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DNA Alkylating Agent Protects Against Spontaneous Hepatocellular Carcinoma Regardless of O⁶-Methylguanine-DNA Methyltransferase Status

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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 O⁶-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 μ mol/g body weight) on day 12 of life with evaluation for

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glucose-6-phosphatase deficient (G6PD) foci at 16, 24, and 32 weeks or N-methyl-N-nitrosourea (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 O⁶-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.

Keywords

O⁶-methylguanine-DNA methyltransferase (MGMT); methylnitrosourea (MNU); hepatocellular carcinoma (HCC); alkylation; C3HeB/FeJ

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 *hMGMT* transgene would also protect against methylating agent-induced hepatocarcinogenesis.

Materials and Methods

Animals

Production, initial characterization, and genotyping of *hMGMT* transgenic mice were described previously (22). The *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 *Helicobacter hepaticus*. All manipulations were approved by the Institutional Animal Care and Use Committee prior to implementation. Mice were fed standard laboratory chow *ad libitum* and housed in micro-isolator-topped cages under 12:12 light:dark cycles.

Spontaneous hepatocellular carcinoma

Animals were group-housed in microisolator-topped cages and fed standard mouse chow *ad libitum*. Each was observed daily to detect signs of illness. Animals were humanely

ethanized 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 ~0.8 to detect a change from 50% prevalence in control mice and 25% prevalence in *hMGMT* transgenic animals (2 tailed test, $\alpha=0.05$).

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 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.6mM NaHCO_3 , 1.0mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 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 $\sim 7 \times 10^7$ /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 5×10^4 viable cells/ 100 μl 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 CO_2 for 4 hours.

MNU (Sigma Chemical, Co.; 10mg/ml) was prepared fresh in solution A (1.4M NaCl, 54mM KCl, 55.5mM Dextrose, 42mM NaHCO_3 ; 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 100 μl 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 $\mu\text{mol/g}$ 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 20 $\times$ 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; *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 *hMGMT* tumor-bearing mice with multiple tumors was similar to MNU treatment (Table II), likely because it was already so low in untreated *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 *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 mm³; 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 *hMGMT* transgenic and wild type mice were isolated, treated with MNU *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 *hMGMT* primary hepatocytes displayed a similar dose-dependent reduction of cell viability with increasing doses of MNU.

Because MNU-treated *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 *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.

