DNA Repair Enzyme, O6-Methylguanine DNA Methyltransferase, Modulates Cytotoxicity of Camptothecin-Derived Topoisomerase I Inhibitors

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Received September 20, 2005; accepted October 27, 2005

ABSTRACT

Two camptothecin-resistant cell lines, CPT30 and KB100, were established and characterized previously in our laboratory. Because enhanced sensitivity to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and decreased expression of O6-methylguanine-DNA methyltransferase (MGMT) protein were observed in these lines, we hypothesized that MGMT may be a determinant of cytotoxicity associated with camptothecin-derived DNA topoisomerase I inhibitors (CPTs). We used the Tet-On system to induce expression of MGMT in Chinese hamster ovary (CHO) cells and RNA interference to knock down MGMT expression in human nasopharyngeal carcinoma HONE-1 cells in order to identify any correlations between MGMT expression and CPTs cytotoxicity. CHO-derived Tet-On-inducible cells (S12+) showed MGMT overexpression and statistically significant more resistance to BCNU, camptothecin, 7-ethyl-10-hydropcamptothecin (SN38), and topotecan than parental CHO cells (p < 0.05), but there was less resistance to CPTs than to BCNU. Knockdown of MGMT expression with small interfering RNA in HONE-1 cells conferred increased sensitivity to BCNU and CPTs compared with mock control. Furthermore, alteration of MGMT expression coincides with CPT-induced cell death and poly(ADP-ribose) polymerase cleavage. There were no differences in protein levels and catalytic activity of topoisomerase I between MGMT-proficient and MGMT-deficient cells from the Tet-On-inducible and small interfering RNA (siRNA) systems. Resistance to CPTs coincided with decreased amounts of protein-linked DNA breaks generated by CPTs in MGMT-proficient cells and vice versa in MGMT-deficient cells. Our data indicate that MGMT can modulate cytotoxicity of CPT-derived topoisomerase I inhibitors.

Topoisomerase I (Top I) plays an essential role in controlling DNA supercoiling and relieving torsional stress that is generated during replication, transcription, recombination, and chromatin remodeling (Lee et al., 1993; Wang, 1996). Top I introduces transient single-stranded DNA breaks in one of the phosphodiester backbones of the duplex DNA, resulting in a reversible Top I/DNA covalent complex (Champoux, 1976). Under normal conditions, religation of the DNA cleavage/religation equilibrium is favored and only a small fraction of DNA is cleaved at any given time. Top I is the primary intracellular target of camptothecin (CPT), a plant alkaloid that was isolated from Camptotheca acumulata. Water-soluble derivatives including irinotecan (CPT-11) and topotecan (TPT) have been widely used in clinical practice for various solid tumors (Gore et al., 2001; Vanhoefer et al., 2001). The cytotoxic mechanism of CPT-derived Top I inhibitors (CPTs) involves at least two successive steps. First, the drug stabilizes the covalent enzyme-DNA intermediate through inhibition of religation without affecting the cleavage reaction (Hsiang et al., 1985). Second, cleavable complexes are converted to DNA double-stranded breaks by interaction with moving replication forks, and these DNA breaks are responsible for observed CPTs cytotoxicity (Hsiang et al., 1989; Zhang et al., 1990).

AABBREVIATIONS: Top I, topoisomerase I; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CHO, Chinese hamster ovary; CPT, camptothecin; CPTs, CPT-derived topoisomerase I inhibitor(s); Dox, doxycycline; MGMT, O6-methylguanine-DNA methyltransferase; PARP, poly(ADP-ribose) polymerase; α-MEM, α-minimal essential medium; PI, propidium iodide; PLDB, protein-linked DNA break(s); S12, a tetracycline-regulatory MGMT expressing clone derived from CHO cells; S12–, S12 cells without doxycycline addition; S12 ±, S12 cells with 1 μg/ml doxycycline addition; SN38, 7-ethyl-10-hydrocamptothecin; siRNA, small interfering RNA; TPT, topotecan; tTs, transcriptional silencer; Vec, vector.
The efficacy of CPTs as cancer therapy is explained only in part by their ability to damage DNA. Although Top I is the primary target of CPTs, a lack of correlation has been demonstrated among Top I levels, CPT-induced protein-linked DNA breaks (PLDB), and CPT cytotoxicity (Eng et al., 1988; Nitas and Wang, 1988; Hsiang et al., 1989). Furthermore, several lines of evidence have shown that a variety of DNA alterations originating from endogenous and xenogenous sources (abasic sites, uracil misincorporations, nicks, oxidized bases, UV photolesions, and carcinogenic adducts) induce the formation of the Top I/DNA-cleavable complex (Pourquier and Pommier, 2001). The influence of defective pathways downstream from the cleavable complex may play a key role in selectivity of CPTs toward cancer cells (Pommier et al., 1994).

