DNA Alkylation Agent Protects Against Spontaneous Hepatocellular Carcinoma Regardless of O\textsuperscript{6}-Methylguanine-DNA Methyltransferase Status

Maryanne C.S. Herzig,*1 Jessica A. Zavadil,*1 Karah Street,2 Kim Hildreth,1 Norman R Drinkwater,3 Traci Reddick,4 Damon C. Herbert,1 Martha Hanes,5 C. Alex McMahan,6 Robert Reddick,6 and Christi A. Walter,1,7,8,9

\textsuperscript{1}Department of Cellular & Structural Biology, \textsuperscript{5}Department of Animal Laboratory Resources, \textsuperscript{6}Department of Pathology, \textsuperscript{7}Barshop Center for Longevity and Aging Studies, \textsuperscript{8}San Antonio Cancer Institute, University of Texas Health Science Center at San Antonio, San Antonio, TX, \textsuperscript{2}Saddleback College, Mission Viejo, CA, \textsuperscript{3}McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, WI, \textsuperscript{4}Transgenic Mouse Facility, Duke University Medical Center, Durham, NC. \textsuperscript{9}South Texas Veteran’s Health Care System, Audie Murphy Hospital, San Antonio, TX

*co-first authors

Running title: Alkylation Damage Protects Against HCC

Key words: O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT), methylnitrosourea (MNU), hepatocellular carcinoma (HCC), alkylation, C3HeB/FcJ.

Financial Support: VA Merit award to CAW

Corresponding Author: Christi A. Walter, Ph.D.
Department of Cellular & Structural Biology
Mail Code 7762
The University of Texas Health Science Center at San Antonio
7703 Floyd Curl Drive
San Antonio, TX 78229-3900
Conflict of interest: All of the authors have no conflict of interest to disclose.

Word count: 3,988

Total number of figures and tables: 6
Abstract

Hepatocellular carcinoma (HCC) is increasingly important in the United States as the incidence rate rose over the last 30 years. C3HeB/FeJ mice serve as a unique model to study HCC tumorigenesis because they mimic human HCC with delayed onset, male gender bias, a ~50% incidence, and susceptibility to tumorigenesis is mediated through multiple genetic loci. Because a human O\textsuperscript{6}-methylguanine-DNA methyltransferase (hMGMT) transgene reduces spontaneous tumorigenesis in this model, we hypothesized that hMGMT would also protect from methylation-induced hepatocarcinogenesis. To test this hypothesis, wild type and hMGMT transgenic C3HeB/FeJ male mice were treated with two monofunctional alkylating agents: diethylnitrosamine (DEN) (0.025 µmol/g body weight) on day 12 of life with evaluation for glucose-6-phosphatase deficient (G6PD) foci at 16, 24, and 32 weeks or N-methyl-N-nitrosurea (MNU) (25 mg MNU/kg body weight) once monthly for 7 months starting at 3 months of age with evaluation for liver tumors at 12-15 months of age. No difference in abundance or size of G6PD foci was measured with DEN treatment. In contrast, it was unexpectedly found that MNU reduces liver tumor prevalence in wild type and hMGMT transgenic mice despite increased tumor prevalence in other tissues. hMGMT and MNU protections were additive, suggesting that MNU protects through a different mechanism, perhaps through the cytotoxic N7-alkylguanine and N3-alkyladenine lesions which have low mutagenic potential compared to O\textsuperscript{6}-alkylguanine lesions. Together these results suggest that targeting the repair of cytotoxic lesions may be a good preventative for patients at high risk of developing HCC.
Introduction

The incidence of hepatocellular carcinoma (HCC) in the United States has been increasing since the 1980s and is linked with nonviral etiologies such as obesity and cirrhosis (1-3). Potentially curative therapies, including resection, liver transplant, and radiofrequency ablation, are available only for patients diagnosed in early stages of disease. Surveillance of cirrhotic patients with imaging has improved early detection somewhat, but many patients are still diagnosed with HCC in advanced stages when chemotherapy is the only available treatment option. Sorafenib, a tyrosine kinase inhibitor, is the only FDA approved chemotherapeutic agent for treatment of HCC at later stages and provides a median overall survival of only 10.3 months (4). Increased understanding of HCC development and identification of targets to prevent progression to HCC in high-risk patients would be invaluable in reducing HCC incidence and death rates.

