled for 5 min, centrifuged and the concentration of the resultant protein extract was determined using the 2-D Quant Kit (GE Lifesciences, Bucks, UK) according to manufacturers' instructions.

Ten micrograms of each protein extract were separated by 10% SDS-polyacrylamide gel electrophoresis. The proteins were then transferred to a nitrocellulose membrane using a vertical cell tank transfer system (Bio-Rad, Hertfordshire, UK). The membranes were incubated with 1:1000 dilutions of primary antibodies specific to MGMT (Abcam, Cambridge, UK), MPG (Sigma–Aldrich, Dorset, UK), p53, phospho^{ser15}-p53 or β-actin (Cell Signalling Tech, New England Biolabs, Herts, UK). They were then stringently washed and a secondary goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate antibody (diluted 1:1000) or rabbit anti-mouse-HRP antibody (diluted 1:20,000; Abcam, Cambridge, UK) were applied as required. Following washes to remove all excess and non-specifically bound antibodies, the membrane was developed with the ECL chemiluminescence detection agent (GE Lifesciences, Bucks, UK) and subsequently viewed under the Chemidoc XRS system (Bio-Rad, Hertfordshire, UK). Average band density analysis was performed on the Quantity One Vs 4.6.3 software (Bio-Rad, Hertfordshire, UK) and all test band densities were normalised against the corresponding β-actin band density to compensate for variations in protein loading.

3. Results

3.1. Quantification of N7-meG DNA adducts

In order to determine if methyl adducts were present in DNA samples below the LOEL, mass spectrometry was utilised. As only 0.3% of MMS adducts occur at O^{6}G it was not considered practical to try and quantify this adduct at such low exposure concentrations. We therefore elected to measure N7-meG adducts, which are substantially more frequent events following exposure to MMS.

To determine sensitivity, a standard curve of MS signal response (normalised to the response obtained from the ethylguanine external standard) was established for concentrations of N7-meG ranging from 10 to 10,000 fM. This curve was linear and was subsequently used to quantify the level of N7-meG adducts in DNA samples from cells treated with MMS for 24 h. As illustrated in Fig. 1,

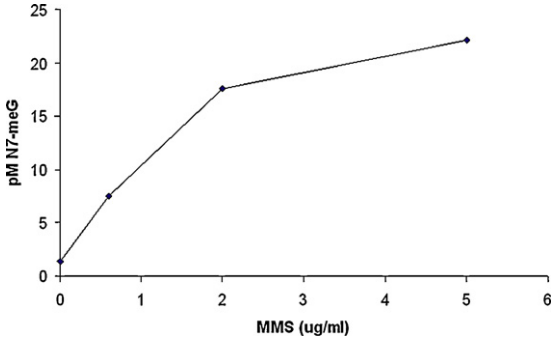


Fig. 1. Concentration of N7-meG DNA adducts detected by mass spectrometry following exposure to MMS.

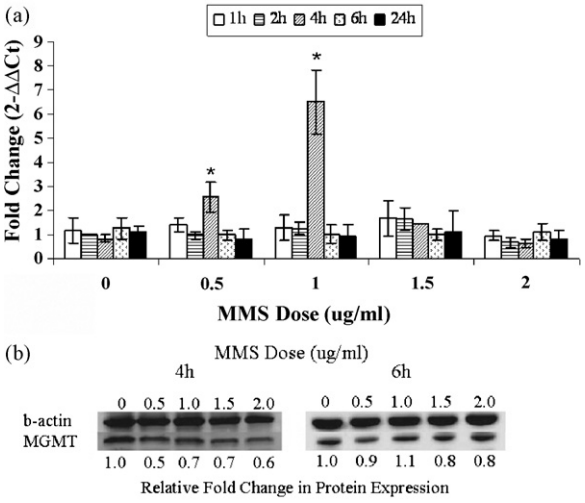


Fig. 2. (a) Relative fold change in MGMT gene expression with time, following exposure to increasing concentrations of MMS. (*) Represents $P < 0.05$. (b) Representative Western blot for MGMT following a 4 and 6 h MMS treatment. Relative fold change in protein expression represents the ratio of the β-actin normalised treated band density to the un-treated control band density.

