Survival and tumorigenesis in \(O^6\)-methylguanine DNA methyltransferase-deficient mice following cyclophosphamide exposure

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\(O^6\)-methylguanine DNA methyltransferase (MGMT) deficiency is associated with an increased susceptibility to alkylating agent toxicity. To understand the contribution of MGMT in protecting against cyclophosphamide (CP)-induced toxicity, mutagenesis and tumorigenesis, we compared the biological effects of this agent in transgenic Mgmt knockout and wild-type mice. In addition, neurofibromin (Nflt/m) background was used to increase the likelihood of CP-induced tumorigenesis. Cohorts of Mgmt-proficient or -deficient mice (either \(Nfl\)/+ or \(Nfl\)/−) were given 6 weekly injections of a maximally tolerated dose of CP (250 mg/kg) or vehicle and followed for 15 months. CP-treated mice had more deaths than control mice but there was no difference in the long-term survival between \(Mgmt\)/+ and \(Mgmt\)/− mice (12 of 83 \(Mgmt\)/+/+ mice died compared to 12 of 80 \(Mgmt\)/−/− mice, disregarding \(Nfl\) status). Lymphomas and adrenal tumours were the most frequent malignancies. Interestingly, CP-treated, Mgmt-deficient mice developed fewer tumours than controls. Ten of 71 (14%) Mgmt-proficient mice developed tumours after CP treatment compared to only 2 of 68 (3%) Mgmt-deficient mice (\(P = 0.02\), \(Mgmt\)/+/−, \(Nfl\)/+/− mice developed fewer tumours (1 of 35, 3%) following CP compared to \(Mgmt\)/+, \(Nfl\)/+ mice (7 of 37, 19%) (\(P = 0.03\). Hypoxanthine–guanine phosphoribosyltransferase mutation assays showed no significant increases in mutant frequencies in \(Mgmt\)/−/− (18.1 \times 10^6) compared to \(Mgmt\)/+/+ mice (12.9 \times 10^6). These data indicate that MGMT deficiency does not protect against long-term toxicity or mutagenicity from CP and appears to attenuate the occurrence of CP-induced tumours in an \(Nfl\)/+/− background.

Introduction

\(O^6\)-methylguanine DNA methyltransferase (MGMT) is a crucial protein in the repair of alkylated DNA adducts that form following exposure to genotoxic agents. MGMT is expressed in many organs, tissues and human tumours with those expressing higher levels being relatively resistant to alkylating agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and temozolomide (1). The \(O^6\)-methylguanine DNA adduct is the critical DNA-damaging lesion primarily responsible for the cytotoxic as well as mutagenic effects of methylating agents (2,3). MGMT repairs the \(O^6\)-methylguanine DNA adduct by transferring the methyl group from the adduct to a cysteine residue on the MGMT protein inactivating itself in the process of repair (4,5). \(O^6\)-methylguanine, if not repaired, preferentially mispairs with thymine during DNA replication, resulting in a G:C → A:T transition mutation. While MGMT overexpression is associated with relative resistance, lack of MGMT is known to cause hypersensitivity to the biological effects of DNA-alkylating agents (6).

MGMT’s role in protecting against the lethal and mutagenic lesions induced by agents that alkylate DNA at the \(O^6\)-position of guanine has been well documented with respect to the alkylamines, dimethylnitrosamine and diethylnitrosamine in vitro and in vivo (7). For example, over-expression of Mgmt in the thymus of mice resulted in protection from thymic lymphomas after exposure to N-methyl-N-nitrosourea (MNU) (8) and skin tumours induced by the chloroethylyating agent, 3-[4-amino-2-methyl-pyrimidin-5-yl)methyl]-1-(2-chloroethyl)-1-nitrosourea (9). Conversely, Mgmt-deficient mice are hypersensitive to the effects of MNU (10,11) and show a higher tumour incidence after nitrosamine exposure (12). These data suggest that Mgmt is critical in protecting against the \(O^6\)-methylguanine pro-carncogenic lesion induced by alkylating agents.