The C3HeB/FeJ mouse strain is a non-transgenic inbred mouse model for non-viral HCC. This model has a number of features in common with human HCC, which makes it a good preclinical model. Human HCC has a 2:1 to 4:1 male gender predisposition (5), and spontaneous HCC arises in male mice of this strain. Hormones seem to play a role in the gender bias of HCC in this mouse model; ovariectomized females injected with androgens display an increased prevalence and castrated males given estrogen injections display a reduced incidence (6-8). In humans, incidence of HCC increases as people achieve middle age, the 40’s and 50’s (9, 10). Male mice develop HCC spontaneously in middle age (9-12 months of age) with a prevalence of ~50%. As in humans, tumors in the C3HeB/FeJ strain vary in their size, location within the liver, and number of tumors per animal (multiplicity). Histologically, HCC in C3HeB/FeJ shows many of the same features as human HCC, including hepatosteatosis, nuclear inclusions, and anisokaryosis, with tumor stage ranging from dysplastic nodules to well-differentiated HCC to poorly-differentiated HCC with vascular invasion (unpublished).
Tumor susceptibility in C3H mice results from differences at multiple genetic loci when compared to mouse strains that are not predisposed to liver tumor development. Drinkwater et al. (11) identified the first “hepatocarcinogen susceptibility locus” (Hcs) by crossing C3H/HeJ males with C57BL/6 females and identified a genetic locus that segregates with tumor prevalence following a challenge with diethylnitrosamine (DEN) or ethylnitrosurea (ENU), both of which produce O6-ethylguanine lesions in DNA (12). Hcs locus 7 (Hcs7), is present on chromosome 1 and accounts for ~85% of the difference in tumor induction between C3H and C57BL/6 strains after challenge with an ethylating agent (11, 13). A total of 7 Hcs loci have been identified using congenic mice (14, 15). The C3HeB/FeJ strain used in our studies was derived from the C3H/HeJ strain by ovarian transplantation to eliminate endogenous MMTV (16). Thus, it is reasonable to expect that there are multiple genetic loci that contribute to the development of HCC in the C3HeB/FeJ mice.

Denlinger et al. (1974) (17) showed that treatment of C3HeB/FeJ mice with N-methyl-N-nitrosurea (MNU) or ENU induced squamous cell carcinoma of the stomach, pulmonary adenomas, neurogenic tumors and others. O6-methylguanine DNA methyltransferase (MGMT) is a stoichiometric-acting DNA repair protein that repairs O6-methylguanine (O6mG) lesions, a highly mutagenic DNA lesion induced by monofunctional methylating agents such as MNU (12, 18, 19). We previously established that C3HeB/FeJ male mice expressing human MGMT (hMGMT) from a transgene driven by a portion of the human transferrin promoter (providing liver and brain expression) have reduced spontaneous tumor prevalence compared to wild type males (20). Furthermore, MGMT must be functional to have this protective effect against spontaneous tumorigenesis because mice expressing the same transgene with an alanine substituted for the active cysteine (C145A), were not protected from HCC (21). The studies
described herein were designed to test the hypothesis that the \textit{hMGMT} transgene would also protect against methylating agent-induced hepatocarcinogenesis.

\textbf{Materials and Methods}

\textbf{Animals}. Production, initial characterization, and genotyping of \textit{hMGMT} transgenic mice were described previously (22). The \textit{hMGMT} transgenic mice (LC26I and LC22I lines) used in this study were produced and maintained in the inbred C3HeB/FeJ strain. Wild-type C3HeB/FeJ males were obtained from Jackson Laboratories or from in-house matings. All animals were maintained in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility and were specific pathogen-free, including \textit{Helicobacter hepaticus}. All manipulations were approved by the Institutional Animal Care and Use Committee prior to implementation. Mice were fed standard laboratory chow \textit{ad libitum} and housed in microisolator-topped cages under 12:12 light:dark cycles.

\textbf{Spontaneous hepatocellular carcinoma}. Animals were group-housed in microisolator-topped cages and fed standard mouse chow \textit{ad libitum}. Each was observed daily to detect signs of illness. Animals were humanely euthanized at 15 (Experiment 1) or 12 (Experiment 2) months of age or when moribund, whichever occurred first. All tumors were scored and assessed for size and the number per animal. Sixty-four animals were assigned to the drug-treatment groups in Experiment 2 to insure sufficient animals to accommodate accidental losses so that at least 58 animals survived in each transgenic line to have a power of \textasciitilde0.8 to detect a change from 50\% prevalence in control mice and 25\% prevalence in \textit{hMGMT} transgenic animals (2 tailed test, \(a=0.05\)).