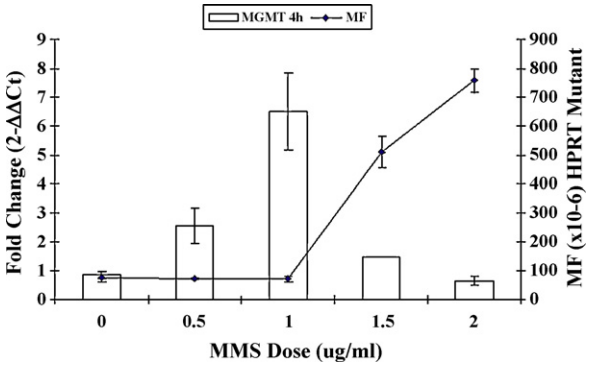
exposure to MMS resulted in measurable increases in N7-meG DNA adducts at all doses. Importantly, 0.6 μg/ml MMS (which lies below the reported LOEL) resulted in 7.55 pM N7-meG. This was a 5-fold increase above the levels observed in the control samples (1.40 pM).

3.2. MGMT and MPG expression

To investigate the MGMT expression induced following exposure to MMS, relative changes in gene expression were quantified by real-time RT-PCR (Fig. 2a). AHH-1 cells were exposed to range of MMS concentrations up to 2 μg/ml for 1, 2, 4, 6, or 24 h in order to determine the speed at which a cellular response was induced. However, as can be seen in Fig. 2a, significant up-regulation of MGMT expression was only observed when cells were treated with 0.5 and 1.0 μg/ml MMS for 4 h. These doses resulted in 2.5- and 6.5-fold increases in MGMT gene expression respectively. Despite the large increases in gene expression at this single 4 h time point, at all further later time points (6 and 24 h), MGMT transcriptional levels returned to base levels. Interestingly, these large increases in expression were not observed at the higher doses utilised in this study (1.5 or 2 μg/ml). When MGMT protein levels were considered, after 4 h exposure to MMS the enzyme levels were reduced at all doses (Fig. 2b), but by 6 h they had increased again, almost to background levels.

When the MGMT gene expression data after a 4 h MMS exposure period was overlaid with the original gene mutation frequency dose–response curve that was previously generated [4], MGMT expression was found to be significantly elevated at doses below the reported pragmatic threshold (Fig. 3). However, at the LOEL dose, the transcriptional status of this repair enzyme returned back to the base level.

When the gene expression patterns of MPG in relation to MMS exposure were investigated just minor fluctuations were observed, with the only significant increase in transcriptional activity occurring at 1.5 μg/ml MMS after 4 h exposure (Fig. 4a). This was a 2-fold up-regulation; however this increase was not maintained at higher doses and at 2 μg/ml MMS for 4 h, MPG expression had returned to basal levels. Additionally, when only the 2 μg/ml dose was considered, there again appeared to be an increasing trend in expression with time that did reach a 1.5-fold up-regulation at 6 h, but this

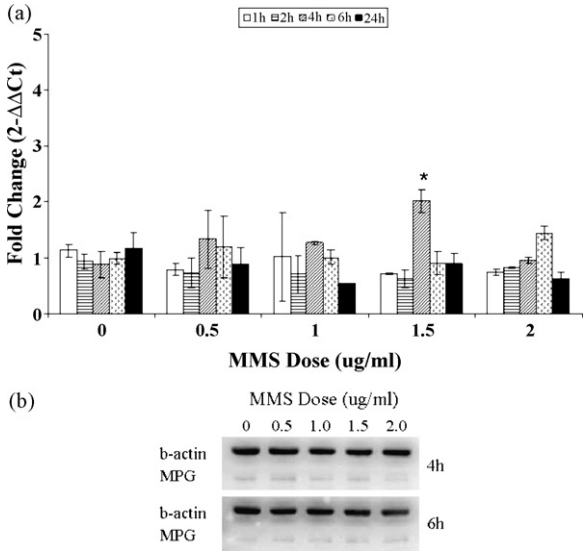


. 3. Relative fold change in MGMT expression after exposure to increasing ncentrations of MMS for 4 h, as compared to the point mutation frequency se–response curve reported in Doak et al. [4]. *MF*, mutation frequency (the number 6-thioguanine resistant clones/10^6 clone-forming cells).

d not reach significance and the 24 h time point was no differ- t to the controls. However, none of these slight variations in gene pression were translated through to the protein level as we found at MPG enzyme levels remained the same across all doses after and 6 h treatment times (Fig. 4b).