The role of MGMT in cyclophosphamide (CP)-induced cytotoxicity and mutagenicity is less clear. CP undergoes metabolic activation in the liver to ultimately form phosphoramide mustard (PM) and acrolein (13). Previous studies demonstrate that AGT plays a role in protecting against the toxic and mutagenic effect of CP; however, the data implicate acrolein, not PM, as the toxic and mutagenic lesions protected by the AGT protein (14). Using human medulloblastoma cell line D283 Med resistant to 4-hydroperoxycyclophosphamide (activated form of CP), Friedman et al. (15) demonstrated that a significant, albeit small, part of the cellular toxicity of CP is due to acrolein and that this toxicity is abrogated in the presence of MGMT. MGMT repair of acrolein would explain clinical data showing a significant decrease in MGMT activity in peripheral blood lymphocytes of patients following treatment with CP (16). However, there are clinical and animal studies that do not show evidence of a correlation between MGMT activity and tumour response to CP (17–19). Specifically, Mgmt-proficient and -deficient mice are equally sensitive to lethal doses of CP in terms of acute survival (20). Taken together, these results suggest further in vitro studies to better understand MGMT’s importance in protecting against CP-induced mutagenicity and toxicity are warranted.

In attempts to understand the role of MGMT in CP’s tumorigenic effects, we produced a compound transgenic...
neurofibromin (Nf1) +/- mouse model that co-expressed and lacked Mgmt. Nf1 +/- mice develop spontaneous, as well as, CP-induced leukaemia at a higher rate compared to Nf1 +/- mice (21). Nf1 is a tumour suppressor gene that encodes a GTPase protein that negatively regulates ras signalling in cells (22). We surmised that a study in Mgmt +/- mice and Mgmt +/+ mice in an Nf1 +/- genetic background, that predisposes mice to higher CP-induced tumours, would help elucidate the role of MGMT in CP-induced toxicity and mutagenicity.

Materials and methods

Drugs

CP monohydrate was purchased from Sigma-Aldrich Corp. (St Louis, MO, USA). The drug was dissolved in 0.9% NaCl.

Transgenic mice

Generation of C57BL/6 mice deficient in Mgmt has been previously described (10). Nf1 +/-, C57BL/6 mice were obtained from Dr Kevin Shannon (University of California, San Francisco, CA, USA) (23). Wild-type C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Nf1 +/-, C57BL/6 mice were crossed with Mgmt +/- to produce Nf1 +/-, Mgmt +/- mice that were used to study CP-induced tumorigenesis in an Mgmt-deficient background. First, Nf1 +/-, Mgmt +/- were crossed with Nf1, Mgmt +/- to produce Nf1 +/-, Mgmt +/- mice then backcrossed with Nf1 +/-, Mgmt +/- mice to produce offspring of four different genotypes (Nf1 +/-, Mgmt +/-; Nf1, Mgmt +/-; Nf1 +/-, Mgmt +/+; Nf1 +/-, Mgmt +/+). The genotyping of the Nf1 and Mgmt genes by polymerase chain reaction (PCR) is described below.

Nf1 and Mgmt genotyping

Offspring of matings were genotyped by using a PCR assay. For Nf1 genotyping, genomic DNA was prepared from tail clippings as previously described (23) using primers Pnf1-wt, Pnf1-neo and Pnf1-com. Primers Pnf1-wt (5’-AGACACTCTTTTGGCTATTGGGACACC-3’) and Pnf1-com (5’-AACTCTTTGTTGCTTTGGGACATCTATATG-3’) detected the wild-type Nf1 allele (248 bp); primers Pnf1-neo (5’-AAGGCCGCGGA- GAACCTGGCTGGAATCATC-3’) and Pnf1-com detected the knockout allele (313 bp). The PCR used Sigma Jump Start RedTaq (Sigma–Aldrich) with the following final concentrations of each component: 0.25 μM for all three primers, 0.1× dNTPs and 2.5 mM Mg+++. Following an initial denaturation at 94°C for 60 sec, 35 cycles of 94°C for 5 sec, 68°C for 20 sec and 72°C for 60 sec followed with a final elongation step of 72°C for 5 min.

