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Modulation of N-Methyl-N-nitrosourea Mutagenesis in Mouse Embryo Fibroblasts Derived from the *gpt* Delta Mouse by an Inhibitor of the O⁶-Methylguanine Methyltransferase, MGMT

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Abstract

DNA methylating agents are abundant in the environment and are sometimes used in cancer chemotherapy. They react with DNA to form methyl-DNA adducts and byproduct lesions that can be both toxic and mutagenic. Foremost among the mutagenic lesions is O⁶-methylguanine (m6G), which base pairs with thymine during replication to cause GC→AT mutations. The *gpt* delta C57BL/6J mouse strain of Nohmi et al. (*Mol. Mutagen.* 1996;28:465–70) reliably produces mutational spectra of many DNA damaging agents. In this work, mouse embryo fibroblasts (MEFs) were made from *gpt* delta C57BL/6J mice and evaluated as a screening tool to determine the qualitative and quantitative features of mutagenesis by N-methyl-N-nitrosourea (MNU), a direct-acting DNA alkylator that serves as a model for environmental N-nitrosamines, such as N-nitrosodimethylamine and therapeutic agents such as temozolomide. The DNA repair protein MGMT (O⁶-methylguanine DNA methyltransferase) protects against environmental mutagenesis by DNA methylating agents and, by removing m6G, limits the therapeutic potential of temozolomide in cancer therapy. The *gpt* delta MEFs were treated with MNU to establish dose-dependent toxicity. In parallel, MNU mutagenicity was determined in the presence and absence of the MGMT inhibitor AA-CW236 (4-(2-(5-(chloromethyl)-4-(4-(trifluoromethoxy)phenyl)-1H-1,2,3-triazol-1-yl)ethyl)-3,5-dimethylisoxazole). With and without the inhibitor, the principal mutagenic event of MNU was GC→AT, but more mutations were observed when the inhibitor was present. Evidence that the mutagenic lesion was m6G was based on mass spectral data collected using O⁶-methyl-d₃-guanine as an internal standard; m6G levels were higher in AA-CW236 treated MEFs by an amount proportional to the higher mutation

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Supporting Information

The supporting information is available free of charge on the ACS Publications website.

This section includes the detailed methods for synthesis of the deuterated m6G, the control mutational spectrum across the 459 nt *gpt* mutational target, the mutational spectra of MNU-500 with and without the MGMT inhibitor, the mutational spectrum of MNU-0 and COSMIC Signature 23 and a cosine similarity matrix comparing the mutational spectra determined in this work with mutational signatures from many human cancers.

The authors declare no competing financial interest.

frequency seen in the same cells. This work establishes *gpt* delta MEFs as a versatile tool for probing mutagenesis by environmental and therapeutic agents and as a cell culture model in which chemical genetics can be used to determine the impact of DNA repair on biological responses to DNA damaging agents.

Graphical Abstract

Keywords

MNU; environmental mutagens; mutational spectra; cancer therapy

INTRODUCTION

DNA methylating agents such as N-methyl-N-nitrosourea (MNU), N-nitrosodimethylamine (NDMA) and temozolomide (TMZ) form DNA lesions that are both toxic and mutagenic. One of those lesions is *O*⁶-methylguanine (m6G), which is potentially mutagenic because it predominantly pairs with thymine during replication.^{1, 2} There is much interest in these agents in the fields of environmental and clinical toxicology, because DNA methylating agents are contaminants found in and around toxic waste sites;^{3–5} they form in preserved foods and beverages;^{6–10} they form during carbon capture processes;^{11, 12} they are contaminants in cardiovascular and other medications;^{13–17} and they are present in tobacco smoke and other tobacco products.^{18, 19} Similarly, there is interest in the field of cancer therapy because the lead agent used in treatment of glioblastoma, TMZ, forms m6G as a critical component of its mechanism of action.^{20–22}

Intact animal models represent the gold standard for characterizing the mutational properties of the aforementioned and many other DNA damaging agents.²³ While several technologies, including CRISPR, are available to make genetically modified animal models, experiments with those animals, however, are expensive, can require many months to perform a single study, and are not easily amenable to chemical manipulation. There is a need for cell culture models that offset some of those limitations. Ideally, these cell lines would be isogenic with the mouse model in which detailed analysis of the mutational properties of DNA damaging agents would be subsequently performed. Nohmi et al. contributed a mouse model in which ~80 copies of the bacterial *gpt* gene - a selectable marker for forward mutagenesis assays - are present on chromosome 17.^{24, 25} The model has been used for many years to

characterize the qualitative features of mutagenesis by methylating agents, UV light, aromatic amines, polycyclic hydrocarbons, mycotoxins and other agents.^{26–32} Immortalized mouse embryonic fibroblasts (MEFs) have been prepared from these mice,³³ creating the possibility of a parallel isogenic cell culture system amenable to chemical or genetic modulation, but little has been done with such cell line models since that early report.^{34–37}