We have established two CPT-resistant cell lines, CPT30 and KB100, from human nasopharyngeal carcinoma HONE-1 and cervical carcinoma KB cells, respectively (Beidler et al., 1996; Chang et al., 2002). We demonstrated that the single amino-acid mutation in E418K causes the observed qualitative changes in Top I that are responsible for CPT resistance in CPT30 cells (Chang et al., 2002). The mechanism underlying CPT resistance in KB100 cells is independent from Top I and involves steps subsequent to the formation of PLDB; it may be attributable to alteration of the poly(ADP-ribose) polymerase (PARP)-related DNA repair system (Beidler et al., 1996). It is interesting that CPT30 and KB100 cells are 4- and 2-fold more sensitive, respectively, to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) than their parental cells. The mechanism of enhanced sensitivity to BCNU in both KB100 and CPT30 cells involves down-regulation of the O6-methylguanine-DNA methyltransferase (MGMT) gene (unpublished observation). In addition, previous studies have shown that cells resistant to BCNU exhibit cross-resistance to CPT (Yamauchi et al., 2003). These findings imply that MGMT may be involved in determining CPT cytotoxicity. In the current research, we used a Tet-On-inducible system to overexpress MGMT in Chinese hamster ovary (CHO) cells and a RNA interference method to silence MGMT expression in human nasopharyngeal carcinoma HONE-1 cells to determine any correlation between expression of MGMT and CPTs cytotoxicity.

Materials and Methods

Materials. CPT and BCNU were purchased from Sigma-Aldrich (St. Louis, MO). SN38 was provided by sanofi-aventis (Bridgewater, NJ). TPT was kindly supplied by GlaxoSmithKline (Welwyn Garden City, Hertfordshire, UK). Plasmids of pTet-On, pTet-tTs, and pTRE2hyg, as well as Tet-On system-approved fetal bovine serum, were purchased from Clontech (Mountain View, CA). Plasmid pBR322 was purchased from Invitrogen (Carlsbad, CA). Monoclonal anti-NGM antibody (clone MT5.1) was purchased from BD Pharmingen (San Diego, CA). Monoclonal anti-Top I antibody (clone 21) was kindly provided by Dr. Yung-Chi Cheng (Yale University, New Haven, CT). Monoclonal anti-PARP antibody was purchased from Trevigen (Gaithersburg, MD). Both G418 sulfate and hygromycin B were purchased from Calbiochem (La Jolla, CA). Cell culture agents were obtained from Invitrogen. N-[3H]-Methyl-N-nitrosourea was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). All other chemicals were from Merck Biosciences (Darmstadt, Germany) or Sigma-Aldrich and were of standard analytic grade or higher.

Cell Culture. Human nasopharyngeal carcinoma HONE-1 cells and colorectal carcinoma HT29 cells were maintained in RPMI 1640 medium supplied with 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. CHO cells were maintained in α-MEM supplemented with 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Insertion of MGMT cDNA Into Tet-On-Responsive Vector. Total RNA was extracted from human colorectal carcinoma HT29 cells using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. RNA was reverse-transcribed using SuperScript II RNase H reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Human MGMT cDNA was amplified using the following primer pairs: forward primer, 5′-AAGGATCCCGTT- TGGCGACTTGGTACTTT-3′, and reverse primer, 5′-CGACGATAT- CAACCGCGCCCGCATGCAGTTGATACACG-3′. Polymerase chain reaction amplification was performed under the following conditions. Preincubation was performed at 94°C for 2 min followed by 30 cycles of 94 (30 s), 64 (45 s), and 72°C (1 min), with a final extension at 72°C for 7 min. The amplified 704-base pair product of MGMT cDNA was digested with BamH1 and NotI restriction enzymes and cloned into the pTRE2hyg vector at multiple cloning sites. The plasmid clones were checked by BamH1 and NotI cutting to contain the 0.7-kb fragment, and potential clones were confirmed by sequencing with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). After verification that the sequences were correct, the recombined plasmid MGMT-pTRE2hyg was grown in large scale and purified using plasmid DNA purification kit (Macherey-Nagel Inc., Easton, PA).