Mgmt genotyping was performed on genomic DNA isolated from tail snips by multiplex PCR analysis as previously described (10) using primers Pwt, Pko and Pcom. Primers Pwt (5’-GGCATCTTTCTCCAATACCTGA-3’) and Pcom (5’-CCCGAGACATCCGAGCCTCT-3’) detected the wild-type Mgmt allele (542 bp); primers Pko (5’-GGTGAGTTGATAAATGGCTCT-3’) and Pcom detected the knockout allele (409 bp). Final primer concentrations were 0.25 μM for Pwt and Pko and 0.5 μM for Pcom. Following an initial denaturation at 97°C for 3 min, the PCR conditions were three cycles of 97°C for 30 sec, 61°C for 60 sec and 72°C for 60 sec; 10 cycles of 94°C for 30 sec, 59°C for 60 sec and 72°C for 60 sec; 20 cycles of 94°C for 30 sec, 57°C for 60 sec and 72°C for 60 sec followed with a final elongation step of 72°C for 5 min.

Animal care

Mice were housed at the University of Chicago’s Animal Care Facility and cared for in accordance with Institutional Animal Care and Use Committee policies. Mice were provided with food and water ad lib and were examined daily by investigators or Animal Care Facility staff for signs of illness. All invasive procedures and euthanasia on animals was carried in accordance with institutional policies with minimal discomfort to the animals.

Maximal tolerated dose determination

Eight- to 10-week-old C57BL/6 wild-type mice were given 6 weekly intraperitoneal (i.p.) injections of CP (100, 200, 250, 375 or 300 mg/kg). Six mice were treated at each drug dose. Animals were weighed prior to the first injection (baseline) and each subsequent injection. All injection volumes were 200 μl/20 g of body weight. Percent weight gain/loss was determined as a percent of pretreatment weight. CP doses were escalated until the 10% lethal dose (LD10) level was reached. LD10 was the lowest dose at which no more than one mouse died within 8 weeks after initial treatment.

Determination of survival and tumorigenesis

Forty mice from each of the four cohorts (Nf1 +/-, Mgmt +/-; Nf1 +/-, Mgmt -/-; Nf1, Mgmt +/-; Nf1, Mgmt -/-), received 6 weekly i.p. injections of 250 mg/kg/week CP. For controls, 40 mice from each cohort received weekly i.p. injections of the vehicle, 0.9% NaCl. All mice were 8–10 weeks old at study entry. Mice were weighed prior to each weekly treatment and the drug dose was adjusted accordingly.

Monitoring for toxicity

Mice were followed for at least 15 months, with regular physical examinations. Weights and complete blood counts (CBCs) were done at baseline and every 2 months on all healthy mice. Mice with morbidity or persistently abnormal CBCs were sacrificed. Mice that survived up to 15 months after treatment without signs of morbidity or tumours were sacrificed after performing a final CBC and peripheral blood smear. CBCs were performed on blood obtained from the tail vein collected in ethylenediaminetetraacetic acid-coated microtainers using a MASCOT 850 (Drew Scientific Group, Oxford, CT, USA) machine counter.

Necropsy and tissue collection

At necropsy, whole body weight was measured and gross examination was conducted on all tissues. Any visible tumours were isolated, gross appearance was noted and sectioned for touch preps, histopathological stains and flash frozen in liquid nitrogen. The spleen, liver and femoral bone marrow were processed similarly. Cytospin preparations from bone marrow and splenic single-cell suspensions were made from 150 000 cells in FACS buffer (Hank’s balanced salt solution (HBSS)), Ca/Mg free, Phenol Red free (Mediatech Inc., Herndon, VA, USA). 2% heat inactivated foetal bovine serum (HyClone, Logan, UT, USA) and 2.5% Gibco-BRL Cell Dissociation buffer (Invitrogen Corp., Carlsbad, CA, USA) spun (18 x g) for 8 min at medium acceleration using a Cytotop 7629 cryocentrifuge (Wescor Inc., Logan, UT, USA) and the remainder of the bone marrow and splenic cell suspensions were cryopreserved in HBSS with 20% foetal bovine serum and 10% dimethylsulphoxide. Remaining tissues were placed in Accustain® formalin solution (Sigma–Aldrich) for future histologic analysis. All paraffin-embedded tumours, spleen and livers, peripheral blood and bone marrow smears, touch preps and cytospins were stained with Wright–Giemsa (Fisher Diagnostics, Middletown, VA, USA).

Statistical analysis

Survival estimates were obtained using Kaplan–Meier survival curves on the different cohorts. Tumour induction comparisons between groups were computed using chi-square statistics or Fisher exact tests. Hypoxanthine–guanine phosphoribosyltransferase (Hprt) mutant frequencies were compared using Student’s t-test.