\textbf{Hepatocyte preparation, culture, and cytotoxicity measurements}. Hepatocytes were isolated by collagenase perfusion as previously described (23). After anesthetization, a longitudinal incision
was made and the liver perfused through the hepatic portal vein with calcium-free Earles balanced salt solution (5.4mM KCl, 116mM NaCl, 0.8mM MgSO₄•7H₂O, 1.6mM NaHCO₃, 1.0mM NaH₂PO₄•H₂O and 5.5mM D-glucose, pH 7.4, 37°C) containing 25mM Hepes and 0.5mM EGTA (Sigma Chemical, Co.). Viable cell yield using 0.05% collagenase (Boehringer Mannheim Biochemica) digestion was routinely ~7 x 10⁷/animal as determined by trypan blue exclusion. Plates and dishes were prepared by pre-treating with a collagen solution (0.5mg/ml, Sigma Chemical, Co.) mixed with Swim-77 medium (Sigma Chemical, Co.), containing penicillin-streptomycin solution (10,000U/ml penicillin and 10mg/ml streptomycin; Sigma Chemical, Co.) at a ratio of 1:8.

Freshly isolated hepatocytes were diluted to a concentration of 5 x 10⁴ viable cells/ 100μl in L-15 medium/ITS (mixed in a ratio of 100:1; Sigma Chemical, Co., and Collaborative Biomedical Products, respectively). One hundred μl of the cell suspension were dispensed into 96-well tissue culture plates. Hepatocytes were incubated at 37°C without CO₂ for 4 hours.

MNU (Sigma Chemical, Co.; 10mg/ml) was prepared fresh in solution A (1.4M NaCl, 54mM KCl, 55.5mM Dextrose, 42mM NaHCO₃; 37°C) each time it was used. All MNU preparations were protected from light. Twenty to twenty-four hours after plating, medium was removed from the 96-well tissue culture plates and washed gently two times with solution A. Hepatocytes were subsequently treated with 100μl of solution A containing 0.0, 0.1, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 7.5, and 10mg/ml MNU. Cells were treated for 60 minutes at 37°C in a sealed modular incubator chamber. Afterward, cells were washed three times with solution A, re-fed with fresh L-15 medium/ITS and incubated at 37°C. Twenty to twenty-four hours after MNU treatment, cell viability was quantified with an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (24) using the cell proliferation kit I (Boehringer Mannheim Biochemica) according to the manufacturer’s protocol. Absorbance was determined at 550 nm with the reference
wavelength at 690 nm using a SLT Lab Instrument 400 ATC microplate reader. Individual data points obtained by the MTT assay represent the mean of a minimum of 8 wells for each MNU treatment. Treatments were performed in duplicate from 3 independent experiments. The surviving fraction of each group was plotted as a function of MNU concentration.

**DEN treatment of mice.** This assay was performed as described in (25). Mice were randomly assigned to untreated control or DEN treatment groups. C3HeB/FeJ wild type and hMGMT transgenic male mice, lines LC26I and LC22I mice were injected i.p. with 0.025 µmol/g body weight on day 12. At 16, 24 and 32 weeks of age, mice were sacrificed, livers excised and histology sections prepared from frozen liver tissue. Foci of glucose 6-phosphatase depleted activity were determined by immunohistochemistry and compared to hematoxylin and eosin stained sections and quantitated as described previously (25).

**MNU treatment of mice.** MNU (Sigma Chemical Co.) was prepared fresh for each use in phosphate/citrate buffered saline at pH 4.2 with constant protection from light. Experiment 1: MNU was delivered by tail vein injections monthly for 7 months starting at 3 months of age. Twenty-five mg MNU/kg body weight was delivered at each injection based on the body weight at the first injection (17). A total of 175 mg MNU/kg body weight or buffer alone was injected into each animal. Mice were humanely euthanized when moribund or at 15 months of age, whichever occurred first. Experiment 2: Mice were treated identically as in Experiment 1 except that MNU was delivered by intraperitoneal (i.p.) injection, the injection was based on the current body weight, and mice were humanely euthanized when moribund or at 12 months of age, whichever occurred first.