Establishment of the MGMT Tet-On-Inducible Cell Line. To establish MGMT Tet-On-inducible cell lines, CHO cells were initially transfected with regulatory plasmids pTet-On and pTet-tTs using SuperFect reagent (Qiagen Operon, Alameda, CA) according to the manufacturer’s instructions. The pTet-tTs plasmid contains the tetracycline-dependent transcriptional silencer (tTs), which binds the tetO-inducible promoter in the absence of doxycycline (Dox), and thus it can potentially reduce high-background activity on the Tet-On-inducible system. The day after transfection, cultures were given medium containing 0.8 mg/ml G418 sulfate. After 14 days, neomycin-resistant clones were collected and combined to make a heterogeneous stable population. Responsive vector MGMT-pTRE2hyg was transfected next into this population to obtain Tet-On-regulated MGMT-expressing cells. Transfected cells were double-selected in medium containing 0.8 mg/ml G418 sulfate and 0.75 mg/ml hygromycin B for 2 weeks, and then various cell clones were isolated. Individual clones were expanded and then incubated with or without 1 μg/ml Dox for 24 h. MGMT-inducible clones were detected by Western blot analysis. After screening, a stable transfectant of CHO cells expressing human MGMT was obtained and named CHO-MGMT-S12. As a control, a mock-transfected CHO-Tet-On cell line named CHO-vector (Vec) (hereinafter referred to as vehicle control) was generated, which was stably transfected with pTet-On, pTet-tTs, and empty pTRE2hyg plasmids. These transfecants were maintained in α-MEM supplemented with 5% Tet-On system-approved fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 400 μg/ml G418 sulfate, and 400 μg/ml hygromycin B.

Silencing of MGMT by Small Interfering RNA Transfection. The siRNA sequence targeting MGMT corresponded to two separate coding regions: region 1, 5′-AAGCTGGAGGCTTCTGTTGTG-3′ (nucleotides 52–71), and region 2, 5′-AAGTTGGTGAATTTCCGAGAA-3′ (nucleotides 310–330). The sequences of both regions are unique to MGMT as indicated by a sequence search. The siRNA sequence targeting luciferase corresponded to the coding region 5′-GCCATCTATCCCTAGAAGATG-3′, and it was used for mock control. Selected annealed RNA was synthesized by Ambion (Austin, TX). HONE-1 cells in exponential growth phase were plated in 6-cm plates at 5 × 10⁴ cells/plate, grown for 24 h, and then transfected with MGMT siRNA duplex using RNAiFect transfection kit (QIA
GEN, Valencia, CA), according to the manufacturer's instructions. The concentrations of siRNA were chosen based on dose-response studies. Silencing was examined approximately 12 to 60 h after transfection. In addition, control cells were transfected with luciferase siRNA duplex (mock).

**Growth Inhibition Assay.** Cells in logarithmic growth phase were cultured at a density of 5000 cells/ml/well in 24-well plates. Cells were exposed to various concentrations of test drugs for three days. The methylene blue dye assay was used to evaluate the effect of test drugs on cell growth as has been described previously (Finlay et al., 1984).

**Clonogenic Survival Assay.** Cells in logarithmic growth phase were cultured in six-well plates (250 cells/well) for 24 h. Cells were treated next with various concentrations of drugs for the indicated times. Cells were then washed with prewarmed phosphate-buffered saline twice and maintained in drug-free complete medium for 10 to 14 days. At the end of the incubation period, cells were fixed and stained with 50% ethanol containing 0.5% methylene blue for 30 min and then washed with water. The number and size of methylene blue-stained colonies were then recorded. The 50% lethal concentration (LC50) was defined as the drug concentration that inhibited colony formation by 50% compared with formation seen with a vehicle-treated control.

**Western Blot Analysis.** Crude cellular extracts were prepared for Western blot analysis as described previously (Kuo et al., 2004). Detection of immunoreactive signals was accomplished with the Western Blot Chemiluminescent Reagent Plus (PerkinElmer Life and Analytical Sciences, Boston, MA). Band-specific intensity was quantitated using an AlphaImager 2000 system (Alpha Innotech, San Leandro, CA).

