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Role of MGMT in Protecting against Cyclophosphamide-Induced Toxicity in Cells and Animals

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Abstract

*O*⁶-Methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein that protects cells from the biological consequences of alkylating agents by removing alkyl groups from the *O*⁶-position of guanine. Cyclophosphamide and ifosfamide are oxazaphosphorines used clinically to treat a wide variety of cancers; however, the role of MGMT in recognizing DNA damage induced by these agents is unclear. In vitro evidence suggests that MGMT may protect against the urotoxic oxazaphosphorine metabolite, acrolein. Here, we demonstrate that Chinese hamster ovary cells transfected with MGMT are protected against cytotoxicity following treatment with chloroacetaldehyde (CAA), a neuro- and nephrotoxic metabolite of cyclophosphamide and ifosfamide. The mechanism by which MGMT recognizes damage induced by acrolein and CAA is unknown. CHO cells expressing a mutant form of MGMT (MGMT^{R128A}) known to have >1,000-fold less repair activity towards alkylated DNA while maintaining full active site transferase activity towards low molecular weight substrates exhibited equivalent CAA- and acrolein-induced cytotoxicity to that of CHO cells transfected with plasmid control. These results imply that direct reaction of acrolein or CAA with the active site cysteine residue of MGMT, i.e. scavenging, is unlikely a mechanism to explain MGMT protection from CAA and acrolein-induced toxicity. In vivo, no difference was detected between *Mgmt* ^{-/-} and *Mgmt* ^{+/+} mice in the lethal effects of cyclophosphamide. While MGMT may be important at the cellular level, mice deficient in MGMT are not significantly more susceptible to cyclophosphamide, acrolein or CAA. Thus, our data does not support targeting MGMT to improve oxazaphosphorine therapy.

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Overview

Alkylating agents in common clinical use today (e.g., methylating agents, chloroethylating agents and oxazaphosphorines) produce their biological effect by transferring alkyl groups to cellular molecules, such as DNA, RNA and proteins [1–4]. These agents react with nucleophilic sites including the oxygen and nitrogen of nucleic acids, as well as the oxygen of the phosphodiester groups within the backbone of DNA. Methylating agents most commonly react with the N7-position of guanine, although evidence implicates the *O*⁶-alkylguanine as the critical biological lesion eventually leading to mutations and cellular death [1]. Chloroethylating agents, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), and oxazaphosphorines, including cyclophosphamide and ifosfamide, eventually lead to DNA interstrand cross-links and subsequent death [5,6]. Unfortunately, tumor resistance to alkylating agents results in eventual clinical failure. A prominent mechanism of resistance against the biological effects of methylating and chloroethylating agents is the DNA repair protein, *O*⁶-methylguanine-DNA methyltransferase (MGMT). Although there is evidence for some role of MGMT as a mechanism of resistance against the biological effects of cyclophosphamide and ifosfamide, the exact mechanism is not clear.

*O*⁶-Methylguanine-DNA methyltransferase

MGMT is a 22-kDa protein that repairs *O*⁶-alkylguanine adducts on DNA without the aid of other cofactors or proteins [7,8]. MGMT binds to DNA via a helix-turn-helix motif in the C-terminal domain while a second helix, containing an arginine “finger,” flips out the alkylated base into the active site of the protein [9]. The conserved active site, IPCHRV/I, is involved in the direct transfer of the alkyl group from the alkylated base to the cysteine, Cys145, acceptor residue [7,8]. This transfer results in “suicide” inactivation of MGMT by the irreversible alkylation of the cysteine moiety and by changing the conformation of the DNA-binding domain of MGMT [9,10]. MGMT then becomes detached from DNA and is targeted for degradation by ubiquitination [11]. MGMT activity is restored in the cell after *de novo* synthesis [7,8].

MGMT is expressed in all normal tissues but varies in expression levels with very low levels observed in bone marrow cells [12–14]. Approximately 20% of human tumor cell lines exhibit low MGMT activity and are sensitive to the cytotoxic and mutagenic effects of *O*⁶-alkylating agents [8,15]. Unfortunately, the majority of tumor cells have high levels of MGMT [16,17] resulting in the limited usefulness of *O*⁶-alkylating agents as therapeutic agents [8,15,18–20].

