Increased expression of the MGMT repair protein mediated by cysteine prodrugs and chemopreventative natural products in human lymphocytes and tumor cell lines

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**O**-Methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein which protects the cellular genome and critical oncogenic genes from the mutagenic action of endogenous and exogenous alkylating agents. An expedited elimination of **O**-alkylguanines by increasing MGMT activity levels is likely to be a successful chemoprevention strategy. Here, we report for the first time that cysteine/glutathione enhancing drugs and certain plant antioxidants possess the ability to increase human MGMT expression beyond its steady-state levels that may afford protection. The non-toxic cysteine prodrugs, 2-oxothiazolidine-4-carboxylic acid (OTC) and N-acetyl-L-cysteine (NAC), metabolized, respectively by 5-oxoprolinase and acylases, increased the MGMT protein and its repair activity levels in a dose- and time-dependent manner in several cancer cell lines and peripheral blood lymphocytes with a maximum of 3-fold increase by 72 h. The natural antioxidants, namely, curcumin, silymarin, sulforaphane and resveratrol were also effective in raising the MGMT levels to different extents. Among the synthetic agents, olitpaz and N-(4-hydroxyphenyl) retinamide (4-HPR) also increased MGMT expression, albeit to a lesser extent. Augmented mRNA levels accounted at least, in part, for the increased activity of MGMT in this setting. However, evidence from cysteine/methionine deprivation, acivicin treatment, and protein synthesis measurements in OTC-treated cells suggested that an increased cysteine flux also contributed significantly to enhanced MGMT expression. Many of these treatments increased the glutathione S-transferase-P1 (GSTP1) levels as well. These findings raise the possibility of MGMT-targeted chemoprevention strategies through dietary supplementation of OTC and herbal antioxidants. Further, the studies reveal the antioxidant responsiveness of the human MGMT gene.

Introduction

Human genome is constantly exposed to complex alkylating carcinogens and electrophilic compounds that can evoke mutagenic, recombinogenic, toxic, and teratogenic effects unless the critical lesions are effectively repaired (1). These structurally diverse group of compounds that cause direct damage to DNA may originate from exogenous (dietary, environmental and therapeutic) or endogenous sources. For example, high levels of DNA-reactive heterocyclic amines, nitrosoamines and polyaromatic hydrocarbons are present in cooked meat (2,3) and tobacco smoke (4). The tobacco-specific nitrosoamines 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosomorornicotine are potent carcinogens in animal models and humans (5). Several anticancer drugs alkylate the chromatin, and introduce mutagenic adducts and DNA cross links to elicit their cytotoxic effects (6), through the reactions similar to those performed by the dietary and environmental alkylators. Alkylation of DNA can occur at various sites, however, the **O**'-alkylguanines, which account for ~8–10% of total alkylations bear strong mutagenic potential (7). This is because **O**'-alkylguanines exhibit distorted base pairing characteristics in pairing with thymine, thereby, resulting in G:C to A:T transitions upon DNA replication (8). S-adenosylmethionine, the endogenous methyl group donor has been estimated to generate 10–30 **O**'-methylguanine residues per day in a mammalian cell (9). Other endogenous methylaing agents may arise from nitrosation of amino acids or bile acids; betaine and choline are other sources (10). Consistent with these estimates, low, but measurable amounts of **O**'-methylguanine have been found in the DNA of both normal and tumor tissues (11).

Cellular repair of **O**'-alkylthymine, **O**'-methylguanine and **O**'-alkylguanines in DNA including the tobacco-specific carcinogen, **O**'-pyridylloxobutyylguanine is mediated by **O**'-methylguanine-DNA methyltransferase (MGMT) in human tissues (1,12). MGMT remains the first line defense against alkylation damage of DNA. In MGMT-deficient cells, **O**'-alkylguanines are processed by the mismatch repair system to trigger genotoxicity and/or apoptosis (12). This protein functions by a unique stoichiometric and suicidal reaction mechanism in which the alkyl groups bound to the **O**'-position of guanine are transferred to a cysteine in its active site, resulting in the direct restoration of the normal base and self-inactivation of MGMT protein (1,12,13). MGMT, which is highly expressed in human cancers is also a central determinant of tumor resistance to many clinically used anticancer alkylating agents, because the **O**'-methylguanine and **O**'-chloroethylguanine lesions induced by the methylating (temozolomide, dacarbazine and procarbazine) and chloroethylyating (BCNU and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea) agents, respectively, are excellent substrates for MGMT (14). In the case of bifunctional alkylators like BCNU, the removal of chloroethyl adducts by...
MGMT prevents the production of cytotoxic DNA interstrand cross links (14). Therefore, inhibition of MGMT by powerful pseudosubstrates such as \(O^6\)-benzyguanine (BG) has emerged as a prominent strategy to enhance the antitumor activity of alkylating agents (15).

The literature on MGMT’s role in preventing carcinogenesis and oncogenic mutations is vast and has been extensively reviewed (12–14, 16, 17). Briefly, mice over-expressing the MGMT gene display significant reduction of spontaneous tumors and marked resistance for developing cancers (brain tumors, lymphomas and liver cancers) after alkylator treatments (18–20). Animals and mouse lines deficient in MGMT gene, which were established by gene targeting, exhibit increased sensitivity to alkylating carcinogens (18). Reduced MGMT activity or silencing of the MGMT gene through promoter methylation in human cancers is associated with frequent mutations in \(K\)-ras, \(p53\) and \(\beta\)-catenin genes (21–24). In contrast, reductions of oncogenic mutations in cells with MGMT over-expression have been reported (25, 26). Further, several studies have demonstrated significant associations between MGMT genotype and lung cancer risk among cigarette smokers and those exposed to second-hand smoke (27–29). Increased prevalence of risk among cigarette smokers and those exposed to second-hand smoke (27–29). Increased prevalence of risk among cigarette smokers and those exposed to second-hand smoke (27–29). Increased prevalence of risk among cigarette smokers and those exposed to second-hand smoke (27–29). Increased prevalence of risk among cigarette smokers and those exposed to second-hand smoke (27–29). Increased prevalence of risk among cigarette smokers and those exposed to second-hand smoke (27–29).