**MGMT Activity Assay.** MGMT activity was measured by transfer of [3H]-labeled methyl groups from the O6-position of guanine in DNA to the MGMT protein as described previously (Myrnes et al., 1984). Cell extracts were prepared by sonication in MGMT assay buffer (50 mM Tris-HCl (pH 8.3), 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitor mixture of 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin, and 50 µg/ml leupeptin) followed by centrifugation at 10,000g for 10 min. Extracts (400 µg of protein) were supplemented with [3H]DNA enriched for O6-methylguanine (200 µg; 5000 cpm) incubated for 60 min at 37°C. Reactions were quantitated after acid hydrolysis of DNA substrate, collection of protein precipitates, and radioactivity counting.

**Top I Catalytic Activity Assay.** Top I DNA-catalytic activity using whole-cell lysates was measured by relaxation of supercoiled pBR322 plasmid DNA in vitro as described previously (Liu and Miller, 1981). The reaction mixtures were performed at 37°C for 30 min and terminated by adding 1% SDS and separated in a 1% agarose gel. Photographs of the resulting ethidium bromide-stained agarose gels were taken under UV light, and band intensities were quantitated using an AlphaImager 2000 system (Alpha Innotech, San Leandro, CA).

**Measurement of PLDB by Potassium-SDS Coprecipitation Assay.** DNA in logarithmically growing cells (2.5 × 10^6 cells/ml) was treated with 1 µg/ml MGMT-targeted siRNA and labeled by adding [3H]thymidine into medium to a final concentration of 0.5 µCi/ml. After a 24-h incubation, cells were trypsinized and resuspended in fresh medium at a density of 2.5 × 10^6 cells/ml. Cells were aliquoted (1 ml each) into a reaction tube and incubated for another 1 h at 37°C with gentle shaking. Cells were then treated with various concentrations of drugs for 30 min and collected and analyzed for PLDB by the potassium-SDS precipitation method, as described previously (Rowe et al., 1986).

**Annexin-V/PI Binding Assay.** Cells were treated with various concentrations of CPT for 24 h, and the Annexin-V-FLUOS staining kit (Roche Diagnostics, Mannheim, Germany) was used according to manufacturer's instructions to evaluate Annexin V/PI positivity. Control cells stained with Annexin-V or PI alone were used to compensate for flow-cytometric analysis (FACSVantage; BD Biosciences, San Jose, CA). Annexin-V- and PI-double-negative cells were defined as live cells. Annexin-V-positive, PI-negative cells were defined as early apoptotic cells, and Annexin-V- and PI-double-positive cells were defined as late-arising apoptotic/necrotic cells.

**Statistical Analysis.** All assays were carried out in triplicate. Data were expressed as mean ± S.D. Student's t test was used to compare the mean of each group with that of the control group. A p value <0.05 was considered statistically significant.

**Results**

**Establishment of a Stable Cell Line Expressing MGMT Induced by Dox.** To investigate the role of MGMT in determining CPTs cytotoxicity, the Tet-On-inducible system was chosen to overexpress MGMT conditionally by exposure to Dox. We successfully established Dox-regulated MGMT-inducible cell lines in CHO cells, which lack MGMT protein expression because of homozygous gene deletion. As shown in Fig. 1A, there was undetectable MGMT expression in CHO-MGMT-S12 (S12) cells without Dox treatment. MGMT protein was induced in a concentration-dependent manner in S12 cells after the addition of Dox and reached a plateau concentration of 1 µg/ml at 24 h. In addition, MGMT expression was not detectable in parental CHO and CHO-Vec cells, which were transfected with Tet-On-response vector but without inserted MGMT cDNA (data not shown). With the use of the MGMT-enzymatic activity assay, we found that MGMT activity of CHO and Vec cells as well as S12 cells without Dox induction (S12−) was not detectable. However, the level of MGMT-enzymatic activity increased significantly after treating S12 cells with 1 µg/ml Dox for 24 h (S12+) (Fig. 1B), rising to a level similar to that seen in HT29 cells. Thus, we chose this condition to detect changes in cellular events in the following experiments.