Dose-finding studies

Preliminary studies were done to determine the maximal tolerated dose (MTD) of CP in Mgmt-proficient and -deficient mice. Mgmt-deficient mice are known to be sensitive to myelosuppression and weight loss, in Mgmt-proficient versus Mgmt-deficient mice. Baseline haemograms (CBCs) were not significantly different between the Mgmt +/- versus Mgmt -/- mice, regardless of Nf1 status. Neutropenic nadir occurred at 4
days after the first CP dose with recovery by day 7 in both cohorts. The degree of neutropenic nadir was also not statistically significantly different between the two groups. Baseline and post-treatment haemoglobin and platelet counts were, likewise, not different (data not shown). Among mice that survived to study termination, there were no significant differences in weight or haematopoietic parameters (data not shown).

**Hprt mutation assay**

We have previously shown that Mgmt protects against *in vitro* mutations induced by the CP metabolite acrolein (14). *In vivo* using Nf1+/+ mice, we previously found that the Hprt mutation frequency significantly increased from $1.8 \times 10^6$ in vehicle-treated mice (both Mgmt+/+ and Mgmt−/−) to $12.9 \times 10^6$ in Mgmt+/+ mice and $18.1 \times 10^6$ in Mgmt−/− mice ($P < 0.005$ compared to control) following a single dose of CP (26). The increased mutation frequency after CP treatment observed in Mgmt−/− as compared to Mgmt+/+ was not significant.

**Event-free survival**

A total of 323 mice were enrolled on the study. The Mgmt+/+ and Mgmt−/− mice were the cohorts of interest. However, to exploit the known occurrence of spontaneous and CP therapy-induced tumours in Nf1−/− mice, compound transgenic mice that were Nf1+/+ or Nf1−/− in addition to their Mgmt genotypes were included. Eight cohorts of mice were treated with either CP or vehicle (control) and followed for at least 15 months for survival outcome and tumour incidence. The number of mice enrolled for each cohort is shown in Table II. CP-treated mice had more deaths than control mice, across all cohorts. Overall survival of control (vehicle treated) Mgmt−/− mice was not different from Mgmt+/+ mice (Figure 1). There was no statistically significant difference in the overall survival of CP-treated Mgmt+/+ mice and Mgmt−/− mice. These comparisons were true regardless of Nf1 genotype. After CP treatment, 12 of 83 Mgmt+/+ mice died compared to 12 of 80 Mgmt−/− mice. This suggests that Mgmt deficiency does not contribute to late deaths after CP therapy. Median survival of CP-treated Mgmt−/− mice was also not different from Mgmt+/+ mice.

**Long-term organ toxicities**

There were no distinct differences in the gross morphologic appearance of organs at necropsy, between the Mgmt+/+ and Mgmt−/− mice that survived to study end point of 15 months.

**Table I.** Toxicity of 6 weekly doses of CP in Mgmt+/+ or Mgmt−/− C57BL/6 mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>No. of mice</th>
<th>Mgmt+/+</th>
<th>Mgmt−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Weight change (%)</td>
<td>Deaths</td>
</tr>
<tr>
<td>CPb</td>
<td>100</td>
<td>6</td>
<td>2.4 0</td>
<td>n.d. 0</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>6</td>
<td>−2.9 0</td>
<td>n.d. 0</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>6</td>
<td>−2.9 0</td>
<td>−0.8 0</td>
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<td></td>
<td>275</td>
<td>6</td>
<td>−11.9 0</td>
<td>n.d. 0</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>6</td>
<td>−12.5 2</td>
<td>n.d. 0</td>
</tr>
</tbody>
</table>

*Per cent weight change is from pretreatment weight.

*CP was administered in normal saline.

*n.d.: not determined.