**Tissue collection, immunohistochemistry, and histological analyses.** A board-certified veterinary pathologist performed complete necropsies on all animals in Experiment 1 of MNU treatment.
Visible tumors were removed, formalin-fixed, paraffin-embedded, and sectioned; adjacent sections were processed for (1) hematoxylin-and eosin (H&E)-staining, (2) immunohistochemistry with monoclonal anti-hMGMT (1:100; Kamiya Biomedical Company), (3) polyclonal antiserum against hMGMT and mMgmt (1:200; Novus Biologicals) or (4) antibody against mouse Ki67 (1:50; Abcam). The pathologist reviewed all H&E slides. Six 20x fields of view of each Ki67 stained tumor were examined for cells with positive staining. Whenever possible, 300 tumor cells and 300 nearby normal hepatocytes were scored for the presence of brown precipitate in the nucleus to quantify the proportion of hMGMT, hMGMT/mMgmt, or Ki67 positive cells. Slides were evaluated and imaged using Zeiss Axio Imager A1 and Zeiss AxioCam HRc with Zeiss AxioVision 4.5 Sp1 software.

**Statistical analyses.** Comparisons of tumor prevalence were carried out using the Chi-square test for independence. Where required, because of low expected frequencies, exact P-values were computed using StatXact 3.0.2 (Cytel Corp., Cambridge, MA) or NCSS 9 (NCSS, Kaysville, Utah). Means and standard errors were computed using the fraction of immunohistochemical-positive cells. Afterward, the fractions of positive cells were compared among cell lines, treatments, and tissues using analysis of variance for repeated measurements. Comparisons among means were Bonferroni-adjusted. P values < 0.05 were considered significant. Cytotoxicity data were analyzed using analysis of variance (ANOVA) for randomized complete blocks. Each experiment was considered as a block. Logarithms were analyzed to better satisfy the assumptions underlying the ANOVA. Means and standard errors were computed from untransformed data. The MNU concentration of 0 was not included in the ANOVA.
Results

Male mice were treated perinatally with DEN, and areas of glucose-6-phosphatase deficient (G6PD) foci, pre-neoplastic liver lesions that correlate with subsequent liver tumors if allowed to continue to grow (25), were identified, counted, and measured for each liver. The number and volume fraction of G6PD foci were similar in the livers of two independent lines of hMGMT (LC26I and LC22I lines) transgenic and wild type mice (Supplementary Table SI). DEN-treated mice displayed more G6PD foci, an increased volume fraction, number of foci per cubic centimeter of liver, number of foci per liver, and number of mice with foci compared with control mice.

DEN induces 12% N7-ethylguanine, 8% O6-ethylguanine (O6eG), 4% N3-ethyladenine lesions, and ethylphosphate lesions, the most common adduct formed (53%) (12, 18) as a proportion of total damage resulting from alkylation of DNA. MNU induces a similar percentage of O6-methylguanine lesions (O6mG) (7.5%) but a much greater percentage of N7-methylguanine (N7mG) (68%) and N3-methyladenine (N3mA) (8%) adducts (12, 18) as proportions of total damage. O6mG lesions have one of the highest mutagenic potentials of the lesions produced by monofunctional methylating agents, while N7-alkylguanine and N3-alkyladenine adducts are more likely to be cytotoxic if they go unrepaired (12). O6eG lesions such as those produced by DEN are repaired by human MGMT at ~1/10 to 1/150th of the rate as for O6mG lesions produced by MNU (26, 27). Therefore, mice were treated with MNU to analyze the effects of an alkylating agent that produces DNA damage that is efficiently repaired by MGMT, namely O6mG. Reduced tumor prevalence after MNU treatment was observed for wild type (Figure 1; from 60% spontaneous development to 12% after MNU treatment) and hMGMT transgenic mice (Figure 1; 0% in LC22I and 11% in LC26I lines after MNU treatment). An increase in prevalence of other cancers was observed with MNU treatment in wild type and hMGMT transgenic mice.
Stomach cancers were the most prevalent (Figure 1), followed by intestinal, lung and then lymphatic cancers (Supplementary Figure S1).