In attempts to increase the sensitivity of tumor cells to the cytotoxic effects of *O*⁶-alkylating agents, potent, irreversible inactivators of MGMT have been developed. Since MGMT is inactivated upon removal of alkyl groups from the *O*⁶-position of guanine, pseudo-substrates (*O*⁶-substituted guanines) have been synthesized and tested. *O*⁶-Benzylguanine (BG) has been found to rapidly deplete MGMT activity to undetectable levels at micromolar concentrations and to increase the sensitivity of human tumor cells and xenografts to the cytotoxic effects of *O*⁶-alkylating agents [21–23]. More recently, McElhinney *et al.* [24] have developed *O*⁶-4-bromothienylguanine (PaTrin-2). Similar to BG, PaTrin-2 increases the sensitivity of human tumor cells and xenografts to the cytotoxic effects of *O*⁶-alkylating agents [25–28]. The promising results in xenograft studies combining MGMT inhibitors, either BG or PaTrin-2, plus *O*⁶-alkylating agents have led to numerous clinical trials aimed at increasing the efficacy of temozolomide or BCNU.

Clinical utility of cyclophosphamide and ifosfamide

Cyclophosphamide has been used in the treatment of many primary malignancies, including hematopoietic and a variety of solid tumors (breast, lung, ovarian and prostate cancer), and at

high doses in bone marrow transplantations because of its immunosuppressive activity [4,29–32]. Cyclophosphamide is also used as an immunosuppressive agent to treat autoimmune diseases. Ifosfamide has shown antitumor activity in many of the same cancers as those sensitive to cyclophosphamide but is also used to treat bladder and cervical cancer, neuroblastoma, Ewing sarcoma and uterine sarcoma [4]. While both cyclophosphamide and ifosfamide are used alone, most often each is a component of multi-drug regimens used against breast cancer (mainly cyclophosphamide-containing regimens) or lymphoma and lung cancer (ifosfamide).

Role of MGMT in clinical response to cyclophosphamide

While the role of MGMT in protecting against toxicity induced by methylating and chloroethylating agents is well established, the role of MGMT in protecting against the genotoxic effects of cyclophosphamide is less clear. Correlative clinical studies conducted on ovarian cancer [33–35], diffuse large B-cell lymphoma [36–38] and breast cancer patients [39,40] conflict on the relationship between MGMT levels and response to cyclophosphamide. Chen *et al.* [33] found that, in general, poor responders to chemotherapy (cyclophosphamide plus cisplatin or carboplatin) had tumors with measurable pre-therapy MGMT activity levels (> 10 fmol/mg protein), while another study did not find this association [35]. In line with the latter, Codegani *et al.* [34] did not find a correlation with MGMT mRNA expression in tumors from ovarian cancer patients and response to cyclophosphamide with doxorubicin plus or minus cisplatin. Several studies have found tumors negative for MGMT immunohistochemical staining [38] or with MGMT promoter methylation [36–38] to be significantly associated with increased overall survival of diffuse large B-cell lymphoma patients following cyclophosphamide and other drugs. On the other hand, Cayre *et al.* [39] did not find MGMT mRNA in tumor biopsies from breast cancer to be predictive of response to cyclophosphamide.

Role of MGMT in resistance to cyclophosphamide in xenograft studies

Equally conflicting are results describing the role of MGMT in resistance to the antitumor effects of cyclophosphamide in mice xenograft experiments [41–43]. Mattern *et al.* [43] showed lung tumor xenografts with high MGMT activity were less sensitive to the growth-inhibitory effects of cyclophosphamide than those with low MGMT activity ($p < 0.05$). Interestingly, 2 of the 5 tumor xenografts in this study that had the greatest response to cyclophosphamide had considerable MGMT activity levels (> 370 fmol/mg protein) [43]. Additionally, using a BCNU-resistant glioblastoma cell line, Friedman *et al.* [42] showed that the antitumor activity following cyclophosphamide was slightly increased after MGMT depletion with BG compared to cyclophosphamide alone ($p = 0.07$). The conclusions of the authors (i.e., cyclophosphamide activity was modulated by MGMT activity) must be re-examined in light of more recent studies showing an MGMT-independent role for BG in enhancing the cytotoxicity of some alkylating agents [44–47]. Specifically, BG was shown to significantly enhance the cytotoxicity and decrease the mutagenicity of nitrogen mustards [i.e., phosphoramide mustard (PM), melphalan, and chlorambucil], a group of alkylating agents not known to produce O^6 -adducts in DNA. The enhancement is observed in cells irrespective of AGT activity. The data suggests that treatment with BG causes G1 arrest and drives non-cycling cells treated with nitrogen mustards into apoptosis [44]. Lastly, a study on 23 different xenograft tumors showed no correlation between the antitumor activity of cyclophosphamide and tumor MGMT activity levels [41]. Although 5/23 of the tumor cell lines used to establish xenografts in this study had low MGMT levels, only one of the 5 was responsive to cyclophosphamide-induced growth inhibition [41]. Taken together, the above studies illustrate the role of MGMT in resistance to cyclophosphamide in vivo has yet to be fully defined.