3. p53 Activity

As it was unexpected that MGMT up-regulation was not induced MMS at doses above the reported LOEL, we investigated whether 53 activation may have been responsible for inhibiting MGMT pression at the higher doses, as it has been suggested that p53 n repress MGMT expression. To investigate, we initially examined e p21 transcriptional profile, a central p53 effector molecule and erefore an indicator of p53 activity. As illustrated in Fig. 5, a sig- ficant elevation in p21 gene expression was only detected after h exposure to 2 μg/ml MMS. This suggests p53 activation, but to rther corroborate this event, p53 and phospho^{ser15}-p53 protein vels were quantified in extracts obtained from cells treated with 2.5 μg/ml MMS for 1–24 h. Over all the doses at each time point, e level of p53 and phospho^{ser15}-p53 remained steady. Thus, no



. 4. (a) Relative fold change in MPG expression with time, following exposure to reasing concentrations of MMS. (*) Represents $P < 0.05$. (b) Representative West- blot for MPG following a 4 h (top panel) and 6 h (bottom panel) MMS treatment.

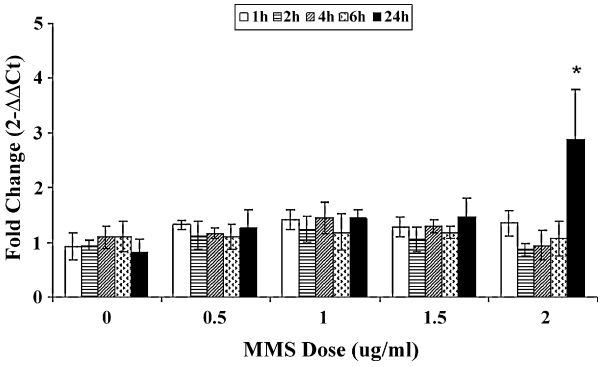


Fig. 5. Relative fold change in p21 expression with time, following exposure to increasing concentrations of MMS. (*) Represents $P < 0.05$.

apparent measurable increases in p53 expression or phosphorylation were observed with time following increasing exposure to MMS up to 2.5 μg/ml (Fig. 6).

4. Discussion

MMS is a model alkylating agent with a high *s*-value. Consequently, it predominantly reacts with N7G, in fact over 80% of DNA adducts induced by MMS occur at this position, with the remainder arising at N3A (10%) and O^6G (0.3%) [6]. This distribution in DNA lesions is likely to govern the biological consequences following exposure to MMS as O^6-alkyG is considered to be mutagenic, while N7-alkylG has a lower genotoxic potential as it is prone to spontaneous depurination (due to the weakened glycosidic bond) resulting in an abasic site that if detected is subsequently corrected by BER. This repair mechanism is error prone, so point mutations and chromosome aberrations can result as a by-product of the correction process. However, if these lesions are not repaired the abasic sites themselves represent single-strand breaks that can readily turn into irreparable double-strand breaks by stalling replication forks [15].

Molecular dosimetry studies have shown that DNA adduct formation in general shows no threshold, with a linear correlation between adduct formation and exposure dose for several compounds including aflatoxin B1, benzo(a)pyrene, methylating and ethylating agents [20–23]. However, there is some controversy over this as theoretically it is possible that chemical agents may be prevented from entering the nucleus due to membranes (both cell and nuclear) and binding to other biomolecules, which may act to limit the quantity of compound that reaches the DNA and thus DNA adduct formation may be expected to demonstrate a NOEL itself. As yet, this has not been demonstrated experimentally. Indeed in the current study we were able to detect N7-meG adducts within the

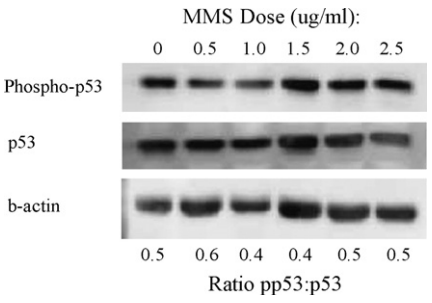


Fig. 6. Representative Western blot for phospho^{ser15}-p53, p53 and β-actin following treatment with 0–2.5 μg/ml MMS for 4 h. The ratio of phospho^{ser15}-p53 to p53 average band density was calculated after normalisation to β-actin.