**Table II.** Mice enrolled in long-term study by cohort

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>No. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nf1+/+</td>
<td>Vehicle</td>
<td>39</td>
</tr>
<tr>
<td>Nf1+/+</td>
<td>CP</td>
<td>41</td>
</tr>
<tr>
<td>Nf1−/−</td>
<td>Vehicle</td>
<td>40</td>
</tr>
<tr>
<td>Nf1−/−</td>
<td>CP</td>
<td>40</td>
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<tr>
<td>Nf1+/+</td>
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<td>40</td>
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<tr>
<td>Nf1+/+</td>
<td>CP</td>
<td>42</td>
</tr>
<tr>
<td>Nf1−/−</td>
<td>Vehicle</td>
<td>40</td>
</tr>
<tr>
<td>Nf1−/−</td>
<td>CP</td>
<td>40</td>
</tr>
</tbody>
</table>

**Fig. 1.** Survival of mice treated with CP or vehicle. (A) Kaplan–Meyer survival curve of Nf1+/+ mice treated with vehicle (Mgmt+/+, n = 39; Mgmt−/−, n = 41) or 250 mg/kg CP (Mgmt+/+, n = 41; Mgmt−/−, n = 40). (B) Kaplan–Meyer survival curve of Nf1−/− mice treated with vehicle (Mgmt+/+, n = 40; Mgmt−/−, n = 40) or 250 mg/kg CP (Mgmt+/+, n = 42; Mgmt−/−, n = 40).

Gross body weight and spleen weight (normalized to total body weight) were not significantly different between the two cohorts (data not shown). In addition, no histopathological differences in the spleen or liver were observed between the two groups.

**Tumour incidence**

Tumours were noted in 24 (out of 294) mice that survived through the observation period of at least 15 months. Two Nf1+/+ Mgmt+/+ mice, one treated with vehicle (saline) and the other with CP, were sacrificed earlier (at 12.5 and 13.8 months, respectively) after observation of visible tumour masses. Tumours in 22 other mice were observed during necropsy at the end of the observation period. Spontaneous tumour rates (tumour incidence in vehicle-treated mice) were comparable among Nf1+/+ mice (0 of 37 in Mgmt+/+ and 1 of 41 in Mgmt−/−; Figure 2), demonstrating that Mgmt deficiency does not enhance the spontaneous tumour rates in mice. Consistent with the literature (23), Nf1+/− mice had
a significantly greater incidence of spontaneous tumours compared to Nfi+/+ (16.2% in Nfi+/-, Mgmt+/+ compared to 0% in Nfi+/-, Mgmt+/+, P < 0.01 as determined by Fisher exact t-test) and were not significantly altered with Mgmt status. In an Nfi+/- background, Mgmt-deficient mice treated with CP had a slightly lower tumour incidence than wild-type Mgmt mice (3.0 versus 8.8%). Interestingly, the tumour incidence following CP in Nfi+/-, Mgmt+/- mice (2.9%) was significantly lower than Nfi+/-, Mgmt+/+ mice (18.9%, P < 0.03). This statistically significant difference suggests that the lack of Mgmt has an attenuating influence on the Nfi+/- genotype’s propensity to developing CP treatment-induced tumours.

**Tumour types**

A variety of soft tissue tumours were noted including adrenal tumours, lung tumours, soft tissue sarcoma and lymphomas (Table III). The spectrum of tumours observed in our mice is consistent with other reports in the literature except that our tumour incidence rates were lower than those previously reported (23,25). While lymphomas occurred in both the Mgmt cohorts (Mgmt+/+ and -/-), no adrenal tumours were noted in CP-treated Mgmt-deficient mice. The only observed leukaemia occurred in an Nfi+/-, Mgmt+/- CP-treated mouse.

Fig. 2. Tumour incidence following treatment with CP or vehicle. (A) At time of necropsy, tumours were noted in Nfi+/- mice treated with vehicle (Mgmt+/+, 0/37; Mgmt-/-, 1/41) or 250 mg/kg CP (Mgmt+/+, 3/34; Mgmt-/-, 1/33). (B) At time of necropsy, tumours were noted in Nfi+/- mice treated with vehicle (Mgmt+/+, 6/37; Mgmt-/-, 5/40) or 250 mg/kg CP (Mgmt+/+, 7/37; Mgmt-/-, 1/35). *Indicates P < 0.03.