Recently we have utilized the *gpt* delta mouse as a model for mutagenic profiling^{38–40} and have improved the back-end of the method by adding duplex consensus sequencing - a highly sensitive method by which mutational spectra are recorded from heterogeneous cell mixtures, without phenotypic selection (i.e., unselected mutational spectra).⁴¹ Duplex sequencing itself, however, is laborious, so there is a need for a rapid screening method to be used to define the relevant parameters of dosing, adduct analysis and mutational scoring. As a next step in enhancing the *gpt* delta system, described here, we have prepared immortalized MEFs and used a chemical-genetic approach to compromise m6G repair. This work shows the potential of the *gpt* delta MEFs for mutational profiling of DNA methylating agents, using MNU as the model genotoxicant. The work also shows the utility of the model for probing the effect of agents that perturb pathways that protect mammalian cells from DNA alkylation.

EXPERIMENTAL PROCEDURES

Experimental System.

Figure 1 presents an overview of the strategy used to investigate MNU mutagenesis and toxicity in MEFs derived from the *gpt* delta C56BL/6J mouse.

MEF isolation and immortalization.—Homozygous *gpt* delta C57BL/6J mice were a gift from T. Nohmi.²⁴ The use of the transgenic animals and all experimental protocols of this study were approved by the Massachusetts Institute of Technology Committee on Animal Care. Primary cultures of 13-day old mouse embryos were prepared as described in Liu et al.³³ Cells were maintained in high glucose DMEM containing 200 mM GlutaMax (Gibco), 10% FBS (VWR Life Sciences), 1 mM sodium pyruvate (Gibco), 100 IU Penicillin, 100 µg/mL streptomycin (Corning) in a humidified incubator at 37 °C in an atmosphere containing 5% CO₂. Aliquots of cells were prepared in growth medium supplemented with 10% DMSO and stored in liquid nitrogen (LN₂).

Primary MEFs were immortalized by lentivirus mediated transformation with the Ef1a_Large T-antigen_Ires_Puro plasmid.⁴² The lentiviral vector was prepared by cotransfection of 293T cells with plasmids pMD2.G (Addgene plasmid #12259), psPAX2 (Addgene plasmid #12260) and Ef1a_Large T-antigen_Ires_Puro (Addgene plasmid #18922). After 48 h, lentivirus was harvested from the cell medium and filtered through a 0.45 µm syringe filter.^{43, 44} Primary cells were grown to 70–80% confluence and infected with lentivirus in growth medium containing 1X polybrene (Millipore, TR-1003-G). Following infection, cells expressing Large T antigen were selected in media containing 6 µg/mL puromycin. Surviving cells were used to produce stocks that were cryopreserved in LN₂ in growth medium supplemented with 10% DMSO.

Relative *gpt* Copy Number Verification.—qPCR analysis was used to verify the presence of the lambda EG10 transgenic reporter in the immortalized *gpt* MEF cells. The following primers were used for these analyses:

Forward: 5'-TGGAAACTATTGTAACCCGCCTG-3'

Reverse: 5'-TCCGCTGGTTGATGACTATGT-3'.

Genomic DNA was extracted from the MEFs by using the DNeasy Blood&Tissue kit (Qiagen, 69504). The relative copy number of transgenic integrated *gpt* sequences in the MEF genomes was estimated by the ratio between *gpt* copy number determined in the MEFs and that in liver DNA obtained from the *gpt* delta C57BL/6J mouse. qPCR was performed with the QuantiTect SYBR Green PCR kit (Qiagen, 204143) by pre-incubating at 95 °C for 15 min, followed by 40 cycles with 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s.⁴⁵

Growth Inhibition by MNU.—MNU was a gift from G. Buchi of MIT. Before use, the NMR spectrum of the MNU was obtained in DMSO: 300 MHz δ : 3.03 (s, 3H, -CH₃), 7.8 (s, 1H, (Z) -NH₂), 8.12 (s, 1H, (E) -NH₂). NMR integration indicates that the molecule exists as an equal mixture of (E):(Z) isomers (50:50). The spectrum is consistent with those reported for MNU and other nitrosamines. For the treatment of cells in culture, stock solutions of 12.5, 25 and 50 mM MNU were prepared in 0.1 M citrate buffer (pH 4.5).