Metabolism of cyclophosphamide and ifosfamide

As shown in Scheme 1, cyclophosphamide and ifosfamide are prodrugs that are activated by oxidation at the C-4 position via the hepatic cytochrome P450 enzymes [4,32] resulting in the formation of 4-hydroxycyclophosphamide and 4-hydroxyifosfamide, respectively. Each 4-hydroxy intermediate rapidly interconverts with an acyclic aldehyde, aldophosphamide or aldoifosfamide. These aldehydes spontaneously (non-enzymatically) fragment leading to acrolein and either phosphoramidate mustard (PM) or isophosphoramidate mustard (IPM). Competing with C-4 oxidation is the P450-mediated, oxidative *N*-dechloroethylation of a side chain resulting in the production of chloroacetaldehyde (CAA). This pathway accounts for less than 10% of the metabolism of cyclophosphamide [29–31] but can be a significant contributor in the metabolism of ifosfamide (up to 50%) [48].

The therapeutic effects of cyclophosphamide and ifosfamide are attributed to PM and IPM, respectively, while acrolein and CAA are associated with unwanted side effects [29]. PM and IPM have been shown to produce interstrand cross-links between the N7-positions of guanines on opposite DNA strands in a G-N-C/C-N-G orientation [49,50]; these cross-links are believed to be the major cytotoxic lesion [5]. The interstrand cross-link produced from PM is slightly shorter, five-atoms, than the seven-atom linkage produced from IPM [50]. Acrolein is thought to be responsible for the dose limiting urotoxicity of cyclophosphamide [51], with the contribution of PM being very little [52]. Acrolein concentrations (~ 40 nM) peak 1 – 12 hr following cyclophosphamide or ifosfamide treatment [53]. Mesna (2-mercaptoethane sulfonate), which has a nucleophilic thiol, is used to protect against cyclophosphamide- and ifosfamide-induced bladder toxicity by directly reacting with acrolein (and, possibly, other metabolites) in the bladder [54,55]. Recently, the thiols (or thiol-equivalent) amifostine and glutathione were shown to prevent acrolein induced hemorrhagic cystitis in the bladder of mice [56]; however, the efficacy of these agents in combination with oxazaphosphorines in humans has yet to be determined.

CAA is thought to be responsible for the neuro- and nephrotoxicity associated with ifosfamide [57–61]. Studies have begun to show that the P450 isoenzymes necessary for *N*-dechloroethylation (primarily CYP 3A4/5 and, to a lesser extent, 2B6) are present and active in kidneys of children and adults [62–64]. Pharmacokinetic modeling, using data from renal tubule cells exposed to 100 μ M ifosfamide, estimated the steady-state concentration of CAA could reach 80 μ M [65]. Together, these studies suggest that localized production of CAA from ifosfamide in the kidney could contribute to the nephrotoxicity associated with ifosfamide therapy. The mechanism of CAA-induced neurotoxicity has been primarily attributed to glutathione depletion and therefore impaired detoxification of ifosfamide and its metabolites (reviewed in [66]). Neurotoxicity can last up to several days even following multiple doses of methylene blue [67,68]. Glutathione has been shown to decrease CAA- and acrolein-induced cell death in vitro [69,70] and attempts to increase glutathione levels clinically would most likely decrease the unwanted side effects from both of these metabolites; however, glutathione has been linked to detoxification/inactivation of cyclophosphamide and ifosfamide and their metabolites [71] and may impair antitumor activity.