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 *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 *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 *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 *hMGMT* transgene, which was a marginally significant effect. Furthermore, with MNU, tumor prevalence is reduced an additional 20.9% in *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 *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 *hMGMT* transgenic C3HeB/FeJ mice. Additionally, DEN produces O6eG lesions, which are repaired less efficiently overall than O6mG lesions by human MGMT (26, 27). Low efficiency repair of O6eG could also have contributed to the similar outcomes for the wild type and *hMGMT* transgenic mice, whether or not *hMGMT* was expressed at the time of treatment. Unrepaired O6mG lesions can be recognized by mismatch repair and lead to apoptosis (33). On the other hand, the toxicity of O6eG 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 O6mG lesion and its repair may not be the mechanism for MNU's cancer preventative effect. While O6mG 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 N7-alkylguanine and N3-alkyladenine adducts, occur in a greater proportion but do not have the high mutagenic potential of the O⁶-methylguanine adduct. Instead, they are more cytotoxic if they are not repaired (33). Interestingly, while DEN produces O6eG in the same proportion as MNU produces O6mG, DEN generates a much lower proportion of N7-alkylguanine and N3-alkyladenine adducts compared to MNU (12). The differences in proportion of N7-alkylguanine and N3-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 N7- and N3-adducts may have a similar preventative effect. Reduced efficiency of base excision repair should yield cell death from N7- and N3-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 O⁶-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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Altekruse SF, McGlynn KA, Reichman ME. Hepatocellular carcinoma incidence, mortality, and survival trends in the United States from 1975 to 2005. *J Clin Oncol*. 2009; 27:1485–1491. [PubMed: 19224838]
2. El-Serag HB, Mason AC. Risk factors for the rising rates of primary liver cancer in the United States. *Arch Intern Med*. 2000; 160:3227–3230. [PubMed: 11088082]
3. Larsson SC, Wolk A. Overweight, obesity and risk of liver cancer: a meta-analysis of cohort studies. *Br J Cancer*. 2007; 97:1005–1008. [PubMed: 17700568]
4. O'Neil BH, Venook AP. Hepatocellular carcinoma: the role of the North American GI Steering Committee Hepatobiliary Task Force and the advent of effective drug therapy. *Oncologist*. 2007; 12:1425–1432. [PubMed: 18165619]
5. Keng VW, Largaespada DA, Villanueva A. Why men are at higher risk for hepatocellular carcinoma? *J Hepatol*. 2012; 57:453–454. [PubMed: 22425699]
6. Agnew LR, Gardner WU. The incidence of spontaneous hepatomas in C3H, C3H (low milk factor), and CBA mice and the effect of estrogen and androgen on the occurrence of these tumors in C3H mice. *Cancer Res*. 1952; 12:757–761. [PubMed: 12988207]
7. Poole TM, Drinkwater NR. Strain dependent effects of sex hormones on hepatocarcinogenesis in mice. *Carcinogenesis*. 1996; 17:191–196. [PubMed: 8625437]
8. Kemp CJ, Leary CN, Drinkwater NR. Promotion of murine hepatocarcinogenesis by testosterone is androgen receptor-dependent but not cell autonomous. *Proc Natl Acad Sci U S A*. 1989; 86:7505–7509. [PubMed: 2798421]
9. Ramirez AG, Weiss NS, Holden AE, Suarez L, Cooper SP, Munoz E, et al. Incidence and risk factors for hepatocellular carcinoma in Texas Latinos: implications for prevention research. *PLoS One*. 2012; 7:e35573. [PubMed: 22530052]
10. Bosch FX, Ribes J, Cleries R, Diaz M. Epidemiology of hepatocellular carcinoma. *Clin Liver Dis*. 2005; 9:191–211. @v. [PubMed: 15831268]
11. Drinkwater NR, Ginsler JJ. Genetic control of hepatocarcinogenesis in C57BL/6J and C3H/HeJ inbred mice. *Carcinogenesis*. 1986; 7:1701–1707. [PubMed: 3757172]
12. Pegg AE. Methylation of the O6 position of guanine in DNA is the most likely initiating event in carcinogenesis by methylating agents. *Cancer Invest*. 1984; 2:223–231. [PubMed: 6733565]
13. Bilger A, Bennett LM, Carabeo RA, Chiaverotti TA, Dvorak C, Liss KM, et al. A potent modifier of liver cancer risk on distal mouse chromosome 1: linkage analysis and characterization of congenic lines. *Genetics*. 2004; 167:859–866. [PubMed: 15238534]
14. Gariboldi M, Manenti G, Canzian F, Falvella FS, Pierotti MA, Della Porta G, et al. Chromosome mapping of murine susceptibility loci to liver carcinogenesis. *Cancer Res*. 1993; 53:209–211. [PubMed: 8417808]
15. Manenti G, Binelli G, Gariboldi M, Canzian F, De Gregorio L, Falvella FS, et al. Multiple loci affect genetic predisposition to hepatocarcinogenesis in mice. *Genomics*. 1994; 23:118–124. [PubMed: 7829059]
16. Festing, MFW. Inbred strains in biomedical research. New York: Oxford University Press; 1979.
17. Denlinger RH, Koestner A, Wechsler W. Induction of neurogenic tumors in C3HeB-FeJ mice by nitrosourea derivatives: observations by light microscopy, tissue culture, and electron microscopy. *Int J Cancer*. 1974; 13:559–571. [PubMed: 4366972]
18. Kondo N, Takahashi A, Ono K, Ohnishi T. DNA damage induced by alkylating agents and repair pathways. *J Nucleic Acids*. 2010; 2010:543531. [PubMed: 21113301]
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. [PubMed: 2233815]
20. Zhou ZQ, Manguino D, Kewitt K, Intano GW, McMahan CA, Herbert DC, et al. Spontaneous hepatocellular carcinoma is reduced in transgenic mice overexpressing human O6-methylguanine-DNA methyltransferase. *Proc Natl Acad Sci U S A*. 2001; 98:12566–12571. [PubMed: 11606727]