Cells were seeded in 6-well plates at 2.5×10^4 cells per well and allowed to attach overnight. Stock solutions of MNU were diluted 1:100 in HBSS (pH 7.4) and used to treat cells for 1 h (final MNU concentrations in media were 125, 250 and 500 μ M). Control cultures received HBSS only. After 1 h, HBSS was replaced with growth medium and cells were incubated for another 48 h before analysis. Cell growth was measured by trypsinizing cells and determining cell number using a Coulter Counter.

To examine the effects of inhibition of the m6G DNA methyltransferase by AA-CW236 (Sigma-Aldrich), cells were pretreated with 3 μ M AA-CW236 in HBSS for 1 h prior to exposure to MNU.

6-TG-Selected *gpt* Mutation Assay and Sequencing of Mutants.—For mutational analyses, 4×10^5 cells were seeded in 15 cm tissue culture plates and incubated for 48 h. Cells at 60–70% confluence were treated for 1 h with 125, 250 or 500 μ M MNU alone or following pretreatment for 1 h as described above with 3 μ M AA-CW236. After 48 h, cells from four plates for each treatment condition were removed by scraping with a rubber spatula and pooled together into a 50-mL falcon tube. The cells were washed twice with PBS and approximately 2×10^6 cells per group were used for DNA isolation.

Genomic DNA was prepared using the MegaLong DNA isolation kit (G-Biosciences) following the manufacturer's directions. The lambda EG10 phage was rescued from genomic DNA using the Transpack Packaging Extract (Agilent Technologies). The lambda phage were then used to infect *E. coli* YG6020, expressing the Cre recombinase, which produced a 6.4-kb plasmid carrying the *gpt* and chloramphenicol acetyltransferase genes. The infected *E. coli* YG6020 were incubated on selective media containing 25 μ g/mL chloramphenicol (CHL) with or without 25 μ g/mL 6-thioguanine (6-TG) for 72 h. The 6-TG

phenotypically resistant colonies (6-TG^r mutant colonies) were confirmed by re-streaking on selective media containing CHL and 6-TG. The mutational frequency was calculated by dividing the total number of 6-TG-resistant colonies by the average number of CHL-resistant colonies for each group.

Sanger sequencing of the 459 bp *gpt* gene was performed at BGI Tech Solutions Co., Ltd. (Tai Po, Hong Kong) by using the forward primer: 5'-GCGCAACCTATTTTCCCCTCGA-3'. All sequences were aligned with the *E. coli gpt* gene (GenBank M13422.1) using NCBI Nucleotide Blast.

O⁶-Methylguanine DNA Adduct Analysis.—The relative levels of m6G in DNA isolated from cells treated with either MNU or with MNU following AA-CW236 treatment were estimated by using the protocols described by Vanden Bussche et al.⁴⁶ and Hemeryck et al.⁴⁷ In brief, the internal standard of 2-amino-6-methoxy-d₃-9H-purine (O⁶-methyl-d₃-guanine) synthesized as indicated in Supporting Information was added to each sample at the final concentration of 0.33 ng/mL (2.0 pmol/mL). Then, 55 µg of DNA was hydrolyzed in 0.1 M formic acid (80 °C for 30 min). The hydrolysate was cooled on ice and then applied to an Oasis® HLB cartridge (SPE) (30 mg, 1 mL), which was conditioned with 2 mL of 100% methanol and equilibrated with 2 mL of Milli-Q water. After loading the hydrolysate, a vacuum suction was applied on the SPE cartridge, followed by the elution step with 2 mL of 100% methanol. The collected fraction was evaporated to dryness (30 °C for 90 min) using a CentriVap (Labconco, USA). Finally, the dried residue was re-dissolved in a total volume of 150 µL of methanol containing 0.05% (v/v) formic acid.

Analysis of m6G was performed by HPLC-MS/MS on an Agilent 1200 system equipped with an Agilent 6410 triple quadrupole mass spectrometer. The chromatography was performed on a Zorbax SB-Aq column (2.1 mm × 150 mm, 3.5 µm; Agilent) at 25 °C eluted at a flow rate of 0.2 mL/min by the following program: A = 0.1% (v/v) aqueous formic acid and B = methanol; the gradient was 0–8 min, 5% to 90% B; 8–11 min, 90% to 5% B; 11–19 min, 5% B isocratic. The separation temperature was 20 °C. The MS/MS system was operated with the electrospray source in the positive ion mode (capillary voltage 4 kV) and using the multiple reaction monitoring (MRM) mode. The transitions m/z 166.1→m/z 149.1 (quantifier ion) and m/z 166.1→m/z 134.0 (qualifier ion) were chosen for m6G and m/z 169.2→m/z 152.2 for the synthesized internal standard O⁶-methyl-d₃-guanine.^{48, 49} The calibration curve was prepared using standard m6G solutions in the range of 0.05 to 5 ng/mL (0.30 to 30.3 pmol/mL) (r = 0.9997). The limit of detection of spiked m6G standards was 0.001 ng/mL (0.006 pmol/mL) with a signal to noise ratio of 3:1.