Despite these well-established experimental and etiological data on the protection endowed on the human genome, MGMT has received little attention as a target for cancer prevention. Further, recent studies have highlighted an important role for MGMT in chemoprotection. Although the BG and subsequent alkylator combinations increase the antitumor efficacy significantly, the MGMT depletion in rapidly proliferating tissues, particularly, the hematopoietic system, results in severe myelosuppression. This problem has necessitated a gene therapy approach involving the transfer of BG-resistant MGMT genes (G156A and P140K) into hematopoietic stem cells and make the bone marrow resistant to alkylators (14, 33). Moreover, cancer patients treated with alkylating agents are at significant risk for developing secondary malignancies (34). Therefore, availability of dietary and non-toxic synthetic compounds that enhance MGMT expression for chemoprevention and chemoprotection is highly desirable. Because a deficiency of cysteine and/or methionine have been shown down-regulate MGMT expression and lead to increased toxicities by alkylating agents (35, 36), we surmised that cysteine or glutathione augmenting drugs may promote cellular MGMT activity. Consequently, we choose L-2-oxothiazolidine-4-carboxylic acid (OTC), which is metabolized to cysteine by \(5\)-oxoprolinase (37), an essential enzyme in the \(\gamma\)-glutamyl cycle, and \(N\)-acetyl-L-cysteine (NAC), degraded by cellular acylases to cysteine for these studies; both these compounds have been demonstrated to be effective and non-toxic cysteine delivery agents (38, 39). Numerous phytochemicals such as the curcumin, silymarin, sulforaphane and others which possess established antioxidant effects and abilities to increase enzyme activities in phase-II metabolism (40) were also tested. Fenretinide (4-HPR) and olitraz, the two agents currently undergoing chemoprevention trials (40) were also included in this group. We report a significant elevation of MGMT activity in human lymphocytes and cancer cell lines by many of these compounds, and show that the MGMT gene may be modulated at both transcriptional and translational levels by these treatments.

### Materials and methods

#### Chemopreventive agents

1L(-)2-Oxothiazolidine-4-carboxylic acid (OTC, 97% purity) was purchased from Sigma Chemicals (St Louis, MO). The following chemopreventive agents were purchased from the LKT Laboratories (St Paul, MN): Curcumin (diferuloylmethane), silymarin, N-acetyl-L-cysteine (NAC), 6-[N-(4-hydroxyphenyl) retinamide, fenretidine], acivicin (2-amino-2-(3-chloro-4,5-dihydrooxazol-5-yl)-acetic acid), N-acetyl-L-cysteine (NAC), S-(N-3-phenylpropylthiocarbamoyl)-L-cysteine (PPTC), S-allyl-L-cysteine (SAC), and epigallocatechin gallate (EGP). All other chemicals and biochemicals used were of highest grade and obtained from Sigma Chemical Co.

#### Cell lines and their culture

The human medulloblastoma cell line UW228 was established and provided by Dr Francis Ali-Osman (Duke University, Durham, NC, USA). The human colon carcinoma cell lines, HT29 and HCT116 and T98G glioblastoma were purchased from the American Type Culture Collection. All cell lines were cultured in Dulbecco’s Modified Eagles Medium (DMEM) in the presence of antibiotics and 10% bovine serum in humidified atmosphere containing 5% CO2 at 37°C. In some experiments, cells were grown in methionine, cysteine-deficient DMEFM (MP Biomedicals, Costa Mesa, CA) for testing the effect of these amino acids on MGMT expression.

#### Isolation of human peripheral blood lymphocytes (HPBLs)

Buffalo coat preparations from healthy volunteers were purchased from the local blood bank. They were fractionated by Histopaque-1077 (Sigma) gradient centrifugation according to the manufacturer’s instructions for isolating HPBLs. The cells were washed twice with RPMI-1640 medium, suspended in the same medium containing 5% serum at 5x10⁶ cells/ml, and cultured for 5 h prior to the addition of compounds. HPBLs were not stimulated with a mitogen.

#### Assay of MGMT activity

MGMT activity was measured by the transfer of \(^1\)H-labeled methyl groups from the \(O^6\)-position of guanine in DNA to the MGMT protein as described previously (41). Briefly, cell-free extracts were prepared by sonication in MGMT assay buffer (40 mM Tris–HCl, pH 8.0, 5% glycerol, 1 mM EDTA, 20 \(\mu\)M spermidine and 0.5 mM DTT) followed by centrifugation at 10000 g for 10 min. The extracts (50-200 \(\mu\)g of protein) were supplemented with \(^1\)H DNA enriched for \(O^6\)-methylguanine (3 \(mg\) 10 000 c.p.m.), and incubated for 30 min at 37°C. The reactions were quantitated after acid hydrolysis of the DNA substrate and the collection of protein precipitates and counting of radioactivity. The activity in the linear portion of the curve was used to calculate the specific activity (pmol CH3 groups removed/mg protein).