**Table III. Distribution of tumour types in Mgmt-proficient and -deficient mice**

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>Mgmt+/+ mice</th>
<th>Mgmt-/- mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (saline)</td>
<td>Lymphoma, 4</td>
<td>Lymphoma, 3</td>
</tr>
<tr>
<td>Adrenal, 1</td>
<td>1 Adrenal, 1</td>
<td></td>
</tr>
<tr>
<td>Lung, 1</td>
<td>Lung, 1</td>
<td></td>
</tr>
<tr>
<td>Sarcoma, 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>Adrenal, 6</td>
<td>Leukaemia, 1</td>
</tr>
<tr>
<td>Lymphoma, 2</td>
<td>Other, 1</td>
<td></td>
</tr>
<tr>
<td>Lung, 2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Discussion**

In this study we used Mgmt-deficient mice to determine the role of Mgmt in protecting against CP-induced mutations, tumorigenesis and long-term toxicities. The MTD of CP, as measured by myelosuppression and weight loss, was similar in Mgmt-proficient and -deficient mice. Additionally, CP-induced in vivo mutations were not significantly increased in the absence of Mgmt. To exploit the natural propensity of Nfi+/- mice to developing spontaneous and CP-induced malignancies (21,23), we evaluated in Mgmt-proficient and -deficient mice following treatment with 6 weekly doses of CP. With at least 15 months of follow-up, we found that there were no differences in the long-term survival of CP-treated Mgmt-deficient mice compared to Mgmt-proficient mice. Surprisingly, we observed a trend towards fewer tumours in Mgmt-/- mice compared to Mgmt+/+ mice, which was significant in an Nfi+/- background.

Preclinical studies in cell lines indicated a role for MGMT in protecting against the cytotoxic and mutagenic effects of CP (14,15). For example, CHO cells transduced with wild-type human MGMT were treated with an activated form of CP (4-hydroperoxy-CP) that spontaneously formed acrolein and PM. Cell lines over-expressing MGMT were significantly less sensitive to the toxic and mutagenic effects of 4-hydroperoxy-CP as well as a derivative of CP that only produced acrolein. In contrast, PM had similar survival curves and exhibited no difference in mutation frequency in cell lines expressing and lacking MGMT. We concluded that MGMT plays an important role in protecting against the toxic and mutagenic effect of CP and suggested that this was due to acrolein generating a toxic and mutagenic lesion repaired by the MGMT protein (14). In addition, a human medulloblastoma cell line resistant to CP in which cells became sensitive to 4-hydroperoxy-CP and a derivative that generated acrolein upon depletion of MGMT (15). Direct reaction of acrolein with DNA followed by incubation with MGMT resulted in a decrease in MGMT levels, consistent with the hypothesis that acrolein reacts with DNA to produce a lesion that is repaired by MGMT (15).

In addition to the preclinical evidence for MGMT’s role in CP resistance, clinical evidence emerged that lack of MGMT was associated with better survival of diffuse large B-cell lymphoma (DLBCL) patients treated with multi-drug regimens including CP (27–29). Initial studies demonstrated that MGMT gene hypermethylation, leading to gene silencing and lack of MGMT protein, was associated with a statistically significant increase in overall survival and progression-free survival in DLBCL patients and was both independent of, and stronger than, established prognostic factors (28). In addition to
measuring MGMT promoter methylation status, MGMT protein expression was analysed by immunohistochemistry in a group of Saudi Arabian (27) and Japanese (29) DLBCL patients treated with drug regimens that included CP. Both studies showed that MGMT promoter methylation was associated with a statistically significant increase in the overall survival and was a strong prognostic factor for DLBCL patients (27,29). In contrast to the data obtained in DLBCL patients, tumour MGMT expression was not predictive of response following treatment with induction chemotherapy including CP in breast cancer patients (30). Survival was longer when breast tumour MGMT expression was high compared to low (P < 0.005). Therefore, the role of MGMT in patient response to CP might be tumour specific or confounded by multi-drug treatment. These studies, along with the preclinical data, led us to rationalize that identifying the role of MGMT in resistance to the toxic and mutagenic effects of CP was worthwhile.

We found that CP at 250 mg/kg was equitoxic between Mgmt-proficient and -deficient mice suggesting that Mgmt deficiency does not increase sensitivity to the acute effects of CP, consistent with previous observations in mice (20). Our study also evaluated long-term toxicities of CP in Mgmt-proficient and -deficient mice and found no differences in long-term toxicities or survival.