Bioinformatics Analyses.—Mutational signature data were obtained from the COSMIC (Catalogue of Somatic Mutations in Cancer) database (https://cancer.sanger.ac.uk/cosmic/signatures_v2), as accessed on October 15th, 2019. Since the COSMIC mutational signatures are derived from human tumors, the proportion of mutations at each sequence context also reflects the distribution of the trinucleotide sequences across the human genome. To enable valid comparisons between these signatures and our data, the COSMIC data was transformed (normalized) to reflect equal probabilities of trinucleotide sequences. Specifically, the proportion of mutations at each triplet nucleotide was divided by the

frequency of occurrence of that triplet in the human genome, and then the resulting vector was unit-normalized. The distribution of trinucleotides throughout the human genome was obtained from reference 50.⁵⁰ The cosine similarity values, the dendrograms and the heatmap plots were constructed using Python 3.7 and the Seaborn 0.9.0 and Fastcluster 1.1.25 function libraries.

Statistical Analysis.—All data were calculated as mean and standard deviation (S.D.) and evaluated by the Student's *t*-test. The differences between groups were considered significant when the p-value was less than 0.05.

RESULTS AND DISCUSSION

Transgenic *gpt* Delta Mouse Embryo Fibroblast Immortalization and *gpt* Delta Transgene Verification.

To establish a cell-based mutational assay from our animal model, a *gpt*-MEF cell line was constructed from embryonic cells of the transgenic *gpt* delta C57BL/6J mouse. An immortalized cell line was obtained by lentivirus-mediated transformation of primary cells with the SV40 large T antigen. The presence of the lambda transgenic reporter in the new cell line was verified by qPCR analysis. This analysis revealed that the *gpt*-MEF cells harbored approximately 400 copies of the lambda reporter, which was about twice the number of copies present in liver tissue of the *gpt* delta C57BL/6J mouse. The difference in copy number likely reflects the highly aneuploid genotype of immortalized cell lines.

MNU-induced Growth Inhibition in Mouse Embryonic Fibroblast Cell Line.

The growth inhibitory properties of MNU on the newly constructed MEF line were evaluated to help determine the doses to be used for subsequent mutational analysis. Cells were treated for one hour with either MNU or MNU plus the MGMT inhibitor AA-CW236 at the doses shown in Figure 2 and maintained in culture medium for 48 h to allow recovery. At the 500 μ M dose of MNU, there was an almost two-fold reduction in survival. Cells experiencing this ~50% reduction in numbers were analyzed for mutations. To partially disable MGMT, a preincubation period of 1 h with 3 μ M AA-CW236 was implemented. This preincubation resulted in a modest effect on survival (Fig. 2A) at the doses tested.

MNU-induced Point Mutations in the *gpt* Reporter Gene.

Point mutations were phenotypically detected by the acquisition of 6-TG^r resistance (6-TG^r) in bacterial DNA sequences recovered from *gpt* delta MEFs (Fig. 2B). Mutations in the 459 bp *gpt* gene that generate the 6-TG^r phenotype are detected by this assay. A dose-dependent increase in the number of 6-TG^r mutants was observed, with a strongly enhanced mutagenic response at the highest dose of MNU (500 μ M) in cells previously treated with AA-CW236.

The 6-TG^r mutants from control (vehicle-treated) MEFs (106 independent mutants), 500 μ M MNU-treated MEFs (208 mutants) and 500 μ M MNU plus MGMT inhibitor-treated MEFs (200 mutants) were sequenced. The control spectrum (Supporting Information Figure S4) was distinct from the mutational spectra of MNU and MNU plus AA-CW236. We examined

mutational patterns three ways. First, the types of mutations (transitions, transversions, etc.) were tabulated (Table 1). Second, the positions, types and frequencies of independent mutations were plotted along the 459 bp *gpt* gene that was sequenced (Supporting Information Figs. S1–S3); this presentation mode allows, among other things, comparison of the position of mutations to functional domains of the GPT protein. Third, each mutation was presented in the context of its 5'- and 3'- neighbors; mutagenesis can be context dependent and plotting the data as three-base contexts reveals patterns that often allow one mutagen to be distinguished from another. This sequence context dependence analysis of the MNU, and MNU plus the MGMT inhibitor, data is presented in Figure 3A and B. The patterns thus generated can be processed by informatic algorithms that allow comparison with patterns from human cancers (Fig. 3C and D and Supporting Information Fig. S5).