#### GSTP1 assay

GSTP1 enzyme activity was determined in 100 mM potassium phosphate buffer (pH 6.5) containing 1 mM EDTA, 1 mM GSH and 1 mM chlorodinitrobenzene (CDNB) in a final volume 1 ml using the well-established spectrophotometric assay (42). The reactions were initiated by addition of control or treated cell extracts (100–200 \(\mu\)g) and changes in absorbance at 340 nm were monitored over two minutes in a Labomed spectrophotometer. The rates of the spontaneous reactions of GSH with CDNB, determined with reaction mixtures in which the cell extract was replaced with buffer were subtracted from the rates of the enzyme catalyzed reactions. The linear part of the reaction kinetics was used to calculate enzyme activity taking the extinction coefficient of the CDNB-GSH conjugate as 9.6 mM.

#### Western blotting

Following trypsinization, the cell pellets were washed with PBS, and cell extracts were prepared by sonication in 50 mM Tris–HCl buffer (pH 8.0) containing 1% glycerol, 1 mM EDTA, 0.5 mM PMSF and 2 mM benzamidine followed by centrifugation. Equal protein amounts (40 \(\mu\)g) were electrophoresed on 12% reducing SDS–polyacrylamide gels. Proteins were electrotransferred to Immobilon-P membranes. The membranes were blocked with 3% non-fat dry milk in Tris-buffered saline (TBS; pH 8.0)
containing 0.1% Tween-20 for 5 h, and subsequently incubated with mouse anti-MGMT monoclonal antibody (Chemicon International, Temecula, CA) or GSTP1 rabbit polyclonal antibodies (EMD Biosciences, San Diego, CA), or polyclonal antibodies to metallothionein-I (Cayman Chemicals, Ann Arbor, MI) at 1 μg/ml. Antigen–antibody complexes were visualized by enhanced chemiluminescence. In most cases, the blots were reprobed with antibodies to β-actin to assess equal protein loading. Band intensities were quantitated using a VersaDoc Imaging system (Bio-Rad, Richmond, CA).

RNA isolation and RT–PCR
Total RNA was isolated from human tumor cells and lymphocytes using the RNA-Zol reagent (Sigma) by standard procedures. The integrity of RNA was verified by electrophoresis on 1% non-formaldehyde agarose gels at pH 8.5. For RT–PCR, 1 μg RNA was reverse-transcribed using Access Quick RT–PCR kit and MuLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer’s instructions. The cDNA obtained was amplified using Taq DNA polymerase using the following primers. MGMT, sense 5’-CCTTGGTACTTAAGCTTATGGACAAG and antisense 5’-CTAC- TGCACGAATTTCAG; actin, sense 5’-ACGATGGAGGG. PCR involved 5 min of denaturation at 94°C, followed by 34 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min (45) using a Biometra thermal cycle. The amplified DNA sizes were 800 bp for MGMT and 650 bp for actin. The PCR products were analyzed by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining under UV light.

Determination of cellular cysteine levels and quantitation of oxoprolinase activity
Quantitation of cysteine levels in OTC-treated tumor cells was accomplished by a specific ninhydrin-based colorimetric assay (44, 45). Aliquots of perchloric acid supernatants from cells were mixed with equal volumes of ninhydrin color mix (2.5% dissolved in acetic acid and con. HCl, 24:16 vol/vol). The mixtures after heating at 100°C for 10 min were cooled, mixed with 0.5 ml of ethanol to stabilize the pink color followed by their reading at 566 nm. A standard curve generated for cysteine (5–100 nmols) under the same conditions was used for calibration. For oxoprolinase assays, cell-free extracts (prepared in 50 mM Tris–HCl/pH 8.0, 1 mM DTT, 0.5 mM EDTA, and 0.5 mM PMSF) were supplemented with 1 mM ATP and 5 mM OTC, and the reactions incubated at 37°C for 30 min (45). The cysteine generated in these reactions was measured by the colorimetric assay described above. The pink color generated with cell extracts alone served as the blanks.

Determination of rate of MGMT protein synthesis in OTC-treated tumor cells
HT29 cells in their exponential phase of growth were exposed to 10 mM OTC for 48 h, and subsequently incubated in leucine-free medium containing 0.1% Tween-20 for 5 h, and subsequently incubated with mouse anti-MGMT monoclonal antibody (Chemicon International, Temecula, CA) or GSTP1 rabbit polyclonal antibodies (EMD Biosciences, San Diego, CA), or polyclonal antibodies to metallothionein-I (Cayman Chemicals, Ann Arbor, MI) at 1 μg/ml. Antigen–antibody complexes were visualized by enhanced chemiluminescence. In most cases, the blots were reprobed with antibodies to β-actin to assess equal protein loading. Band intensities were quantitated using a VersaDoc Imaging system (Bio-Rad, Richmond, CA).

Results
To determine and validate the influence of cysteine delivery agents, natural and synthetic chemopreventative agents on MGMT, much of this study used well characterized human colon cancer (HT29, HCT116) and brain tumor (UW228, T98G) cell lines, which are MGMT-proficient and possess a robust metabolic system for glutathione synthesis and appropriate antioxidant responses to psychochemicals (46). Initial experiments explored the metabolism of OTC, demonstration of increased intracellular cysteine levels followed by studies of dose and time-dependent changes in MGMT activity.