NOEL for mutagenicity and this is in agreement with a very recent report by Swenberg et al. [22] who have demonstrated a linear DNA adduct response across a wide dose range of MMS in this same cell line. Consequently, the non-linear mutagenic response observed for MMS is not due to lack of DNA adducts within the NOEL dose range and therefore indicates that those adducts present are probably not being fixed as permanent genetic alterations.

DNA adducts represent biomarkers of exposure, but the fact that they are present at doses that do not cause genetic damage indicates they can be biologically insignificant perhaps as they are removed before the lesions are processed into genetic aberrations. Indeed O^6-alkyG is directly repaired by the one-step "suicide" enzyme, MGMT, with pre-existing MGMT levels in the cell determining its capacity for repairing O^6-alkylG lesions [24]. N7-alkylG adducts that have not suffered spontaneous depurination are subject to enzymatic hydrolysis of the N-glycosydic bond by MPG to remove the adduct leaving an apurinic site that is repaired by BER [25]. In the present study we therefore assessed the gene expression of MGMT and MPG in relation to MMS exposure.

With regards to MPG, a lack of inducibility following MMS treatment was observed at both the gene and protein expression levels. This is not unexpected as it has been well documented that over-expression of MPG increases the cytotoxicity of alkylating agents as it accelerates the rate of formation of apurinic sites and/or strand breaks resulting in an imbalance in the BER pathway such that apurinic sites are generated faster than they can be repaired [13,26]. Our findings therefore confirm that MPG is tightly regulated during exposure to alkylating agents, but despite its expression not being implicated in contributing to the NOEL of MMS, it still remains possible that BER is enhanced at low doses through another of the enzymes involved in this repair pathway.

In contrast, an increasing trend in MGMT expression with dose (after 4 h exposure) was observed within the previously reported NOEL for MMS. A 2.5- and 6.5-fold MGMT up-regulation occurred at 0.5 and 1 μg/ml MMS respectively, with the highest level of expression at the dose just below that of the LOEL for MMS induced point mutations (1.25 μg/ml). Thus, this boost in MGMT expression at low doses may be responsible for repairing O^6methylG lesions at a faster rate than they appear resulting in the observed NOEL. Indeed after 4 h exposure to MMS, MGMT protein levels were depleted by a factor of approximately 50%, suggesting their involvement in removing methyl groups from DNA and subsequently undergoing proteosomal degradation. However, by 6 h the quantity of enzyme in the cells was elevated to a level similar to un-treated cells, likely due to the transcriptional up-regulation. The increase in MGMT gene expression was not maintained after 4 h as we detected no further up-regulation so the initial burst may result in the synthesis of enough enzyme for the cell to cope with the damage, this is supported by the fact that after a 24 h dose with 1 μg/ml MMS no point mutations result (Fig. 3) [4]. However, this is not the case for chromosomal damage as the LOEL for MMS is 0.85 μg/ml. The evidence therefore indicates that O^6-alkylG is unlikely to be primarily responsible for the clastogenicity observed at these lower doses, because despite the high MGMT up-regulation at 1 μg/ml, at this dose significant chromosomal damage can be detected. This observation supports previous findings where the MMS non-linear dose–response curve for chromosomal damage was the same in an MGMT deficient cell line (MT1) as compared to cells that express the enzyme [4]. Thus removal of O^6-alkyG by MGMT has little influence on the chromosomal damage related NOEL dose range for MMS, suggesting that O^6-alkylG is not a key clastogenic adduct. Consequently, it appears that at low doses O^6-methylG lesions are responsible for MMS mutagenicity, while N7-methylG may be the predominant cause of MMS clastogenicity.