21. Herzig MC, Hildreth K, Huamani J, Perez M, Goins BA, McMahan CA, et al. Human O(6)-methylguanine-DNA methyltransferase containing C145A does not prevent hepatocellular carcinoma in C3HeB/FeJ transgenic mice. *Mol Carcinog*. 2011
22. Walter CA, Lu J, Bhakta M, Mitra S, Dunn W, Herbert DC, et al. Brain and liver targeted overexpression of O6-methylguanine DNA methyltransferase in transgenic mice. *Carcinogenesis*. 1993; 14:1537–1543. [PubMed: 8353838]
23. Van Remmen H, Williams MD, Heydari AR, Takahashi R, Chung HY, Yu BP, et al. Expression of genes coding for antioxidant enzymes and heat shock proteins is altered in primary cultures of rat hepatocytes. *J Cell Physiol*. 1996; 166:453–460. [PubMed: 8592006]
24. Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, et al. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res*. 1988; 48:4827–4833. [PubMed: 3409223]
25. Moore MR, Drinkwater NR, Miller EC, Miller JA, Pitot HC. Quantitative analysis of the time-dependent development of glucose-6-phosphatase-deficient foci in the livers of mice treated neonatally with diethylnitrosamine. *Cancer Res*. 1981; 41:1585–1593. [PubMed: 7214332]
26. Liem LK, Lim A, Li BF. Specificities of human, rat and *E. coli* O6-methylguanine-DNA methyltransferases towards the repair of O6-methyl and O6-ethylguanine in DNA. *Nucleic Acids Res*. 1994; 22:1613–1619. [PubMed: 8202360]
27. Coulter R, Blandino M, Tomlinson JM, Pauly GT, Krajewska M, Moschel RC, et al. Differences in the rate of repair of O6-alkylguanines in different sequence contexts by O6-alkylguanine-DNA alkyltransferase. *Chemical research in toxicology*. 2007; 20:1966–1971. [PubMed: 17975884]
28. Newlands ES, Stevens MF, Wedge SR, Wheelhouse RT, Brock C. Temozolomide: a review of its discovery, chemical properties, pre-clinical development and clinical trials. *Cancer Treat Rev*. 1997; 23:35–61. [PubMed: 9189180]
29. Zhang YJ, Chen Y, Ahsan H, Lunn RM, Lee PH, Chen CJ, et al. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation and its relationship to aflatoxin B1-DNA adducts and p53 mutation in hepatocellular carcinoma. *Int J Cancer*. 2003; 103:440–444. [PubMed: 12478658]
30. Kaina B, Christmann M, Naumann S, Roos WP. MGMT: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents. *DNA Repair (Amst)*. 2007; 6:1079–1099. [PubMed: 17485253]
31. Vesselinovitch SD, Rao KV, Mihailovich N. Neoplastic response of mouse tissues during perinatal age periods and its significance in chemical carcinogenesis. *Natl Cancer Inst Monogr*. 1979:239–250. [PubMed: 384263]
32. Lu Y, Cox LA, Herbert DC, Weaker FJ, Walter CA, Adrian GS. Expression of chimeric human transferrin-chloramphenicol acetyltransferase genes in liver and brain of transgenic mice during development. *Dev Biol*. 1993; 155:452–458. [PubMed: 8432399]
33. Shrivastav N, Li D, Essigmann JM. Chemical biology of mutagenesis and DNA repair: cellular responses to DNA alkylation. *Carcinogenesis*. 2010; 31:59–70. [PubMed: 19875697]
34. Claij N, van der Wal A, Dekker M, Jansen L, te Riele H. DNA mismatch repair deficiency stimulates N-ethyl-N-nitrosourea-induced mutagenesis and lymphomagenesis. *Cancer research*. 2003; 63:2062–2066. [PubMed: 12727820]

Figure 1. Prevalence of hepatocellular carcinoma is reduced with MNU treatment, despite an increase in gastric squamous cell carcinoma, in wild type and *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 (mm³) 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 mm³ for untreated wild type mice, 2 to 2274 mm³ for MNU-treated wild type mice, 3 to 916 mm³ for untreated *hMGMT* mice, and 8 to 2624 mm³ for MNU-treated *hMGMT* transgenic mice. An exceptionally large tumor was recovered from an MNU-treated wild type mouse (7704 mm³). There were no statistical differences with MNU treatment for wild type or *hMGMT* transgenic mice.

Figure 3. *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 *hMGMT* transgenic mice (open circles) that were matched for age and sex. Data are presented as means $\pm$ 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=20 μ m

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)

Treatment group	# Tissues Analyzed		% Positive Cells for hMGMT/mMgmt		% Positive Cells for hMGMT	
	Normal	Tumor	Normal	Tumor	Normal	Tumor
C3HeB/FeJ	6	6	73.9±4.3	50.1±12.9	-32%	0
+MNU	2	2	77.7±11.6	46.0±36.8	-41%	ND
LC22I	2	3	77.8±8.9	60.9±11.7	-22%	76.2
+MNU	0	0	ND	NA		18.5* -75%
LC26I	5	5	59.6±7.9	33.2±11.9	-45%	80.2
+MNU	4	4	80.6±4.0	28.8*#±14.2	-64%	21.7* -72%
						ND

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.

Tumor prevalence is reduced with i.p. MNU treatment in both wild type and *hMGMT* (LC26I line) C3HeB/FeJ mice (MNU Experiment 2).

Table II

Treatment group	Total # mice analyzed	# mice with tumors	% with tumors	# mice with multiple tumors	% of tumor bearing mice with multiple tumors	% of all mice with multiple tumors	Total Tumor Burden (mm ³)	
							Mean	Median
C3HeB/FeJ	88	50	56.8% ^a	18	36.0% ^b	20.5% ^c	424	136
+MNU	74	24	32.4% ^a	5	20.8%	6.8% ^c	726	111
LC26I	32	14	43.8%	1	7.1% ^b	3.1%	208	66
+MNU	57	14	24.6%	3	21.4%	5.3%	400	217

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

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

^c Indicates 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)