Table 1 shows that MNU selectively induced GC→AT mutations (76% of all mutations), which was expected^{51–53} and helps to validate the MEF assay. MNU generates a highly reactive methylating agent (methyl diazonium ion), which has an S_N1-like product distribution and forms m6G as ~6% of its total DNA lesion burden.⁵⁴ m6G is well known to base pair with thymine during replication, leading to GC→AT mutations *in vivo*.¹ MNU also induced 7% AT→GC and 8% GC→TA mutations, which may be attributed to O⁴-methylthymine (m4T)⁵⁵ and 7,8-dihydro-8-oxoguanine,⁵⁶ respectively. MEF cells grown in atmospheric oxygen may have acquired a significant burden of oxidative damage, resulting in some background (also seen in the control) of oxidized bases. Looking at the distribution of mutations in cells treated serially first with the MGMT inhibitor AA-CW236 and then with MNU, one sees a similar pattern. Taken together, the pattern of mutations seen in Table 1 looks like that of a classical S_N1 alkylating agent.

We note that a recent report using a human induced pluripotent stem cell line indicates that the nearly-exclusive mutation of MNU at a dose similar to the one used here is the T→C transition.⁵⁷ The authors posit that m6G formed by MNU may have been repaired in their cell system, leaving m4T to be the dominant mutagenic lesion. Our results differ and can possibly be explained by the differences in the role that MGMT plays in modulating mutagenesis in the different cell culture systems. Of possible relevance is work showing that single m6G lesions replicated in MGMT-proficient mammalian cells are vanishingly mutagenic, whereas m6G is very mutagenic in cells lacking or compromised in the activity of MGMT.^{55, 58} A rationalization of the present work and that of Kucab et al.⁵⁷ is that MGMT levels differ from cell line to cell line; in our work, ambient MGMT levels were not sufficient to fully suppress m6G mutagenesis (Fig. 3) but, in their work, the cells perhaps had a higher level of MGMT. If MGMT removed the mutagenic m6G from the genomes of their cells, it would not have been present to drive the m6G-induced G→A mutations. The use of an MGMT inhibitor in the present study (*vide infra*) demonstrates how powerful MGMT is as a modulator of m6G mutagenesis.

Further evidence that the mutation assay in our *gpt* delta MEFs is working as expected comes from an examination of the contexts in which GC→AT mutations occurred in the *gpt* transgene. The mutational pattern we observed with MNU is likely influenced by a combination of chemical and biochemical factors including: (1) context-selective alkylation of bases, (2) context-specific preferential DNA repair and (3) context-determined mispairing

abilities of modified bases during replication.^{59, 60} To probe the sequence-specificity of MNU mutagenesis, we compared our data (Table 2, and Supplemental Information Figs. S2 and 3) with several studies that have examined the role of sequence context on the formation of m6G by alkylating agents, including MNU. Treatment of double stranded oligonucleotides derived from the *H-ras* or *E. coli gpt* gene sequences with MNU revealed a strong preference for m6G formation at the middle G in the trinucleotide sequences GGT, GGA and GGG.^{61, 62} There are 20 GGT, 12 GGA, 14 GGC and 7 GGG trinucleotide sequences in the sense and antisense strands of the 459 bp *gpt* gene. In cells treated with 500 μ M MNU, out of a total 151 6-TG-selected G $\rightarrow$ A *gpt* mutations, 118 (78%) occurred at 12 of these trinucleotide sites at which mutation of the central guanine resulted in non-conservative amino acid substitutions at critical sites within the protein or via the formation of a stop codon. Only one such mutation was found in control (untreated) cells, representing only ~1% of the total mutations analyzed. In addition to the aforementioned chemical studies on m6G formation in specific sequence contexts, there are additional data in the literature that has examined the context-dependent mutational patterns of MNU in various biological systems. Consistent with a previous report examining mutagenesis by MNU, we observed a strong strand preference for MNU-induced GC $\rightarrow$ AT transition mutations at the middle G in GGT, GGA or GGG sequences, with no mutations found in the sense strand.⁵¹ Taken together, the mutational patterns observed in the MNU treated MEFs matched expectations from the chemical and biological literature.

The Amount of m6G in the MEF Genome Correlated with Mutational Outcomes.

A chemical-genetic approach was used to establish that most of the mutations observed, specifically the GC $\rightarrow$ AT transitions, were attributable to m6G. As shown in Figure 2B, pretreatment of the MEF culture with AA-CW236 increased substantially the mutation frequency of MNU at the MNU dose of 500 μ M. The chloromethyl triazole AA-CW236 is a strong, cell permeable inhibitor of MGMT, showing an approximately ten-fold greater inhibition potential as compared to the classical inhibitor, *O*⁶-benzylguanine.⁶³ The low level of mutagenesis observed at lower doses of MNU was expected because MGMT is very effective at repairing m6G in cells treated with low doses of alkylating agents.^{1, 55, 64, 65} Our data suggest a saturation point for MGMT in the 300–500 μ M range of MNU. That dose corresponds to 20–40 pM m6G per mg DNA (Fig. 2).