Biological effects of acrolein and CAA

Several mechanisms by which acrolein and CAA result in cellular toxicities have been described [42,72–75]. In addition to being a metabolite of cyclophosphamide and ifosfamide, acrolein is a by-product of lipid peroxidation [76]. Studied as such, acrolein has been shown to induce expression of heme oxygenase-1, a cytoprotective enzyme, which can be blocked by tyrosine kinase inhibitors [72]. Acrolein has also been shown to result in neuronal cell death following a 100 μ M dose for 4 h, with apparent changes to the cytoskeletal structures [73].

Acrolein is also in tobacco smoke and can accumulate in vivo bound to proteins. The etiology and consequence of these acrolein-protein conjugates is under study. Both acrolein and CAA have been described to alter Ca^{2+} homeostasis [74] and disruption of mitochondria oxidative phosphorylation [75]. At concentrations as little as 10 μM , acrolein increases intracellular Ca^{2+} resulting in increased nitric oxide levels, which may explain the mechanism of how acrolein exposure results in apoptosis in human endothelial cells [77]. In vitro, it appears cellular death following acrolein and CAA is primarily by necrosis with only a small extent of death via apoptosis [69,73].

Acrolein and CAA have been shown to alkylate DNA [78–81]. Acrolein has been shown to form cyclic adducts between the N1 and exocyclic amino nitrogen of guanine in DNA [78, 79]. Fibroblasts from xeroderma pigmentosum patients (nucleotide-excision repair deficient) are more susceptible to acrolein-induced cytotoxicity compared to fibroblasts from normal patients, suggesting that nucleotide-excision repair is important in protecting against toxicity induced by acrolein [82]. CAA is reported to form two exocyclic guanine adducts, $N^2,3$ -ethenoguanine and $1,N^2$ -ethenoguanine [80,81,83]. Both base- and nucleotide-excision repair systems have been shown to be involved in repair of these CAA-induced adducts [83] and, in neuronal cells, it appears both base- and nucleotide-excision repair pathways are important in protecting against CAA-induced cell death [84]. A role for MGMT in protecting against acrolein and CAA has been proposed [42,45].

Role of MGMT in protecting against cyclophosphamide in vitro

Most cells grown in culture lack the P450 enzymes necessary for cyclophosphamide or ifosfamide activation; therefore, pre-activated forms of cyclophosphamide or ifosfamide are used including 4-hydroperoxycyclophosphamide (4-HC) and 4-hydroperoxyifosfamide (4-HI), respectively. Synthetic 4-HC and 4-HI provide facile access to the active metabolites of cyclophosphamide and ifosfamide, respectively (Scheme 1). In solution, the hydroperoxides spontaneously (non-enzymatic) produce the corresponding 4-hydroxy metabolites which then provide the aldehydic intermediates and, ultimately, acrolein and PM or IPM. Using CHO cell lines expressing high levels of MGMT and cell lines lacking MGMT, Cai *et al.* [45] demonstrated that the sensitivity of CHO cells expressing MGMT to the cytotoxic and mutagenic effects of 4-HC was lower than in CHO cells lacking MGMT. These studies further evaluated the metabolites of cyclophosphamide using authentic acrolein, PM and 4-hydroperoxydidechlorocyclophosphamide (4-HDC, a compound that mimics the chemistry of 4-HC but produces acrolein and a non-alkylating, non-toxic analog of PM ($^-\text{OP}(\text{O})(\text{NH}_2)\text{N}(\text{CH}_2\text{CH}_3)_2$) [85]). MGMT expressing CHO cells were protected against the cytotoxicity and mutagenicity of 4-HDC and acrolein, but not PM. Together, these experiments indicated that MGMT protects from toxicity and mutagenicity induced by acrolein but not PM. Consistent with a role for MGMT in cyclophosphamide resistance, Friedman *et al.* [42] reported incubation of purified recombinant MGMT with DNA previously reacted with 4-HC or acrolein, but not PM, resulted in decreased residual MGMT activity levels, suggesting that acrolein produces a DNA adduct that MGMT recognizes and repairs. Additionally, Preuss *et al.* [86] showed that MGMT proficient cells were protected against sister chromatid exchange induction, compared to MGMT deficient cells, following microsomally activated cyclophosphamide; however, this protection was only significant at higher doses of activated cyclophosphamide. Conversely, this same group found cells with high MGMT activity were not protected against cytotoxicity and sister chromatid exchanges induced by mafosfamide, an activated form of cyclophosphamide that produces mesna in addition to PM and acrolein [86]. The discrepancy between the two studies could possibly be that mafosfamide additionally produces mesna and reaction of mesna with acrolein would reduce the amount of acrolein available for reaction with DNA. These studies suggest that some portion of the cytotoxicity