We previously demonstrated that a large fraction of tumor cells in spontaneous tumors of hMGMT transgenic mice lacks detectable hMGMT expression compared to normal liver of these transgenic mice (20). To test if this also occurs in tumors that develop with MNU treatment, immunohistochemistry was used to detect MGMT expression in liver tumors vs. normal liver from MNU treated mice. Polyclonal MGMT antibody detected total MGMT expression (mouse and human proteins) (20) while the monoclonal antibody directed against hMGMT enabled us to detect expression of hMGMT only. Tumors that arose spontaneously and in MNU treated mice all displayed a lower proportion of cells staining positive for hMGMT/mMgmt with the polyclonal antibody; however, only tumors arising in MNU treated LC26I line of hMGMT transgenic mice displayed significantly lower positive staining compared to matched normal liver (Table I). Tumors that arose spontaneously in hMGMT transgenic mice displayed an approximately 70% lower proportion of cells positive for hMGMT (Table I) but this did not reach statistical significance, likely due to the small group size.

Because the reduction in tumor prevalence with MNU treatment in wild type mice was unexpected, a second experiment was conducted to test reproducibility and to gather more detailed information on the changes in liver tumor size and multiplicity, using wild type and hMGMT (LC26I line) transgenic mice. Treatment with MNU reduced the HCC prevalence in wild type (from 56.8% spontaneous to 32.4% after MNU; p=0.00178) and hMGMT transgenic (43.8 to 24.6%; p=0.0614) mice (Table II). These results are similar to what was seen with the first experiment, although the tumor prevalence in untreated hMGMT transgenic mice (43.8%) was higher than previously reported (18-19%) (20), and therefore was not significantly different from wild type tumor prevalence. Untreated hMGMT transgenic mice displayed significantly lower
spontaneous tumor multiplicity compared to wild type (Table II; \textit{hMGMT}: 7.1%; wild type: 36.0%; \(p=0.0367\)). MNU treatment significantly reduced the multiplicity of tumors in wild type mice (Table II; 20.5 to 6.8%; \(p=0.01022\)). The percentage of \textit{hMGMT} tumor-bearing mice with multiple tumors was similar to MNU treatment (Table II), likely because it was already so low in untreated \textit{hMGMT} mice. While there was a decrease in tumor prevalence with MNU treatment, there was an increase in the mean and median total tumor volume with MNU treatment in \textit{hMGMT} transgenic mice (Table II). In wild type mice, there was a similar increase in the mean total tumor volume (Table II); however this is due to one mouse with a total tumor volume of 10 times the average (7704 mm\(^3\); Figure 2). In general, tumor burden is statistically similar across groups (Table II), although this may be due to the large variability in total tumor volume within the groups (Figure 2).

One possible explanation for the lower liver tumor prevalence with MNU treatment is that the DNA damage induced by MNU resulted in cell death in pre-neoplastic cells thereby precluding carcinogenesis. To directly test the effect of MNU treatment on cell viability, primary hepatocytes from \textit{hMGMT} transgenic and wild type mice were isolated, treated with MNU \textit{in vitro}, and cell viability was measured (Figure 3). Similar sensitivities to MNU treatment were detected and both demonstrated hormesis, a common dual phased response to exogenous agents with initial stimulation followed by growth inhibition or cell death. Overall, wild type and \textit{hMGMT} primary hepatocytes displayed a similar dose-dependent reduction of cell viability with increasing doses of MNU.

Because MNU-treated \textit{hMGMT} transgenic mice displayed greater median total tumor volume despite lower overall prevalence, we hypothesized that cellular proliferation was greater in MNU-treated tumors compared to untreated tumors. To test this, immunohistochemistry for Ki67 was performed on fixed tumor tissues. Very few cells were positive for Ki67 staining, regardless
of whether they were from mice treated with MNU or solvent control, or from \textit{hMGMT} transgenic or wild type mice (Figure 4A). One tumor displayed foci of positive nuclear staining in cells near the periphery of the tumor (Figure 4B). A few mitotic figures seen within the tumors stained positively for Ki67 (Figure 4C) while in the positive control, a mouse spleen, a high proportion of cells with Ki67 nuclear staining (Figure 4D) was detected.

\textbf{Discussion}

Approximately 40\% of human HCC tumors tested for MGMT expression are deficient in the protein (29), thereby suggesting that MGMT and the O6mG lesion it repairs may play a role in the etiology of HCC. The reduction of spontaneous HCC in \textit{hMGMT} transgenic mice in the C3HeB/FeJ background supports the hypothesis that O6mG contributes to hepatocarcinogenesis (20); O6mG is mutagenic, procarcinogenic, and cytotoxic (30). Mice in the present study were treated with MNU, an agent that produces O6mG lesions, with the expectation that the prevalence of HCC would increase in wild type mice compared with \textit{hMGMT} transgenic mice that have an enhanced capacity to repair O6mG via transgene expression (20). Instead, the antitumor benefit of alkylating agents was detected. However, unlike traditional use of alkylating agents to treat existing tumors, we found that MNU prevented tumors from arising.