Ability of OTC to increase the levels of cysteine and MGMT activity in HT29 and UW228 cell lines
5-Oxoprolinase (OPase) which catalyzes the conversion of oxoproline to glutamic acid in the γ-glutamyl cycle also accepts OTC, a thiazolidine analog of oxoproline as a substrate to generate cysteine (37). First, the presence of 5-oxoprolinase activity in HT29 and UW228 cells was verified by incubating the cell free extracts with OTC and determining the production of cysteine (45). HT29 cells showed ~1.7-fold higher levels of OPase activity than UW228 cells, and OPase levels remained constant when these cells were incubated in the presence of 10 mM OTC (Figure 1A). This treatment led to an increase in intracellular cysteine content with maximal levels reached as early as 12 h, and it remained at similar levels at 72 h of continuous incubation (Figure 1B). The level of cysteine enhancement achieved was roughly proportional to the oxoprolinase content in these cell lines. After validating the cysteine production from OTC, we tested the hypothesis that greater availability of the thiol amino acid may drive the synthesis of cysteine-rich and cysteine-sufficient proteins including the MGMT. As a first step, the UW228 and HT29 cells were exposed to different OTC concentrations (5–25 mM) for 24 h and the DNA repair activity of MGMT was measured. Figure 1C shows that MGMT activity was slightly increased, with a highest 1.6–fold augmentation occurring at 10 mM OTC. Higher OTC amounts reduced the MGMT activity which appears to be related to elevated GSH content (K.S. Srivenugopal unpublished results).

Dose-dependent up-regulation in MGMT activity induced by N-acetylcysteine and plant antioxidants in human cancer cell lines
Next, the UW228 and HT29 cells were exposed for 24 h to varying doses of NAC (another widely used cysteine prodrug), curcumin, and silymarin, the chemopreventative compounds well known to modulate glutathione metabolism (40), and alterations of MGMT activity determined. NAC was effective in enhancing the MGMT activity, with a 3-fold increase at an optimal concentration of 7.5 mM (Figure 2A). Both curcumin and silymarin also raised the MGMT activity consistently and reproducibly in tumor cells, with a 1.6-fold increase at 20 μM after 24 h (Figure 2B and C). All the four compounds (OTC, NAC, curcumin, silymarin) inhibited the activity of purified recombinant MGMT (Figure 2D), suggesting that intracellular metabolism and/or signaling events triggered by the compounds, and not their direct interaction was responsible for MGMT activation in tumor cells.

Kinetics of enhancement in MGMT activity by OTC, NAC, curcumin and silymarin in colon cancer and brain tumor cell lines
The extent and duration of MGMT activation by the chemopreventative compounds were examined in two each of colon cancer (HT29, HCT116) and brain tumor (UW228, T98G) cell lines. Cells were exposed at concentrations which elicited maximal increase of MGMT activity, and the dealkylation reaction was determined at 12, 24, 48 and 72 h. The rank order of MGMT content (fmols/mg protein) in untreated tumor cells was HT29 > HCT116 > UW228 > T98G. The relative increase in MGMT activity by the compounds compared to the untreated controls is shown in Figure 3A–D. OTC enhanced the MGMT levels in a time-dependent manner.
reaching a 2.1-fold increase at 72 h in HT29 and UW228 cells (Figure 3A). In contrast, the other cysteine prodrug NAC regulated MGMT expression differently, with a spurt in activity at 24 h (3-fold in HT29 and HCT116 cells) followed by its decline at later times (Figure 3B). Curcumin and silymarin, which respectively possess anti-inflammatory and hepatoprotective properties, induced the MGMT activity in a linear fashion with a 2.6-fold increase at 72 h (Figure 3C and D). No changes in cell morphology or signs of cell death were evident during the 3-day incubation with the compounds.

Fig. 1. Detection of OPase activity, and OTC-induced increases in cysteine content and MGMT activity levels in HT29 and UW228 tumor cells. (A) Tumor cell monolayers were exposed to 10 mM OTC for 12–72 h. Oxoprolinase activity in cell extracts was measured using OTC as the substrate. (B) Intracellular cysteine levels were measured in acid supernatants from cells treated with 10 mM OTC. C, control. (C) Effect of OTC dose on MGMT activity. HT29 and UW228 cells were treated for 24 h with OTC at 5–25 mM concentrations. MGMT activity was determined as described in Materials and methods. Results shown are means ± SD from four independent experiments. *, Significant at P < 0.05.

Fig. 2. Dose dependent influence of NAC, curcumin and silymarin on MGMT activity in human cancer cell lines. HT29, HCT116 and UW228 cells were used as indicated in the inset of this figure. Tumor cells were exposed to NAC (A), curcumin (B) or silymarin (C) at concentration ranges shown for 24 h followed by determination of the DNA repair activity of MGMT. At least three separate experiments were performed to obtain the data shown (means ± SD). *, Significant at P < 0.05. (D) Effect of cysteine prodrugs and chemopreventative compounds on the activity of recombinant MGMT (rMGMT) protein. Purified human rMGMT protein of 200 ng (of the ref. 37) was incubated either alone or with NAC (7.5 mM), OTC (10 mM), silymarin (20 μM) and curcumin (20 μM) for 20 min at 37°C, and the DNA repair activity was measured.
These data demonstrate that compounds with dissimilar structures and mechanisms have the ability to up-regulate and maintain the MGMT activity at levels compatible for chemoprevention.