and mutagenicity induced by cyclophosphamide is due to acrolein and the presence of MGMT may protect from that metabolite.

Proposed mechanism for role of MGMT in protecting from cyclophosphamide and ifosfamide-induced biological effects

The mechanism by which MGMT protects against acrolein is not understood. As MGMT is a DNA repair protein, MGMT could be recognizing and repairing an acrolein-induced DNA adduct. Acrolein has been shown to result in the cyclic adducts on guanine and MGMT may be involved in their removal; however, as described above, these adducts do not involve the O^6 -position of guanine and, therefore, are not classic substrates for MGMT. A possibility is that acrolein may react at the O^6 -position of guanine, producing an O^6 -propyl aldehyde moiety ($O\text{-CH}_2\text{CH}_2\text{CHO}$) or a hemiacetal ($O\text{-CH(OH)CH=CH}_2$), as suggested in Friedman *et al.* [42]. MGMT would most likely recognize and repair these potential adducts.

Acrolein is a highly reactive molecule and has been shown to react directly with nucleophilic sites in glutathione, mesna and other thiols such as cysteine containing proteins [87]; therefore, direct reaction of the acceptor site cysteine residue of MGMT with acrolein, i.e. scavenging, is a reasonable possibility particularly since the Cys¹⁴⁵ acceptor site of MGMT has a very low pK_a (4–5) and is very reactive [88]. MGMT activity levels are reduced in peripheral blood mononuclear cells from patients receiving cyclophosphamide treatment [89]. In addition, MGMT activity in cell lysates is reduced 60% following exposure to acrolein (although this was observed at concentrations greater than 3-fold above physiological concentrations of acrolein) [90]. Furthermore, purified recombinant MGMT incubated with increasing concentrations of acrolein for 2 h results in a dose dependent decrease in residual MGMT activity [89]. Approximately 60% and 10% MGMT activity remained following incubation with physiological achievable concentrations of 10 and 100 μM acrolein, respectively [89]. These studies suggest that acrolein can lead to a reduction in MGMT activity likely by direct reaction with MGMT; however, whether direct reaction of acrolein with MGMT is a mechanism by which MGMT protects cells from acrolein toxicity has not been examined. In studies presented below, we determined whether direct reaction of MGMT with acrolein is the likely mechanism.

MGMT protects from acrolein and CAA toxicity in vitro

To determine whether CAA and/or acrolein reacted directly with the active site cysteine of MGMT, we treated CHO cells expressing an MGMT mutant containing an alteration at a crucial amino acid required for DNA binding, MGMT^{R128A} [91]. MGMT^{R128A} has > 1,000-fold reduced repair activity, compared to wtMGMT, towards a methylated DNA substrate, while retaining its functional active site and the capacity to react with BG [91,92]. Due to the fact that MGMT^{R128A} lacks the ability to repair O^6 -alkylguanine DNA lesions, we probed the transfected lines with MGMT monoclonal antibody to verify the presence of MGMT protein (Figure 1). MGMT protein was detected in wtMGMT and MGMT^{R128A} transfected cells, while MGMT could not be detected in 50 μg of whole cell lysate from pcDNA3 (vector). MGMT activity (as measured using a methylated DNA substrate) was observed in wtMGMT transfected cells (931 fmol/mg total protein) while activity in CHO cells transfected with vector or MGMT^{R128A} were below detectable levels (Figure 1). Thus, MGMT^{R128A} expressed protein was unable to repair methyl groups from O^6 -methylguanine within a DNA substrate.