**Cells Overexpressing MGMT Causing a Low-Level Resistance to CPTs.** The cell-doubling times for CHO, Vec, S12−, and S12+ cells were similar on the order of 13.5 h. The 50% inhibitory concentration (IC50) values for BCNU and CPTs as tested in CHO, Vec, S12−, and S12+ cells with growth inhibition assay are presented in Table 1. S12+ cells were 3.1-fold more resistant to BCNU and approximately 1.3- to 1.4-fold more resistant to CPTs, including CPT, SN38, and TPT, than seen with CHO cells. Furthermore, clonogenic results for BCNU, CPT, SN38, and TPT in CHO and S12− cells displayed a similar pattern to that seen with the growth inhibition assay (Fig. 2A). In addition to resistance to BCNU, S12+ cells exhibited low-level but significant resistance to CPT, SN38, and TPT compared with CHO cells. S12+ cells were 5.1-fold more resistant to BCNU and approximately 1.4- to 1.7-fold more resistant to CPTs than CHO cells (LC50 of BCNU, 15.63 ± 8.2 versus 80.0 ± 6.9, p < 0.05; LC50 of CPT, 40.5 ± 5.4 versus 69.1 ± 5.2, p < 0.05; LC50 of SN38, 42.0 ± 6.8 versus 57.2 ± 6.6, p < 0.05; and LC50 of TPT, 112.5 ± 10.2 versus 155.4 ± 9.1, p < 0.05).

**Efficient Silencing of MGMT Gene Expression in HONE-1 Cells Using siRNA.** To further confirm that MGMT may participate in determining CPTs cytotoxicity, siRNA in the form of two independent nonoverlapping 21-base pair RNA duplexes that target MGMT was used to silence expression. As shown in Fig. 1C, treatment of HONE-1 cells with MGMT-targeted siRNA oligonucleotides was quite effective, resulting in significant knockdown of cellular MGMT protein levels (Fig. 1C). The greatest inhibition of MGMT protein expression was observed in HONE-1...
cells treated with 1.0 μg/ml MGMT-targeted siRNA for 12 h, with silencing observed up to 60 h. No silencing of the MGMT gene was seen either in the presence of luciferase siRNA oligonucleotides or in the absence of RNA duplexes from transfection medium (data not shown).

**Silencing of MGMT Expression Rendering Cells More Sensitive to CPTs.** HONE-1 cells were transiently transfected with MGMT-targeted siRNA at a concentration of 1 μg/ml for 9 h and then treated with various concentrations of BCNU and CPTs. Clonogenic survival assay was used to ascertain cellular sensitivity toward those drugs. As shown in Fig. 2B, cells transfected with MGMT-targeted siRNA were 4-fold and approximately 1.5- to 1.9-fold more sensitive to BCNU and CPTs, respectively, than mock controls (LC50 of BCNU, 19.1 ± 3.2 versus 76.1 ± 6.5, p < 0.05; LC50 of CPT, 6.9 ± 0.4 versus 13.1 ± 2.2, p < 0.05; LC50 of SN38, 6.2 ± 0.5 versus 10.8 ± 1.6, p < 0.05; and LC50 of TPT, 5.1 ± 0.2 versus 7.5 ± 0.8, p < 0.05).

**Alteration of MGMT Expression Coincides with CPT-Induced Cell Death and PARP Cleavage.** To determine further whether alteration of sensitivity to CPT in both MGMT-proficient and MGMT-deficient cells is through apoptosis, an Annexin-V/PI binding assay was performed. As shown in Fig. 3A, significant increases in both early apoptotic and late-arising apoptotic/necrotic cells were clearly seen in CHO cells compared with S12− cells after treatment with various concentrations of CPT. In addition, a significant increase in late-arising apoptotic/necrotic cells was also clearly observed in siRNA-transfected cells compared with mock controls. However, the magnitude of increase in early apoptotic cells to mock-treated cells was similar to that seen with siRNA-transfected cells (Fig. 3B).

Because proteolytic cleavage of PARP has been considered an early marker of apoptosis, we evaluated the status of PARP cleavage in cells treated with CPT in both Tet-On-inducible and siRNA systems. As shown in Fig. 3C, there was markedly decreased PARP cleavage in S12+ cells compared with CHO cells after CPT treatment. Conversely, HONE-1 cells transfected with MGMT-targeted siRNA showed significantly increased PARP cleavage after the addition of CPT (Fig. 3D).