Contrary to our hypothesis that Mgmt deficiency would lead to increased tumorigenesis following CP treatment, Mgmt-deficient mice had a reduced tumour incidence compared to wild-type mice. The difference in tumour incidence following CP treatment between Mgmt+/+ and Mgmt−/− mice was only significant in an Nf1+/− background. This may be due to accumulation of active Ras in the Nf1+/− setting, which likely contributes to cell proliferation and malignant transformation of a variety of cell types (31). The tendency of the Nf1+/− background towards tumorigenesis is enhanced after genotoxic treatment with CP alone or CP plus radiation (25). The exact reason why the lack of Mgmt attenuates the Nf1+/− susceptibility to tumour incidence in this study is unclear but could be due to increased cellular toxicity due to a multiplicity of DNA adducts formed after exposure to CP. While O6-methylguanine DNA adducts are known to be mutagenic as well as tumorigenic, they are also capable of inducing chromosomal instability, apoptosis and cell death (2). Thus, a possible explanation is that the multiplicity of genotoxic adducts (DNA cross-links due to PM or O6-guanine DNA adducts formed with acrolein) generated as a result of CP treatment combined with the lack of repair by MGMT protein may result in increased cytotoxicity and cell death rather than the stabilization and propagation of mutations through successive cell divisions. That these mechanisms did not lead to acute toxicities or animal deaths in the Mgmt-deficient cohorts is likely explained by the fact that the MTD of CP dose that we used was equally well tolerated by Mgmt-proficient and -deficient mice.

In support of our plausible explanation that there could be more cytotoxicity than mutagenicity, we noted no significant increase in spontaneous or CP-induced mutagenicity, in Mgmt-deficient mice, as measured by in vivo Hprt mutant frequency assays (26). Our results do indicate significant mutation induction following CP treatment in both Mgmt-proficient and -deficient mice compared to control mice. These results indicate that the primary mutagenic lesion incurred following CP treatment is not recognized by Mgmt, thus mutations observed in CP-treated mice in both Mgmt wild-type and -deficient mice are likely the result of PM-incurred damage (26).

Hprt mutation frequencies were not determined in an Nf1+/− background and our hypothesis that there is a reduced number of mutations in Nf1+/−, Mgmt−/− mice has not yet been tested. Additional complications in attempting to equate mutation frequencies in the spleen to tumour incidence in this study are that there is a known propensity for tumours to occur preferably in certain tissue types than others, especially in the Nf1 heterozygous background (23,25). Lastly, the C57BL/6 mouse strain used in our study could have had a modulating effect on tumorigenesis. C57BL/6 mice have been shown to have an attenuated leukaemia phenotype in previous studies (21) as observed with F1 offspring (129/Sv and C57BL/6) mice having a lower incidence of spontaneous and CP-induced myeloid malignancies compared to 129/Sv mice. The MTD of CP is also higher in these F1 mice (200 mg/kg) compared to 150 mg/kg in 129/Sv mice. This is consistent with a higher MTD for CP (250 mg/kg) in our study using C57BL/6 mice.

The role of MGMT in protecting against cytotoxicity from genotoxic agents that produce an O6-alkylguanine lesion is well known (32,33). Mice depleted of Mgmt have been shown to be hypersensitive to the lethal and toxic effects of BCNU and temozolomide, requiring dramatic dose reductions in their MTD (8,10,24). Studies comparing tumorigenesis in mice depleted of or deficient in Mgmt, with that of Mgmt over-expressing mice, establish that the O6-alkylguanine lesion, which is repaired by Mgmt, plays a critical role in initiating and propagating the toxic and mutagenic effects of nitrosamines and nitrosoureas (7,8,10–12). Our current studies demonstrate that Mgmt deficiency does not result in increased in vivo toxicities, as the MTD of CP was equivalent in Mgmt+/+ and Mgmt−/− mice. Importantly, Mgmt deficiency did not result in increased mutations following CP and CP-induced tumorigenesis was actually reduced. The lack of increased tumorigenesis following CP is intriguing and requires further investigation. A possible place to begin is determining why in vivo mutation frequencies are reduced in the Nf1+/− setting. In addition, one might consider evaluating for chromosomal rearrangements since this arises from exposure to PM, while acrolein is more likely to cause point mutations. CP is likely mutagenic primarily as a result of the effect of PM.

In conclusion, our study demonstrates that Mgmt deficiency does not enhance acute toxicities after CP therapy and also does not affect long-term survival of CP-treated mice.

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