Using a colony-forming assay, we determined the ability of wtMGMT and MGMT^{R128A} to protect from increasing physiological levels of acrolein. As seen in Figure 2, it was confirmed that wtMGMT provides significant protection from acrolein-induced cytotoxicity at 20 μM compared to vector expressing cells ($p < 0.05$). At this dose, the percentage of cell survival of

wtMGMT cells was 25% compared to 11% in pcDNA3 cells. This is consistent with previously published results [45]. To determine whether DNA binding of MGMT is required for this protection, cytotoxicity assays were also conducted on CHO cells expressing MGMT^{R128A}. Percent cell survival in MGMT^{R128A} at 20 μ M acrolein (12.5%) was similar to that observed in vector expressing cells (Figure 2). While wtMGMT expressing cells appear slightly protected at 10 and 15 μ M acrolein, this increase in percent survival is not statistically significant from CHO cells expressing pcDNA3 or MGMT^{R128A}.

We also determined the ability of wtMGMT and MGMT^{R128A} to protect from cytotoxicity induced by CAA. Doses of CAA used have been observed physiologically [57]. Following exposure of cells to 12 μ M CAA, percent survival was significantly greater in wtMGMT expressing cells (44%) compared to MGMT^{R128A} (27%) and pcDNA3 (31%) expressing cells ($p < 0.03$ and $p < 0.003$ when wtMGMT was compared to MGMT^{R128A} and pcDNA3, respectively) (Figure 3). Percent survival of CHO cells expressing wtMGMT (13%) following treatment with 24 μ M CAA was also significantly higher compared to survival in MGMT^{R128A} (2%) and pcDNA3 (4%) (both $p < 0.01$). These results are the first to demonstrate that MGMT protects against CAA toxicity in vitro.

Our results show survival of CHO-MGMT^{R128A} cells was similar to vector expressing cells following exposure to acrolein or CAA, demonstrating that DNA binding of MGMT is required to protect against both metabolites. This is also suggestive that the mechanism by which MGMT provides cytotoxic protection is not via a scavenging mechanism. Therefore, the most likely explanation is that MGMT is protecting against acrolein/CAA cytotoxicity by recognizing and repairing a DNA adduct(s). Whether this acrolein or CAA adduct recognized by MGMT is an *O*⁶-guanine adduct, a previously reported *N*-alkylguanine adduct or a yet to be identified DNA adduct is not known. Regardless, these data support a role for MGMT in protecting from acrolein- and CAA-induced cytotoxicity.

The ability of MGMT to protect from CAA has clinical implications, as CAA is associated with neuro- and nephrotoxicity observed following exposure to ifosfamide [57–60]. CAA is produced to a much greater extent following treatment with ifosfamide as compared to cyclophosphamide, as up to 50% of ifosfamide undergoes *N*-dechloroethylation to produce CAA. Our results are the first to suggest that MGMT may protect against neuro- and nephrotoxicity following ifosfamide treatment. There is a report that CAA may contribute to the antitumor effects of ifosfamide in xenograft models [93]; therefore, MGMT may also play a role in resistance against ifosfamide therapy by reducing the contribution of CAA to the antitumor effect of ifosfamide. Whether ifosfamide would result in greater antitumor effect, enhance unwanted side effects, or both, in the absence of MGMT is not yet clear.

Toxicity of cyclophosphamide, acrolein and CAA in *Mgmt* $-/-$ mice

Studies presented previously and here suggest that MGMT protects against toxic lesions introduced by acrolein as well as CAA in cells. We expanded our studies to determine whether MGMT deficiency results in increased toxicities and death following cyclophosphamide, acrolein or CAA in mice. *Mgmt* $-/-$ mice were not more sensitive to death caused by cyclophosphamide compared to wild type mice [94]. This result was not entirely unexpected as the majority of the toxicity associated with cyclophosphamide has been attributed to PM and we showed previously that the cytotoxicity of PM in vitro was unaffected by MGMT activity [45]. Prior to the work described herein, the role of MGMT in protecting against toxicity induced by acrolein and CAA in *Mgmt* $-/-$ mice had not been determined. Thus, we determined the toxic effects of the maximum tolerated dose (MTD) of acrolein and CAA (determined in *Mgmt* $+/+$) in *Mgmt* $-/-$ mice. The toxic effects of cyclophosphamide, acrolein or CAA, as determined by weight change and animal death, were similar in *Mgmt* $+/+$ and

Mgmt^{-/-} mice (Figure 4). This is in contrast to the dramatic dose reductions required following methylating agents (streptozocin and temozolomide) and the chloroethylating agent BCNU in *Mgmt*^{-/-} mice compared to *Mgmt*^{+/+} mice [95]. These data suggest that MGMT deficiency does not result in an increase in acute cyclophosphamide related toxicities and possibly indicates that MGMT expression does not greatly impact the toxicities associated with cyclophosphamide.