**Alterations in MGMT protein expression induced by cysteine prodrugs, curcumin and silymarin in HT29 colon cancer cells**

Human MGMT functions by a stoichiometric reaction mechanism (1,13), and posttranslational mechanisms such as the phosphorylation inhibits its activity (41). Therefore, to investigate whether the changes induced in MGMT activity were accompanied by corresponding increases in its protein levels, western blot analysis was performed at 12, 24, 48 and 72 h after incubation of different cell lines with the cysteine prodrugs, curcumin and silymarin. The effect of some naturally occurring cysteine conjugates was also tested. The resulting immunoblots are shown in Figure 4A–E. It is clear that most agents increased the MGMT protein levels in a gradual and time-dependent kinetics with the highest levels accumulating at 72 h. In general, the kinetics of MGMT protein accumulation observed in the western blots correlated closely with the time course changes seen for MGMT activity (Figures 3A–D). Thus, while the progressive increase of MGMT activity seen after OTC in different cell lines (Figure 3A) was consistent with the steady enhancement of MGMT protein by this compound over 12–72 h (Figure 4A), the initial increment of MGMT activity at 24 h and its reduction at 48 and 72 h in NAC-treated cells (Figure 3B) was also well reflected in the immunoblots (Figure 4B). In contrast to the agents which generated cysteine in-situ, other cysteine conjugates (found in plants) such as the S-allyl-L-cysteine (SAC), phenylpropylthiocarbamoyl-L-cysteine (PPTC), or cysteine, itself, were unable to increase MGMT expression (Figure 4C); these data suggest that free cysteine or its metabolism to GSH mediated the cellular augmentation of MGMT. The kinetics of MGMT protein accumulation observed in silymarin and curcumin (Figure 4D and E) treated cells also agreed with their effects on MGMT activity (Figure 3C and D). Taken together, the immunoblot analyses confirmed the dose- and time-dependent induction of MGMT by cysteine prodrugs and antioxidant plant compounds.

**Increased levels of MGMT mRNA in HT29 cells treated with cysteine prodrugs and natural products**

To test whether the increased MGMT expression resulted from enhanced gene transcription, we performed RT–PCR in HT29 cells after 24, 48 and 72 h exposure to OTC, NAC, curcumin and silymarin at their optimal concentrations. Figure 5A and B show, respectively the abundance of MGMT gene transcripts and their quantitation by densitometry. All the four compounds increased the MGMT mRNA above control levels. In the case of OTC and curcumin, 2- and 1.5-fold increases were attained at 24 h, which were maintained at later periods. However, in NAC-treated cells, the transcripts rose 2.5-fold at 24 h followed by their decline, which mirrored the alterations in MGMT activity and protein levels induced by this compound. Silymarin, however, induced a steady-increase of MGMT mRNA. Collectively, these findings indicate that at least part of the MGMT upregulation by the different compounds occurred at the transcriptional level. However, a translational regulation also appears to contribute to enhanced MGMT expression as described in a later section.

**Marginal augmentation of MGMT expression by other plant antioxidants, fenretidine (4-HPR) and oltipraz in tumor cell lines**

Encouraged by the above observations, we exposed the HT29 and UW228 cells to other phytochemical antioxidant and...
synthetic chemopreventatives including 4-HPR and oltipraz, at concentration ranges used in literature and determined changes in MGMT activity. Among these, sulforaphane, resveratrol, geldanamycin (chaperone-binding drug), silybin (the main component of silymarin), and 4-HPR caused a marginal 1.3–1.4-fold increase in MGMT activity (Figure 6A). Western blot analysis in HT29 cells treated for 24 h with these compounds (at 5 and 10 μM) confirmed the low-levels of MGMT induction; interestingly, other compounds such as the ellagic acid, β-carotene, EPG and kahweol acetate, the latter two present in green tea and coffee, respectively, did not promote MGMT induction, and even led to a decrease beyond control levels (Figure 6B).

Oltipraz, a substituted dithiolthione, is a well known inducer of phase-II enzymes including the GST, NADPH-quinone reductase, and UDP-glucuronyltransferases, and is structurally related to chemicals found in cruciferous vegetables (47). Therefore, we compared the induction of GST and MGMT activities by oltipraz in HT29 and HCT116 cells. The results show that oltipraz can increase MGMT activity (1.3–1.5-fold), roughly, two times lower than the GSTP1 under identical conditions (Figure 6C). Western analysis showed that oltipraz altered these proteins with different kinetics, with MGMT peaking early (at 24 h) and then declining, whereas the GSTP1 increasing gradually (Figure 6D). Overall, these results suggest for the first time that the MGMT gene is responsive to antioxidant chemicals, either synthetic or those found in higher plants.

**Ability of OTC to enhance GSTP1 expression in tumor cell lines**

GSTP1, a crucial player in cellular adaptation to oxidative stress, is induced by many chemopreventative compounds (48). Because alterations in glutathione levels are known to modulate GSTP1, we examined the changes in its expression in tumor cells treated with OTC and phytochemicals. Figure 7A and B depict the changes in GST activity and GSTP1 protein levels, respectively in OTC-treated HT29 and

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Fig. 4. Kinetics of alterations in MGMT protein expression in human cancer cells by cysteine prodrugs, cysteine conjugates, and chemopreventative phytochemicals. The UW228, HT29, T98G and HCT116 cells were continuously exposed to (A) 10 mM OTC, (B) 7.5 mM NAC, (D) 20 μM curcumin, and (E) 20 μM silymarin for 12–72 h. In panel C, HT29 cells were treated for 24 h with 10 μM S-(N-3-phenylpropyliodocarbamoyl)-t-cysteine (PPTC), 7.5 mM NAC, 5 mM S-allyl-l-cysteine (SAC), or 5 mM l-cysteine. At each time point, 60 μg protein amounts were resolved 12% SDS–PAGE, and processed for western blot analyses using a monoclonal antibody for MGMT as described in methods. Subsequently, the membranes were stripped and probed with a monoclonal antibody to actin to assess equal protein loading.