**Alteration of CPTs Cytotoxicity among Variant MGMT Status Correlates with Level of PLDB Production but Independent of Level and Catalytic Activity of Top I.** To investigate whether alteration of CPTs cytotoxicity is correlated with the amount of Top I-linked DNA breaks, a potassium-SDS coprecipitation assay was used to determine the amount of CPTs-induced PLDB. Results are shown in Fig. 4. After a 30-min exposure to increasing concentrations of CPT, SN38, and TPT, the steady-state level of PLDB increased in a concentration-dependent manner. In the Tet-On-inducible system, S12+ cells were less susceptible to CPT-induced PLDB than CHO cells. At 10 μM, the number of PLDB precipitated was only 2.1-fold that of CHO cells, which was significantly lower than the 57.5-fold increase in S12− cells. Furthermore, the steady-state level of Top I-linked DNA breaks in cells treated with CPT at 10 μM was only 2.6-fold that of CHO cells, which was significantly lower than the 13.1-fold increase in S12− cells. These results indicate that alteration of sensitivity to CPTs is independent of Top I activity, but that the steady-state level of PLDB correlates with the amount of Top I-linked DNA breaks.
Fig. 2. Clonogenic survival curve of tested cells against BCNU and CPTs in both of Tet-On-inducible and RNA interference systems. A, effect of Dox-induced MGMT expression on sensitivity of cells toward BCNU, CPT, SN38, and TPT. B, effect of MGMT siRNA on sensitivity of cells toward BCNU, CPT, SN38, and TPT. S12 cells were first added with 1 μg/ml Dox for 24 h and then exposed to all test drugs for another 24 h. HONE-1 cells were transiently transfected with MGMT-targeted siRNA at concentration of 1 μg/ml for 12 h, and then cells were exposed to all test drugs for another 24 h. After drug treatment, cells were plated in drug-free medium for 10 to 14 days to assess the resulting colony-forming ability of the cells. Each data point represents the mean ± S.D. from at least three separate experiments.
CPT-induced PLDB in S12+ cells was approximately one-half of that seen for parental cells. In addition, S12+ cells were also less susceptible to SN38-induced and TPT-induced PLDB than CHO cells (Fig. 4A). Consistently, increases in levels of PLDB after CPTs treatment in siRNA-transfected populations were observed compared with mock controls. At 10 μM, the numbers of CPT-, SN38-, and TPT-induced PLDB in MGMT siRNA transfectants were approximately 1.4-, 2.0-, and 1.6-fold of those of mock controls, respectively (Fig. 4B).

Therefore, we wanted to check whether alteration of PLDB production is the result of change in protein level and catalytic activity of Top I between MGMT-proficient and MGMT-deficient cells. In the Tet-On-inducible system, protein levels of Top I in CHO, Vec, S12−, and S12+ cells were identical (Table 2). Likewise, we found that expression levels of Top I in MGMT siRNA-transfected cells did not differ from those of mock controls (Table 2). ATP-independent DNA Top I-catalytic activity in cells from both the Tet-On-inducible and RNA interference systems was assessed by adding whole-cell lysates to a reaction mixture containing pBR322 DNA as a substrate; the resulting specific activities are presented in Table 2. The results show that catalytic activity of Top I of CHO, Vec, S12−, and S12+ cells was identical. Similar observations were also found in MGMT siRNA-transfected cells and mock controls.

**Discussion**

Top I has been well characterized as the primary intracellular target of CPTs (Hsiang et al., 1989). CPTs stabilize covalent complexes between Top I and nuclear DNA. When moving replication complexes encounter these Top I/DNA complexes, DNA double-stranded breaks result and subsequently lead to cell death (Holm et al., 1989; Hsiang et al., 1989). Despite the formation of Top I/DNA-cleavable complexes as an essential step for CPTs cytotoxicity, no direct correlation between levels of complex formation and CPT cytotoxicity has been observed. Previous work has demonstrated that sensitivity of cells to Top I poisons is modulated by a variety of factors, including Top I content/activity, cell cycle distribution, DNA repair/recombination, and drug accumulation (Beidler et al., 1996; Fujimori et al., 1996; Larsen and Skladanowski, 1998; Park et al., 2002). Despite the fact Top I poisons display a weak sequence specificity (mainly one or two nucleotides on the 3′ and 5′ sides of the cleavage site, representing the only regulation elements), CPTs induce massive nonspecific DNA damage in cells. Thus, additional cellular events subsequent to DNA-break formation are implicated in CPTs cytotoxicity.