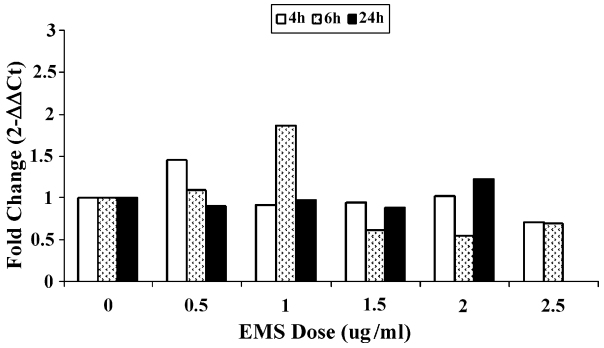


Fig. 7. Representative graph demonstrating fold change in MGMT expression with time following exposure to increasing concentrations of EMS.

It was however unexpected that the increasing trend in MGMT expression did not continue with the longer incubation periods or higher concentrations of MMS. With regards to the lengthier treatment times, it is possible that the increase in expression at 1 μg/ml at 4 h exposure resulted in a dramatic boost in cellular MGMT protein levels that caused a negative feedback loop (based on DNA damage signalling), leading to reduced transcriptional activity of the gene, and thus accounting for the base levels of expression at 6 and 24 h 1 μg/ml treatments. However, this would not account for the lack of MGMT gene response at 4 h with concentrations higher than 1 μg/ml, unless at the much higher concentrations alternative repair pathways are activated in response to the increase in adduct formation, not only in DNA but also on other biomolecules.

One such pathway involves the p53 tumour suppressor gene and is particularly interesting in the context of this study as it has been found to modulate both basal and genotoxic stress induced MGMT expression. However this regulation courts some controversy as p53 appears to be vital for MGMT up-regulation following genotoxic insults [9,27,28], yet p53 over-expression inhibits MGMT expression *in vitro* and such an association has also been indicated *in vivo* [9,29,30].

The AHH-1 cell line we have utilised in this study does harbour a heterozygous p53 mutation at the codon 281/282 interface within exon 8, but it does not influence the molecules DNA damage response [31]. Thus, to determine whether or not p53 was involved in modulating the expression of MGMT in the current study we first monitored the p21 gene expression pattern as it is a key p53 down stream effector molecule. However, no increases in expression were observed until the cells were exposed to 2 μg/ml MMS for 24 h. This indicated that at the higher doses, p53 was activated in response, but this response was delayed as compared to the sharp loss of MGMT expression and so was not a conclusive indicator of p53 activity at lower concentrations. We therefore, assessed p53 and phospho^{ser15}-p53 protein levels, but this again did not demonstrate any measurable increases that could account for modulation of MGMT expression. Hence, the source of failure in maintaining the MGMT transcriptional up-regulation, particularly with increasing MMS dose remains unknown.

Interestingly, a similar MGMT expression pattern was observed when cells were insulted with EMS (representative data shown in Fig. 7). Our preliminary results demonstrated MGMT up-regulation only at the NOEL and below for EMS, not at higher concentrations); but in this case the response was lower (a 2-fold increase in expression) and slightly more delayed as it was only observed after a 6 h exposure time. This difference between MMS and EMS might be largely due to the fact that although MGMT recognises a range of alkyl groups as substrates, larger alkyl adducts are removed less efficiently than methyl adducts [8,32]. Hence, the presence of ethyl adducts may therefore illicit a weaker MGMT response.

In conclusion, the gene expression of MGMT is substantially
-regulated at doses that lie within the reported NOEL for MMS
ereby suggesting that it might play a key role in removing DNA
ducts before they are fixed as permanent mutations. However at
ses higher than the LOEL, MGMT expression drops back to base
vel. The reason for this is unknown; our initial data does not indi-
te p53 activation is involved, but at this stage it cannot be ruled
t and further investigation is required. It is possible that MGMT
ight simply be the more dominant means of adduct removal at
w dose, while at higher exposure levels alternative DNA repair
thways are triggered in the cell by the genotoxic responses.
deed this has been observed in resistance to the chemother-
eutic agent BCNU [33]. Additionally, N7-alkylG and O^6-alkylG
e substrates for mismatch repair and nucleotide excision repair
rticularly when MGMT is overloaded [7,15,34–36], but further
alysis is required to substantiate this theory with regards to
onofunctional alkylating agents.

onflicts of interest

The authors declare that there are no conflicts of interest.

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