Fig. 5. RT–PCR analysis of MGMT mRNA levels in HT29 cells after chemopreventative treatments. Tumor cells were exposed to 10 mM OTC or 7.5 mM NAC or 20 μM curcumin or 25 μM silymarin for 12–72 h. Total RNA isolated from these cells was subjected to quantitative RT–PCR as described in methods. Panel A displays the ethidium bromide staining pattern of PCR products representing the abundance of mRNA for MGMT. The densitometric quantitation of changes in MGMT mRNA levels is shown in panel B. The kinetics of changes in MGMT gene transcripts in other cell lines was similar.
HCT116 cells. It is clear that OTC up-regulated the GST expression significantly, ~2-fold, in a manner similar to MGMT. Silymarin, and to a lesser extent, curcumin also induced the GST activity with a ~2-fold increase (not shown). These data highlight the multiple benefits of chemopreventative agents used in this study in decreasing the genomic DNA damage.

Positive regulation of MGMT and GSTP1 expression by the cysteine prodrug OTC, curcumin, silymarin and other compounds in human peripheral blood lymphocytes

For testing the feasibility of enzyme induction and protection of normal cells against alkylation DNA damage, we used HPBLs isolated from healthy volunteers as a model and determined changes in MGMT and GST activities. Unstimulated HPBLs were exposed for 24 h to OTC, and other chemicals at their effective concentrations, and the protein, activity and mRNA levels for MGMT were determined. As seen with the tumor cell lines, OTC (1.6-fold), 4-HPR (1.3-fold), oltipraz (1.5-fold), curcumin and silymarin (1.7-fold) increased the MGMT activity consistently in different lymphocyte preparations (Figure 8A). Western analysis supported the augmented expression of MGMT in lymphocytes (Figure 8B). Consistent with the apparent translational regulation observed in tumor cell lines (Figure 5), the levels of MGMT mRNA were also significantly enhanced in HPBLs after OTC, silymarin, curcumin and oltipraz treatments (Figure 8D). The GST activity (Figure 8A) and protein levels (Figure 8C) were also elevated after lymphocyte treatment with the chemopreventative agents. Overall, these data strongly support the feasibility of increasing MGMT expression as a viable chemopreventative strategy.

Role of glutathione metabolism and intracellular cysteine content in the regulation of MGMT expression

Previous reports indicate that a deficiency of sulfur amino acids, cysteine and methionine may down-regulate MGMT expression (35,36). In view of the data that cysteine prodrugs promote MGMT expression, we reinvestigated these effects in a cell culture setting. For this, UW228 and HCT116 cells were cultured in serum-free DMEM lacking cysteine and methionine, or regular DMEM for 20 h. In adjacent cultures, the deficient medium was supplemented with cysteine (0.52 mM) and/or methionine (0.2 mM) at concentrations found in the regular medium. The relative MGMT activity and protein levels assessed by western analysis under these conditions were fully consistent with the reduction and repletion of the DNA repair activity after amino acid supplementations (Figure 9A). Absence of both essential amino acids reduced the MGMT activity by 50%, which was replenished by the addition of methionine alone or methionine + cysteine together (Figure 9A, upper panel). MGMT protein levels assessed by western analysis under these conditions were fully consistent with the reduction and repletion of the DNA repair activity after amino acid supplementations (Figure 9A, lower panel). The apparently similar influence of methionine and cysteine can be explained by their precursor–product relationship through the trans-sulfuration pathway (49).
Catabolism of GSH through the reactions in γ-glutamyl cycle provides a significant pool of cellular cysteine, whose availability is critical for protein synthesis, as well as the resynthesis of glutathione (49). γ-Glutamyl transpeptidase (γ-GGT), which catalyzes the hydrolysis of glutathione, and glutathione S-conjugates, is the first enzymatic step in GSH catabolism. To investigate whether the degradation of GSH and the consequent cysteine production plays a role in MGMT expression, we used acivicin, an irreversible inhibitor of γ-GGT (50). HCT116 and UW228 cells were incubated with 5 μM acivicin for 22–64 h, when substantial changes in glutathione homeostasis is expected to occur (46), and MGMT activity and protein levels were determined. Figure 9B (upper panel) shows that in both tumor cell lines, acivicin inhibited the MGMT activity in a time-dependent manner; the lower panel of this Figure shows that MGMT protein expression was markedly reduced in this treatment. Together, the data presented in Figure 9 suggests that intracellular cysteine content significantly affects the level of MGMT gene expression, and supports the notion of using GSH-enhancing drugs for MGMT-targeted chemoprevention. Evidence that cysteine may up-regulate MGMT, at least in part, through an enhanced translation is shown in the next section.

**Glutathione depletion down-regulates MGMT activity in HT29 cells**

The results presented in Figure 9 indicate an important role for GSH in the regulation of MGMT activity and/or its expression. To obtain direct evidence for this hypothesis, HT29 cells were depleted of their GSH by buthionine sulfoximine (BSO; 100 μM for 24 h), or diethylmaleate (DEM; 0.4 mM for 4 h). BSO curtails the biosynthesis of GSH through inhibition of γ-glutamylcysteine synthetase, and diethylmaleate oxidizes GSH inducing its rapid loss and increase in GSSG levels (38). Both these treatments induced ~90% depletion of GSH in HT29 cells. MGMT activity was reduced by 50 and 70% in BSO and DEM–treated HT29 cells (data not shown). A direct inhibition of cellular MGMT by DEM cannot be ruled out however, the BSO–induced down-regulation indicates a direct or indirect role for GSH in the maintenance of MGMT activity in human cells.