A lower tumor prevalence was observed in wild type and \textit{hMGMT} transgenic mice treated with MNU. The effects of the transgene and MNU treatment were additive. When the results from Experiment 1 and 2 are combined (Supplemental Table SII), tumor prevalence was reduced by 13\% with the \textit{hMGMT} transgene, which was a marginally significant effect. Furthermore, with MNU, tumor prevalence is reduced an additional 20.9\% in \textit{hMGMT} transgenic, similar to the 28.5\% reduction seen in wild type mice with MNU treatment (Supplemental Table SII). This additive effect implies the mechanisms through which the \textit{hMGMT} transgene and MNU protect
against tumorigenesis are independent. The hMGMT transgene is thought to provide protection by increasing the repair of mutagenic O6mG lesions (20).

In support of independent mechanisms, there was a significant loss of hMGMT/mMgmt expression in tumors that arose from hMGMT transgenic mice treated with MNU compared to matched normal liver and a tendency toward loss of expression in the untreated transgenic groups (>70% reduction in hMGMT positive staining cells but did not reach significance likely due to group size). These data support that in the transgenic mice, protection from the hMGMT transgene expression is lost during tumorigenesis. However, a significant loss of mMgmt expression is not observed in the wild type mice with or without MNU, which suggests that MGMT expression is not a driving factor for protection against tumorigenesis provided by MNU. This conclusion is further supported by the dose-dependent kill curves of primary hepatocytes from wild type and hMGMT transgenic mice. The hMGMT transgene does not provide protection from the cytotoxicity of MNU, suggesting therefore that death induced by MNU is not due to O6mG lesions in cells.

In contrast to the tumor preventative effect of MNU, DEN caused increased prevalence and volume of preneoplastic G6PD foci in wild type and transgenic mice, with no protective effect imparted by the hMGMT transgene. It was shown previously that all perinatally DEN-treated C3H mice develop liver tumors if allowed to survive long enough and that G6PD foci will develop into tumors if allowed to progress (11). DEN is administered perinatally, while hepatocytes are proliferative, to induce G6PD foci and eventual tumorigenesis (31). However, the hMGMT transgene may or may not have been expressed at this time because it has been shown previously that genes driven by the transferrin promoter are not expressed in the liver until between 10 and 20 days of age (32). If O6-alkylguanine repair is key for preventing DEN induced carcinogenesis, the weak or absent transgene expression may explain the lack of
difference between DEN-treated wild type and \textit{hMGMT} transgenic C3HeB/FeJ mice. Additionally, DEN produces O\textsubscript{6}eG lesions, which are a repaired less efficiently overall than O\textsubscript{6}mG lesions by human MGMT (26, 27). Low efficiency repair of O\textsubscript{6}eG could also have contributed to the similar outcomes for the wild type and \textit{hMGMT} transgenic mice, whether or not hMGMT was expressed at the time of treatment. Unrepaired O\textsubscript{6}mG lesions can be recognized by mismatch repair and lead to apoptosis (33). On the other hand, the toxicity of O\textsubscript{6}eG lesions is independent of mismatch repair (34). This could also potentially explain the differences in response to DEN vs. MNU.

However, the data suggest that the O\textsubscript{6}mG lesion and its repair may not be the mechanism for MNU’s cancer preventative effect. While O\textsubscript{6}mG is one of the most mutagenic lesions induced by monofunctional methylating agents, it makes up a minority of the DNA damage. Other lesions, such as N\textsubscript{7}-alkylguanine and N\textsubscript{3}-alkyladenine adducts, occur in a greater proportion but do not have the high mutagenic potential of the O\textsuperscript{6}-methylguanine adduct. Instead, they are more cytotoxic if they are not repaired (33). Interestingly, while DEN produces O\textsubscript{6}eG in the same proportion as MNU produces O\textsubscript{6}mG, DEN generates a much lower proportion of N\textsubscript{7}-alkylguanine and N\textsubscript{3}-alkyladenine adducts compared to MNU (12). The differences in proportion of N\textsubscript{7}-alkylguanine and N\textsubscript{3}-alkyladenine may contribute to the differential effect on tumorigenesis by these two monofunctional alkylating agents.