We and others have demonstrated that mechanisms responsible for CPT resistance are decreased expression of Top I, altered Top I, and reduced intracellular accumulation of CPT (Chang et al., 1992, 2002; Pommier et al., 1996; Saleem et al., 2000). Additionally, a CPT resistance mechanism involving DNA repair has been suggested based on findings from several studies (Beidler et al., 1996; Fujimori et al., 1996; Larsen and Skladanowski, 1998; Park et al., 2002; Pommier et al., 2003). Moreover, several lines of evidence have shown that collateral increased sensitivity to etoposide or elevated Top II levels is
Fig. 4. Measurement of CPTs-induced protein-linked DNA breaks in vivo from Tet-On-inducible system (A) and siRNA system (B) by a potassium-SDS coprecipitation assay. Cells prelabeled with [14C]thymidine were incubated with each drug for 30 min and then lysed. Covalent DNA-protein complexes were obtained selectively as potassium-SDS precipitates. Points represent triplicate determinations, and vertical bars indicate mean ± S.D.
TABLE 2
Analyses of specific activity and the relative amount of Top I protein in whole-cell lysates

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Relative Amount of Top I Protein</th>
<th>Catalytic Activity of Top I</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>units/μg protein</td>
<td></td>
</tr>
<tr>
<td>Tet-On inducible system</td>
<td></td>
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</tr>
<tr>
<td>CHO</td>
<td>100 ± 4.8</td>
<td>8.2 ± 1.2</td>
</tr>
<tr>
<td>Vec</td>
<td>102 ± 6.6</td>
<td>9.4 ± 0.1</td>
</tr>
<tr>
<td>S12−</td>
<td>97 ± 6.1</td>
<td>8.6 ± 0.5</td>
</tr>
<tr>
<td>S12+</td>
<td>105 ± 3.2</td>
<td>8.9 ± 1.0</td>
</tr>
<tr>
<td>MGMT siRNA systema</td>
<td></td>
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</tr>
<tr>
<td>HONE-1</td>
<td>100 ± 5.9</td>
<td>19.2 ± 2.4</td>
</tr>
<tr>
<td>HONE-1 luciferase-siRNA</td>
<td>108 ± 7.8</td>
<td>20.8 ± 1.8</td>
</tr>
<tr>
<td>HONE-1 MGMT-siRNA</td>
<td>101 ± 4.6</td>
<td>16.9 ± 3.7</td>
</tr>
</tbody>
</table>

a One unit of Top I-catalytic activity was defined as the amount of enzyme that causes the relaxation of 50% of 0.25 μg of supercoiled form of pBR322 DNA, and the specific activity was determined by calculating the number of units of catalytic DNA activity per microgram of whole-cell lysate. Triplicate determinations were used to obtain mean ± S.D. values. Besides, whole-cell lysates were used from each line for Western blot analysis to assess the level of Top I protein. Relative amount of Top I protein was based on the results of three independent experiments.

b HONE-1 cells transiently transfected with MGMT-targeted siRNA at a concentration of 1 μg/ml for 24 h have been used in this study.

observed in some CPT-resistant cell lines (Sugimoto et al., 1990; Chang et al., 1992), although this is not a general phenomenon across CPT-resistant cell lines. Little is known about the relationship between CPT-resistant cells and anticancer drugs other than Top II inhibitors, especially drugs involved in the DNA repair process. We have previously demonstrated that enhanced sensitivity to BCNU in two CPT-resistant cell lines is caused by down-regulation of the MGMT gene (unpublished observation). In addition, previous studies have shown that cells resistant to BCNU exhibit cross-resistance to CPT (Yamauchi et al., 2003). These findings imply that MGMT may be involved in determining cytotoxicity of CPT.

To explore the hypothesis that MGMT may be involved in determining CPT cytotoxicity, the current work employed two methods (e.g., Tet-On-inducible system to induce expression of MGMT in CHO cells and RNA interference technique to knock down MGMT expression in HONE-1 cells) to identify any correlation between MGMT expression and CPTs cytotoxicity. Our results show that overexpression of MGMT in S12+ cells not only correlated with resistance to BCNU but also to CPT, SN38, and TPT. Conversely, the knockdown of MGMT with siRNA led to cells that were sensitized to these drugs (Table 1; Fig. 2). However, the levels of resistance to CPTs were relatively lower than those for BCNU, which is consistent with the finding that cells resistant to BCNU display a low level of resistance to CPT (Yamauchi et al., 2003). In addition, our finding was similar to the result from Okamoto et al. (2002) who demonstrated that the level of MGMT expression closely correlated with sensitivity to the CPT derivatives (Okamoto et al., 2002).