**Evidence for increased synthesis of MGMT and metallothionein in OTC-treated HT29 cells**

As stated previously, we propose that increased intracellular cysteine facilitates enhanced synthesis of proteins, in particular, the stress signaling proteins with moderate to higher cysteine content including the MGMT. To obtain a direct evidence for this premise, we measured the rate of MGMT synthesis in HT29 cells primed with OTC. Tumor cells were exposed to 10 mM OTC for 48 h followed by labeling with [14C] leucine for 2 h. Radiolabeled MGMT protein was immunoprecipitated, the complexes were resolved by SDS–PAGE and autoradiographed. The results showed that OTC-treatment induced a 1.7-fold increase the rate of MGMT synthesis (Figure 10A). OTC-treated human lymphocytes and HT29 cells also had significant increases in their metallothionein protein content; one-third of 60+ amino acids present in this metal binding family of proteins are cysteines. Overall, the data from multiple approaches suggest that OTC up-regulates cellular MGMT production by enhancing the translation and perhaps the transcription steps as well.

**Discussion**

The level of MGMT expression in human normal tissues is quite low making them more susceptible to the toxic and mutagenic effects of alkylating agents. For example, MGMT is constitutively expressed with highest levels found in liver, followed by colon and lung and is very low, ~20-fold lower than liver in brain and myeloid tissues, (12,51). A great deal of variation in MGMT expression between individuals is also known (12). Although its protective role has been long studied, the augmentation of MGMT expression as a strategy for chemoprevention through dietary or synthetic compounds has not been investigated. The notion that human MGMT is largely a constitutive and non-inducible enzyme (13,52), unlike its bacterial counterparts which participate in the adaptive response to methylating agents (53), may explain the lack of effort in this area. However, MGMT, being the primary defense against alklylation DNA damage (1,13), even marginal increases (2–3-fold) of its activity may be sufficient for significant cellular protection. Further, MGMT appears to interact physically with the proliferating cell nuclear antigen (PCNA), and polymerase δ (54), thus, raising the possibility of a repair-related function for this protein during DNA replication.

Development of dietary or synthetic compounds as potential cancer chemopreventive agents against alkylating chemicals is highly attractive because of their safety, low toxicity and ease of oral intake for prolonged periods (40). Our study provides the first evidence for a small, but significant
increase in MGMT activity by cysteine prodrugs (OTC and NAC) and antioxidants (curcumin and silymarin) in human lymphocytes and cancer cell lines. Other chemopreventative agents (oltipraz, 4-HPR, sulforaphane, resveratrol) were also effective to a lesser extent. In our studies, unstimulated HPBLs incubated with the chemopreventative compounds showed increased MGMT expression. It is not surprising to see changes in lymphocyte gene expression without activation,
and autoradiography. The incorporation of $[^{14}C]$-leucine for 2 h into the repair protein was assessed by immunoprecipitation, SDS–PAGE, and autoradiography. (B) OTC-induced enhancement in metallothionein (MT) protein levels in HPBLs and HT29 cells. Cells after 10 mM OTC treatment for 24 h were analyzed by western blot analysis. The antibody detected two protein bands of 6.5 and 8 kDa.

Fig. 10. (A) Increase in the rate of MGMT protein synthesis after OTC treatment. HT29 cells were exposed to 10 mM OTC for 48 h to enhance the intracellular cysteine levels. Then, the incorporation of $[^{14}C]$-leucine for 2 h into the repair protein was assessed by immunoprecipitation, SDS–PAGE, and autoradiography. (B) OTC-induced enhancement in metallothionein (MT) protein levels in HPBLs and HT29 cells. Cells after 10 mM OTC treatment for 24 h were analyzed by western blot analysis. The antibody detected two protein bands of 6.5 and 8 kDa.

because, the serum components may prime the cells for a low level of activation (55), and others have observed increased expression of antioxidant enzymes (GST and quinone reductase) following exposure of resting lymphocytes to antioxidants or xenobiotic compounds (56,57). Nevertheless, it is possible that the extent of MGMT induction could have been higher, if we had used stimulated lymphocytes.

The ability of cysteine prodrugs, in particular of OTC, to enhance MGMT expression was unexpected. Among these drugs, OTC and NAC have been widely studied. Ribocys or [D-ribo-(1'-2'-3'-4'-tetrahydroxybutyl)thiazolidine-4-carboxylic acid)] (58) is another prodrug that undergoes non-enzymatic decomposition to generate cysteine. OTC is a superior intracellular cysteine delivery agent because of the wide distribution of 5-oxoprolinase in human normal tissues (45). Human tumors appear to have lower oxoprolinase content than corresponding normal tissues (45), which may offer therapeutic benefits. Administration of OTC to animals has been shown to result in significant increases of cysteine and GSH levels in various tissues, including the liver, kidney, lung and brain (37,59). This thiol enhancement has been shown to prevent hepatic and other toxicities due to acetaminophen (60), carcinogens (61), anticancer drugs (62) and oxygen (63) in animals. Inhibition of the human immunodeficiency viral replication by oral administration of OTC has been reported (64). The pharmacokinetics and pharmacodynamics of OTC in humans has been studied and high amounts of OTC (0.5 mmol/kg) administered were non-toxic (65,66); in these studies, rapid 3- and 2-fold increases in cysteine and GSH levels, respectively, were observed in lymphocytes within 1 h (65). Many ongoing clinical trials have established OTC as an effective and non-toxic cysteine delivery agent.