To establish further the role of m6G in MNU mutagenesis in the MEFs, we decided to determine if the enhanced mutation level seen in AA-CW236 pretreated cells (Figure 2B) were paralleled by an increased m6G burden in MEF DNA. Figure 2C shows the effect of adding the inhibitor one hour before and during MNU treatment. The data show that the increase in mutation frequency (Panel B; 500 μ M dose of MNU) was matched by an increase in the level of m6G in the genome as determined by LC-MS/MS. Analysis for m6G was enabled by the synthesis and use of a heavy atom labeled derivative of m6G, *O*⁶-methyl-d₃-guanine (Supporting Information – Methods). While addition of the repair inhibitor enhanced the mutagenic potency of MNU, it is noteworthy that the context-dependent mutagenic patterns were unaffected. For example, with addition of AA-CW236, 115 of 144 GC $\rightarrow$ AT 6-TG^r (79%) occurred at the middle G of the GGT, GGA, GGC or GGG trinucleotide sequences, which is similar to the pattern observed without the inhibitor. Taken

together, the biological and chemical results above strongly support the role of m6G in MNU mutagenesis and demonstrate the powerful impact of MGMT on repair of this lesion.

Cosine Similarity Comparisons of MNU-induced Mutational Patterns with Mutational Patterns Seen in Human Cancer.

Tables 1–2 and Figures S2–4 provide a useful way to look at the types of mutations induced by MNU, with and without the MGMT inhibitor, and give information on the contexts in the *gpt* gene most prone to mutation. As indicated above, an additional way to analyze the data is to examine each data set in a manner that allows computational comparison between patterns (e.g., Fig. 3 Panels A and B) and with patterns that have been seen before through sequencing of human tumors (e.g., Fig. 3 Panels C and D and Fig. S4). In early work, we defined the mutational properties of m6G, the lesion believed to cause most of the MNU-induced mutations, in all 16 3-base contexts in which it can form.⁵⁹ Plotting mutational spectra as 3-base contexts is now done routinely because the data lend themselves to comparisons as in the present study. Figure 3 presents the mutational spectra of MEFs treated with MNU (Panel A) and MEFs treated with MNU after treatment with the MGMT inhibitor (Panel B) plotted to show the 3-base contexts in which mutations were detected. For illustration, the tallest bar in Figure 3B shows a GC→AT mutation in a GGA context, where the underscored G is the site mutated. As indicated above, this is an anticipated hotspot for m6G mutagenesis. There are 16 possible 3-base contexts for any given point mutation and six types of point mutation (G→T, G→C, G→A, etc., color coded in the Figure), for a total of 96 contexts in which a point mutation can occur. In all spectra presented, the proportion of mutations presented was normalized to the frequency at which each of the 3-base contexts occurs in the *gpt* sequence analyzed; in addition, the background mutational spectrum was subtracted from the data.

Spectra can be compared by using the metric of cosine similarity. Cosine similarity is a correlation metric (mathematically related to Pearson correlation coefficient) that has been used in many fields⁶⁶ to measure similarity between two multidimensional objects, including the 3-base context, 96-dimensional mutational spectra produced by analyses such as the ones shown here. Mathematically, cosine similarity denotes the cosine of the angle between two vectors of size n , in the n -dimensional space, and thus has a value between 0 and 1; if the two vectors (i.e., mutational spectra) are superimposable, the angle between them is 0, and therefore cosine similarity is 1 (because $\cos(0)=1$). If the vectors are orthogonal (highly dissimilar), then the angle between them is 90° , and therefore, cosine similarity is 0 (because $\cos(90^\circ)=0$). An advantage of cosine similarity is that it is independent of the magnitude of the vectors; the vectors do not need to be unit-normalized to be compared to each other. In other words, we can compare a spectrum with 100 mutations, with another that has 200 mutations directly, without having to convert each vector to proportions that sum up to 1. These analyses provide a quantitative measure of the relatedness among the different spectra and give us the ability to distinguish one mutational pattern from another. Panel C of Figure 3 shows the cosine similarity relatedness of the mutational patterns induced by MNU in MEFs with and without pretreatment with the MGMT inhibitor AA-CW236. The spectra show a similarity of 0.93, which indicates that the patterns are highly related. Neither pattern shows significant relationship to the control (MNU-0; data plotted in Fig. S4).