MNU induces the same lesions and at similar proportions to those induced by temozolomide, the first line therapy for glioblastoma (12, 28). While MNU or temozolomide would not be used for chemoprevention, these studies do reveal a potential target for chemoprevention. Targeting the repair of N\textsubscript{7}- and N\textsubscript{3}-adducts may have a similar preventative effect. Reduced efficiency of base excision repair should yield cell death from N\textsubscript{7}- and N\textsubscript{3}-adducts because these lesions can lead to cytotoxic abasic sites (33). This may reduce the number of preneoplastic lesions,
particularly in high-risk patients. Unlike targeting the repair of O\textsuperscript{6}-alkylguanine lesions, this approach does not carry the same risk of increased carcinogenesis because N7- and N3-adducts do not have as much mutagenic potential (33). Because high-risk patients are already identified to undergo surveillance for development of HCC, there is an opportunity to treat with a preventative agent to reduce incidence of HCC. Since HCC is refractory to chemotherapy in the majority of patients, effort toward prevention in high-risk patients may be more effective.

Acknowledgements

These studies were supported by a VA Merit grant to CA Walter. We thank Wouter Koek, PhD, for performing some statistical analyses. The histochemical staining for the G6PD studies was done by the Experimental Pathology Core of the University of Wisconsin Carbone Cancer Center Cancer Center supported by Grant P30 CA014520. The Ki67 staining conducted by the Histology and Immunohistochemistry Laboratory of the Department of Pathology at University of Texas Health Science Center San Antonio.

References


19. Swann PF. Why do O6-alkylguanine and O4-alkylthymine miscode? The relationship between the structure of DNA containing O6-alkylguanine and O4-alkylthymine and the mutagenic properties of these bases. Mutat Res. 1990;233:81-94.


Table I. Proportion of mMgmt and hMGMT positive cells in tumors compared to normal liver of mice treated i.v. with MNU (MNU Experiment 1)

<table>
<thead>
<tr>
<th>Treatment group</th>
<th># Tissues Analyzed</th>
<th>% Positive Cells for hMGMT/mMgmt</th>
<th>% Positive Cells for hMGMT</th>
<th>% change</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal Tumor</td>
<td>Normal Tumor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3HeB/FeJ</td>
<td>6</td>
<td>73.9±4.3</td>
<td>50.1±12.9</td>
<td>-32%</td>
<td>0</td>
</tr>
<tr>
<td>+MNU</td>
<td>2</td>
<td>77.7±11.6</td>
<td>46.0±36.8</td>
<td>-41%</td>
<td>ND</td>
</tr>
<tr>
<td>LC22I</td>
<td>2</td>
<td>77.8±8.9</td>
<td>60.9±11.7</td>
<td>-22%</td>
<td>76.2</td>
</tr>
<tr>
<td>+MNU</td>
<td>0</td>
<td>ND</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC26I</td>
<td>5</td>
<td>59.6±7.9</td>
<td>33.2±11.9</td>
<td>-45%</td>
<td>80.2</td>
</tr>
<tr>
<td>+MNU</td>
<td>4</td>
<td>80.6±4.0</td>
<td>28.8*±14.2</td>
<td>-64%</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM. *Indicates tumors for which the proportion of Mgmt/MGMT positive cells was ≤ 50% of nearby normal tissue. #Indicates statistically significant. ND denotes not determined. NA denotes not available.
Table II. Tumor prevalence is reduced with i.p. MNU treatment in both wild type and hMGMT (LC26I line) C3HeB/FeJ mice (MNU Experiment 2).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Total # mice analyzed</th>
<th># mice with tumors</th>
<th>% with tumors</th>
<th># mice with multiple tumors</th>
<th>% of tumor bearing mice with multiple tumors</th>
<th>% of all mice with multiple tumors</th>
<th>Mean Total Tumor Burden (mm³)</th>
<th>Median Total Tumor Burden (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3HeB/FeJ</td>
<td>88</td>
<td>50</td>
<td>56.8%α</td>
<td>18</td>
<td>36.0%b</td>
<td>20.5%c</td>
<td>424</td>
<td>136</td>
</tr>
<tr>
<td>+MNU</td>
<td>74</td>
<td>24</td>
<td>32.4%α</td>
<td>5</td>
<td>20.8%</td>
<td>6.8%c</td>
<td>726</td>
<td>111</td>
</tr>
<tr>
<td>LC26I</td>
<td>32</td>
<td>14</td>
<td>43.8%</td>
<td>1</td>
<td>7.1%b</td>
<td>7.1%</td>
<td>208</td>
<td>66</td>
</tr>
<tr>
<td>+MNU</td>
<td>57</td>
<td>14</td>
<td>24.6%</td>
<td>3</td>
<td>21.4%</td>
<td>5.3%</td>
<td>400</td>
<td>217</td>
</tr>
</tbody>
</table>