Interestingly, the kinetics of MGMT induction by NAC was different from that of OTC. While OTC increased the MGMT expression gradually, in NAC-treated cultures, there was an early spurt of MGMT expression followed by its reduction at later times. The reason for this dissimilarity is unclear, but, may relate to their uptake, patterns of enzymatic breakdown, and the times at which cysteine levels peak in cellular milieu. OTC has been shown to be more effective than NAC in generating sustained levels of cysteine and GSH in animal tissues (38). The increased MGMT mRNA levels observed in after OTC or NAC treatments are significant and warrant discussion. Evidence suggests that intracellular free cysteine pools in mammalian cells are tightly regulated in a narrow range, just sufficient for supporting normal metabolism, so as to avoid any toxicity by this amino acid (49,67). In this context, the expression of two enzymes that maintain cysteine homeostasis, namely, the cysteine dioxygenase (CDO) and γ-glutamyl cysteine synthase (γ-GCS) has been shown to be controlled by cellular cysteine levels (67). Thus, addition of cysteine to hepatocyte cultures decreased the γ-GCS mRNA and increased the mRNA for CDO (67), much similar to our observations with MGMT. Therefore, cysteine may act as a metabolic signal for cellular synthesis of MGMT and this raises the possible dietary regulation of MGMT expression in human tissues.

Our studies also identified a role for glutathione in MGMT regulation in human cells. In contrast to GSH augmenting agents which increased cellular MGMT, depletion of GSH reduced the MGMT activity. While GSH may influence protein functions in several ways including the maintenance of protein sulphydryls in a reduced state, our data suggested that cysteine derived from GSH catabolism may regulate the extent of MGMT synthesis. Thus, the inhibition of γ-GGT by acivicin, which creates a cysteine deficient state (68), caused a time-dependent reduction in MGMT activity and protein. Cysteine and/or methionine supplementation to the cell culture media enhanced MGMT expression. Further, $[^{14}C]$-leucine incorporation revealed a marked increase in the rate of MGMT synthesis in OTC-treated HT29 cells. GSH serves as the preferred form of storage and transport for cysteine (49). Therefore, we suggest increased cysteine availability drives the synthesis of MGMT protein, at least in part through a translational activation.

Cellular MGMT expression was also enhanced by the well characterized plant antioxidants, sylimarin and curcumin. Silymarin is a purified extract from the milk thistle herb (Silybum marianum) composed of a mixture of four isomeric polyphenolic flavonolignans: silybin (its main, active component), iso-silybin, silydianin and silchristin (69). Silymarin has been empirically used as a remedy for almost 2000 years, and is widely known for its hepatoprotective anti-inflammatory, and anticarcinogenic properties (69). Topical treatment of silymarin has been shown to retard mouse skin carcinogenesis and inhibit azoxymethane-induced colon carcinogenesis (70,71). The mechanism(s) by which silymarin modulates MGMT is unclear, but, presumably involves its antioxidant activity, and interaction with GSH metabolism. Curcumin is a widely studied polyphenol derived from the herbal remedy and dietary spice turmeric (Curcuma Longa) (72). It possesses diverse anti-inflammatory and anti-cancer properties following oral or topical administration. Inhibition of the transcription factors (AP-1 and NF-κB), cell signaling and cytokine down-regulation are some of the mechanisms attributed to curcumin (72,73). Among the other compounds tested, oltipraz, 4-HPR, sulforaphane, and resveratrol promoted MGMT activation to different extents. Interestingly, a previous study reported a slight up-regulation of MGMT by 4-HPR and in head and neck cancer cells (74), consistent with our observations.

The capacity of cells to maintain homeostasis during oxidative stress resides in the activation or induction of protective enzymes. A common and unifying theme among many of the chemopreventatives studied here is that they
possess ability to activate the transcription of genes encoding phase II metabolic enzymes (40). This activation has been traced to the six-acting transcriptional enhancers called ARE (antioxidant response element) or the electrophilic response element (EpRE) and the nuclear-factor-E2-related factor (Nrf2), a member of bZIP transcription factor widely expressed in human tissues (75). The genes that contain a functional ARE include those encoding GSTs, NAD(P)H:quinone reductase, and others that play a role in defense against oxidative stress. Nrf2 is normally bound to the Kelch-like reductase, and others that play a role in defense against oxidative stress. Nrf2 is normally bound to the Kelch-like reductase, and others that play a role in defense against oxidative stress. Nrf2 is normally bound to the Kelch-like reductase, and others that play a role in defense against oxidative stress. Nrf2 is normally bound to the Kelch-like reductase, and others that play a role in defense against oxidative stress.

In summary, our studies identified two mechanistically different classes of compounds namely, the glutathione augmenting agents and plant flavonoid (silymarin) and non-flavonoid (curcumin) antioxidants that possess the ability to increase MGMT expression beyond its steady-state levels. The agents studied are relatively nontoxic as evident from the literature and/or their long term use by public; many of them have shown efficacy in chemoprevention clinical trials. Thus, increasing the MGMT activity in human tissues through a long-term use of the compounds identified or their superior congeners may facilitate a speedy removal of the mutagenic lesion in normal tissues, and thereby reduce cancer risk. Such approaches may have applications in the ongoing bone marrow protection strategies against the BG plus alkylator combinations (14,33) as well.

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