Another asset of cosine similarity is that it allows comparison of mutational spectra with mutational signatures derived from human tumors. A “mutational spectrum” is the experimentally determined distribution of mutations across a segment of DNA, as measured by enumerating mutations that occur at each base position; the data presented in Figures 3A and 3B and Figures S1–3 are mutational spectra. By contrast, a “mutational signature” is derived by mathematical extraction of mutational patterns from tens of thousands of human cancer genomes; so far, this computational technique involving non-negative matrix factorization of large data sets has identified over 40 different base-substitution mutational signatures.^{50, 67, 68} Figure 3D is a human tumor signature listed as Signature 11 in the COSMIC mutational signatures catalogue.⁶⁸ Signature 11 shows reasonable cosine similarity (Panel C) to our MNU spectra (similarities of 0.70–0.79). While the hot and cold spots for mutation match well across all three spectra (Panels A, B and D), it is noteworthy that the match with Signature 11 is best in cells that have been depleted for the m6G repair protein, MGMT (Panel B). As indicated above, cells depleted for MGMT are disabled for repair of m6G, so mutational spectra in those cells should match well the mutational specificity of m6G. Interestingly, there is reason to believe that Signature 11 could be induced by m6G because tumors displaying this signature are often from people who have been treated with the anticancer agent TMZ. TMZ, like MNU, forms m6G and, if the patients displaying Signature 11 were MGMT deficient (which is often the case in patients treated with this drug), their mutational spectra would be expected to show similarity to that seen in the MEFs treated with AA-CW236, the MGMT inhibitor. Indeed, our spectra from MEFs depleted for MGMT and treated with MNU look very similar to those of people who exhibit Signature 11 (Fig. 3B and 3C). These data further support the potential of the MEF line presented here as a tool to probe medically important responses to DNA damaging agents.

Cosine similarity comparison of the MNU mutational spectra with all cancer signatures in the COSMIC database revealed another human tumor that shows a modest association with our MNU data. The full comparison is shown in a cosine similarity matrix presented as Figure S5; the Signature that shows some association with the MNU data is Signature 23, which was seen in a liver tumor. A 0.42 to 0.57 cosine similarity is seen with the MNU-treated MEFs; that similarity, while modest, could be a hint that an alkylating agent with MNU-like properties may have been involved in the etiology of the tumor displaying Signature 23. One strong caveat regarding Signature 23 is that it was derived from a single human tumor; consequently, we are cautious not to over-emphasize a possible relationship. We do note, however, that the mutational hotspots (denoted in Fig. S4 with broken lines) and cold spots for mutation in Signature 23 match the MNU data fairly well.

Summary.

There is an acute need for simple biological systems that recapitulate mutational patterns seen in animal models and in human cancers. The work presented here involved generation of a cell culture model isogenic with a commonly used mouse model of human cancer. The pattern of mutations seen for a DNA damaging agent, MNU, likely reflects that of m6G as evidenced by the observation of enhanced mutagenesis following application of an inhibitor of the alkyltransferase protein that repairs m6G. This chemical-genetic approach establishes

the potential of the system and acts as a prelude to further development of the MEF strategy in which complementary purely genetic approaches are used to manipulate specific DNA repair and replication systems. Such cell culture models derived from animal models offer much potential for dissection of the intricacies of the origins of genetic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AA-CW236	4-(2-(5-(chloromethyl)-4-(4-(trifluoromethoxy)phenyl)-1H-1,2,3-triazol-1-yl)ethyl)-3,5-dimethylisoxazole
CHL	chloramphenicol
COSMIC	catalogue of somatic mutations in cancer
DMEM	Dulbecco's modified Eagle media
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
<i>E. coli</i>	Escherichia coli
FBS	fetal bovine serum
gpt	guanine phosphoribosyltransferase
HBSS	Hank's balanced salt solution
HPLC-MS/MS	high pressure liquid chromatography coupled with tandem mass spectrometry
LN₂	liquid nitrogen
MEFs	mouse embryo fibroblasts
m6G	6-methylguanine
MGMT	6-methylguanine DNA methyltransferase
MNU	N-methyl-N-nitrosourea
MRM	multiple reaction monitoring

NDMA	N-nitrosodimethylamine
PBS	phosphate buffered saline
qPCR	quantitative polymerase chain reaction (real-time polymerase chain reaction)
SPE	solid phase extraction
6-TG	6-thioguanine
TMZ	temozolomide

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Figure 1.

Experimental system and workflow. Embryos from the *gpt* delta C57BL/6J mouse were converted into a MEF cell line by lentivirus-mediated transformation. MEFs were treated with MNU alone, or with MNU plus AA-CW236, an inhibitor of the MGMT repair protein. Cellular toxicity and mutagenicity were determined. Analytical measurement of m6G, a mutagenic DNA adduct of MNU, was performed at a dose of MNU that showed a strong mutagenic response (500 μ M). Mutation distributions were determined in all possible 3-base contexts. The mutational patterns generated were then compared by cosine similarity with mutational signatures derived from sequencing of human tumors.