Conclusion

The role of MGMT in cyclophosphamide antitumor effect and toxicity has remained elusive. Clinical studies that suggest a role for MGMT have included multiple drug regimens, making it difficult to deduce the role of MGMT in response to cyclophosphamide treatment. In the current studies performed in cell culture, we establish that MGMT significantly protects against CAA-induced cytotoxicity and confirm that MGMT protects from acrolein-induced cytotoxicity. We also present data that shows the importance of DNA binding of MGMT in providing protection against these metabolites. These results suggest that scavenging of acrolein or CAA by MGMT is not a likely mechanism by which MGMT protects cells, a possibility that had not been ruled out until now. Interestingly, MGMT deficiency does not result in a significant increase in toxicities in vivo, as the MTD of cyclophosphamide, acrolein or CAA were essentially equivalent in *Mgmt*^{+/+} and *Mgmt*^{-/-} mice, suggesting that the role of MGMT in vivo may be less important. Other mechanisms of resistance to cyclophosphamide, such as high levels of aldehyde dehydrogenase, glutathione levels and DNA repair mechanisms other than MGMT are current targets of manipulation to reduce tumor resistance to cyclophosphamide and other oxazaphosphorines [4].

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Abbreviations

BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
MGMT	<i>O</i> ⁶ -methylguanine-DNA methyltransferase
PM	phosphoramidate mustard
IPM	

	isophosphoramidate mustard
CAA	chloroacetaldehyde
4-HC	4-hydroperoxycyclophosphamide
4-HI	4-hydroperoxyifosfamide
4-HDC	4-hydroperoxydidechlorocyclophosphamide
MTD	maximum tolerated dose

Figure 1. MGMT expression and activity in transfected CHO lines

MGMT protein expression was determined on 50 μ g of whole cell lysates prepared from transfected CHO lines via Western blot analysis. Lane 1, HT29; lane 2, CHO-pcDNA3; lane 3, CHO-MGMT^{R128A}; lane 4, CHO-wtMGMT. MGMT activity (fmol/mg total protein) was also determined on whole cell lysates. Lysates from HT29 cells were used as positive controls.

Figure 2. Effect of MGMT and MGMT^{R128A} on cytotoxicity induced by acrolein
 CHO lines transfected with pcDNA3, MGMT^{R128A}, or wtMGMT were treated with acrolein in serum-free media for 4 h. Media was replaced with media containing serum. After 16 h, cytotoxicity was measured as colony-forming ability 9–12 days after treatment. The data represents the mean of three or more separate experiments (except for cytotoxicity determined at 15 μM). Bars are standard deviation. * indicates $p < 0.05$.

Figure 3. Effect of MGMT and MGMT^{R128A} on cytotoxicity induced by CAA
 CHO lines transfected with pcDNA3, MGMT^{R128A}, or wtMGMT were treated with 12 or 24 μM CAA in serum-free media. After 4 h drug incubation, the media was replaced with media containing serum. After 16 h, cytotoxicity was measured as colony-forming ability 9–12 days after treatment. The data represents the mean of three or more separate experiments. Bars are standard deviation. ***, ** and * indicate $p < 0.003$, $p < 0.01$ and $p < 0.03$, respectively.

Figure 4. Weight change and deaths in *Mgmt* +/+ and -/- mice within 14 days following a single intraperitoneal injection with vehicle (0.9% NaCl), cyclophosphamide, acrolein or CAA
 Each bar represents mean weight change relative to pre-injection for at least 3 mice/group.
 *Represents death(s) in a treatment group, specifically, 1/6 and 4/6 *Mgmt* +/+ mice died in the 275 and 300 mg/kg cyclophosphamide group, respectively as well as 2/7 and 1/8 mice died in *Mgmt* +/+ and *Mgmt* -/- mice, respectively, treated with 20 mg/kg CAA.