αIndicates statistically significant difference of percentage of mice with tumors between C3HeB/FeJ with MNU compared to untreated wild type mice (p=0.00178)

βIndicates statistically significant difference of percentage of tumor bearing mice with multiple tumors between untreated C3HeB/FeJ and hMGMT (p=0.0367)

γIndicates statistically significant difference of percentage of all mice with multiple tumors between C3HeB/FeJ with MNU compared to untreated wild type mice (p=0.01022)
Figure 1. Prevalence of hepatocellular carcinoma is reduced with MNU treatment, despite an increase in gastric squamous cell carcinoma, in wild type and \textit{hMGMT} (LC22I and LC26I lines) mice (MNU Experiment 1). Tumor prevalence was calculated as the percent of mice with one or more HCC or gastric squamous cell carcinoma tumors for each genotype and treatment. * = significantly different from C3HeB untreated control group.

Figure 2. Total tumor volume for individual animals by genotype and treatment. Each data point represents the total tumor volume (mm$^3$) for a single animal. Non-tumor bearing animals are not represented. Data within the groups are spread on the x-axis to improve visualization. Tumor volumes ranged from 1 to 2895 mm$^3$ for untreated wild type mice, 2 to 2274 mm$^3$ for MNU-treated wild type mice, 3 to 916 mm$^3$ for untreated \textit{hMGMT} mice, and 8 to 2624 mm$^3$ for MNU-treated \textit{hMGMT} transgenic mice. An exceptionally large tumor was recovered from an MNU-treated wild type mouse (7704 mm$^3$). There were no statistical differences with MNU treatment for wild type or \textit{hMGMT} transgenic mice.

Figure 3. \textit{hMGMT} and wild type primary hepatocytes display a similar dose-dependent reduction in cell viability with MNU treatment. Significant differences were not detected between primary hepatocytes obtained from wild type mice (black circles) and primary hepatocytes obtained from \textit{hMGMT} transgenic mice (open circles) that were matched for age and sex. Data are presented as means ± SEM representing 3 separate experiments.

Figure 4. Sparse Ki67 staining is seen in tumors, regardless of treatment or genotype. A subset of 3 tumors per treatment group and genotype collected from i.v. and i.p. routes of MNU delivery were evaluated for mouse Ki67 staining. The fraction of Ki67 positive cells were not determined due to minimal positive staining detected in the tumors. Panel A shows a representative image of the absence of Ki67 staining in most tumor (from i.p. MNU-treated wild
type mouse). Panel B shows one of the tumors from a solvent treated wild type mouse that displayed modest Ki67 nuclear staining (see arrow heads for examples) in some regions of the tumor periphery. Panel C shows a mitotic figure with staining for Ki67 (see arrow head) (from solvent treated hMGMT transgenic mouse), which were sparsely present in most tumors, but unrelated to treatment or mouse background. Panel D shows mouse spleen tissue, a highly proliferative positive control tissue, with a large amount of nuclear Ki67 staining (see arrow head for examples), demonstrating that the lack of staining in tumors was most likely due to modest hepatocyte proliferation. Scale bar=20μm
DNA Alkylating Agent Protects Against Spontaneous Hepatocellular Carcinoma Regardless of O6-Methylguanine-DNA Methyltransferase Status

Maryanne C. Herzig, Jessica A. Zavadil, Karah Street, et al.

Cancer Prev Res  Published OnlineFirst December 14, 2015.

Updated version  Access the most recent version of this article at: doi:10.1158/1940-6207.CAPR-15-0251

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://cancerpreventionresearch.aacrjournals.org/content/early/2015/12/12/1940-6207.CAPR-15-0251.

Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.