CPTs can kill cells through apoptosis. Cleavage of PARP by caspases is a prominent characteristic of apoptosis shown to be induced by CPTs (Whitacre et al., 1999). Studies looking at differences in PARP cleavage and apoptotic response of cells treated with CPT in MGMT-proficient and MGMT-deficient cell populations have found that enhancement of PARP cleavage and apoptotic (as well as necrotic) cell death was observed in CHO cells compared with S12+ cells (Fig. 3, A and C). Conversely, enhancement of PARP cleavage and necrotic cell death was observed in CPT-treated cells with knockdown of MGMT expression (Fig. 3, B and D). Although many reports have demonstrated that PARP-mediated cell death has many features in common with apoptotic forms of cell death (Soignet et al., 1998; Huang et al., 1999), PARP-mediated necrotic cell death has also been addressed in several studies (Ha and Snyder, 1999; Kang et al., 2004). These results suggest that CPT triggers a different form of cell death in Tet-On-inducible and RNA interference systems that might be related to specific cell lines.

According to the formation of Top I/DNA covalent complexes is an essential step in CPTs cytotoxicity, we evaluated whether the function of MGMT is related to PLDB production. Notably, we found that resistance to CPTs in MGMT-proficient cells coincided with decreased production of PLDB and vice versa in MGMT-deficient cells (Fig. 4). The present observation of PLDB production is consistent with the results from cytotoxicity data.

Several studies have demonstrated that the amount of CPTs-induced PLDB is mainly modulated by cellular content of Top I (Beidler and Cheng, 1995). Therefore, to exclude the possibility of changing levels of Top I in both MGMT-proficient and MGMT-deficient cells, the amount and specific activity of Top I were determined in both the Tet-On-inducible and RNA interference systems. The results clearly show that no difference in protein content and catalytic activity of Top I was observed between MGMT-proficient and MGMT-deficient cells (Table 2). In addition, we also evaluated the drug sensitivity of Top I toward CPT, SN38, and TPT by using two units of Top I extracted from each cell line, 0.25 μg of supercoiled form of pBR322 DNA, and various concentrations of CPTs. The result demonstrated that the dose of CPTs to inhibit 50% Top I-catalytic activity from MGMT-proficient and MGMT-deficient cells was identical (data not shown). These findings indicate that altered levels of CPTs-induced PLDB in MGMT-proficient and MGMT-deficient cells are not due to the changing of amount and activity of Top I. MGMT might alter the sequence selectivity of the enzymatic cleavage of Top I (directly or indirectly via DNA structural perturbation), without affecting expression and activity.

From above results, we demonstrate that alteration of MGMT expression could lead to change in the amount of CPT-induced PLDB, suggesting that MGMT might be involved in modulation of the process/repairing of CPT-induced PLDB. Beyond the expression level and catalytic activity of Top I protein, cellular processes downstream from induction and repair of DNA damage may also be important in resistance/cytotoxicity to CPT as well as PLDB production. Our previous work suggested that PARP might participate in CPT resistance in a KB-resistant cell and involve steps subsequent to the formation of PLDB (Beidler et al., 1996). Recently, Boothman et al. (1994) suggested that PARP modification of Top I plays a role in repair of Top I cleavage complexes and/or may inactivate the enzyme to prevent further Top I-mediated illegitimate recombination following DNA damage. Malanga and Althaus (2004) showed that ADP-ribose polymers could target specific domains of Top I and reprogram the enzyme to remove itself from cleaved DNA and close the resulting gap, and they suggested that PARP acts as a poly(ADP-ribose) carrier to stall Top I sites and induce efficient repair of enzyme-associated DNA strand breaks. In present study, we demonstrate that alteration of MGMT expression could lead to a change in the amount of...
CPT-induced PLDB (Fig. 4, A and B) and proteolytic cleavage of PARP (Fig. 3, C and D); this proteolytic cleavage decreases the activity of the PARP form coordinating DNA repair (Nicholson et al., 1995), suggesting that PARP might be involved in MGMT-modulated protective effect on CPT-induced PLDB. Despite the mechanism of cellular sensitivity/resistance to CPTs and CPT-induced PLDB production are largely unknown; however, MGMT might act on the factors controlling topology either directly or indirectly with other parameters. This concept warrants further investigation.

In conclusion, despite the role of Top I as major determinant of CPT toxicity, our data provide strong evidence that MGMT also plays a role in determining CPTs cytotoxicity. The effect of MGMT on CPTs cytotoxicity may arise through the modulation of PLDB production. This result may have a potential clinical implication.

References