Figure 2.

Dose-dependent responses of MEFs to MNU treatment with and without the MGMT inhibitor, AA-CW236. Panel A: effect of MNU and MNU plus AA-CW236 on MEF survival. Panel B: 6-TG^r mutation frequency of MEFs with and without AA-CW236 using the *gpt* locus as the target for mutagenicity. Panel C: levels of m6G in the DNA of *gpt* delta MEFs treated with MNU, with or without AA-CW236 treatment to inactivate the MGMT protein. Error bars indicate mean ± SD; there were 4–6 replicates for each dose. If an asterisk is present over a point, that point is statistically different from the point with which it is vertically aligned (P < 0.05). See text for specific conditions used.

Three-base context mutational spectra of the DNA alkylating agent MNU and their relation to mutational patterns seen in human cancer. Mutation distributions were determined in all 96 possible 3-base contexts (3'-NXN-5', where X is the position of the mutation and N and N are the flanking bases); there are six point mutation types and 16 possible 3-base contexts (6×16=96). Panel A: three-base context mutational spectrum of MNU alone at a dose of 500 μM. Panel B: three-base context mutational spectrum of MNU (500 μM) plus the MGMT inhibitor, AA-CW236. Panel C: heat map depicting the relative similarities among the spectra of MNU at 0 μM (MNU-0; the control), MNU at 500 μM (MNU-500), MNU+AA-CW236 at an MNU dose of 500 μM (MNU-500AA), and two mutational signatures from human cancer (COSMIC Signatures 11 and 23). The darkness of the shading in each box

reflects the cosine similarity of the spectra that intersect in that box (e.g., the spectra in Panels A and B), with the numerical values of the cosine similarities shown in each box. Mutational Signatures 11 and 23 were chosen as they are the most similar among the 30 COSMIC Mutational Signatures (v2) to the MNU spectra (see Supporting Information Figure S5). Mutational Signature 11 is mainly found in glioblastomas that were treated with TMZ (shown in Panel D transformed to reflect equal trinucleotide frequencies (see Experimental Procedures)). Mutational Signature 23 is from a liver tumor. The control spectrum (mutational background) represented in MNU-0 (Figure S4) was subtracted from the raw data of MNU-500 and MNU-500AA, to yield the data shown in Panels A-B.

Table 1.
Mutation Types Induced in the *gpt* Gene of the *gpt* Delta MEF Cell Line after Treatment with 500 μ M MNU alone (MNU-500), or MNU with the MGMT Inhibitor, AA-CW236 (MNU-500AA).

Values in the Table are the number of mutations for each mutation type; the data in parentheses are the percentages of each mutation with respect to the total number of mutants sequenced. The vehicle control and treatment conditions are described in the text. This Table includes insertions and deletions; the plotted data in the Figures do not include those mutations.

Mutation type	MNU-0 (Control)	MNU-500	MNU-500AA
Transitions			
GC→AT	7 (7)	157 (76)	146 (73)
AT→GC	37 (34)	15 (7)	25 (13)
Transversions			
GC→TA	48 (45)	16 (8)	17 (8)
GC→CG	4 (4)	3 (1)	0 (0)
AT→TA	5 (5)	5 (2)	9 (4)
AT→CG	3 (3)	0 (0)	0 (0)
Deletions	1 (1)	6 (3)	2 (1)
Insertions	1 (1)	6 (3)	1 (1)
Total number of mutants	106 (100)	208 (100)	200 (100)

Table 2.
GC→AT Mutations in the 5'-GGN-3' Context Under the Conditions of Table 1.

Values in the Table are the number of mutations for each mutation type; the data in parentheses are the percentages of each mutation with respect to the total number of GC→AT mutations. The vehicle control and treatment conditions are described in the text.

GC→AT mutation at G <u>G</u> N site	MNU-0 (Control)	MNU-500	MNU-500AA
5'-G <u>G</u> T-3'	0 (0)	13 (8.6)	22 (15)
5'-G <u>G</u> G-3'	0 (0)	14 (9.3)	18 (13)
5'-G <u>G</u> C-3'	0 (0)	14 (9.3)	17 (12)
5'-G <u>G</u> A-3'	1 (25)	77 (50)	58 (40)
Total 5'-G <u>G</u> N-3' *	1 (25)	118 (78)	115 (79)
Total number of GC→AT mutants	4	151	144

* In the control, there were 4 GC→AT mutations in the 459 bp *gpt* gene. Only one of those mutations occurs in the 5'-GGN-3' context (Supporting Information, Fig. S1).