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Transcriptional Repression of O^6-Methylguanine DNA Methyltransferase Gene Rendering Cells Hypersensitive to *N,N′*-Bis(2-chloroethyl)-*N*-nitrosurea in Camptothecin-Resistant Cells[S]

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ABSTRACT

O^6-Methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein that removes alkyl-adducts from the O^6-guanine in DNA and is a crucial defense against O^6-alkylating agent-induced cytotoxicity. We demonstrated here that two camptothecin (CPT)-resistant cell lines (CPT30 and KB100) were more sensitive to *N,N′*-bis(2-chloroethyl)-*N*-nitrosurea (BCNU) than their parental cells. Enhanced sensitivity to BCNU in these two CPT-resistant cells involved transcriptional repression of the MGMT gene. The mechanism of MGMT gene down-regulation in CPT-resistant cells was not through gene abnormality, mRNA stability, and CpG island hypermethylation. However, the high level of methyl-CpG-binding protein 2 (MeCP2) and dimethylation of H3K9 in the promoter region were found in CPT30 and KB100 cells. Furthermore, increased MeCP2 binding on MGMT promoter was also found to be correlated with MGMT gene-silencing in short-term CPT treatment; thus, enhanced BCNU sensitivity was found in CPT-treated cells. Taken together, we suggest that CPT is able to suppress the transcription of the MGMT gene through recruiting of MeCP2 and H3K9 dimethylation, thus causing a synergistic interaction with BCNU. These findings provide a possible explanation regarding why the combination of CPT and BCNU results in a better objective response than single-use alone. In addition, this study supports a new indication for treating patients who are receiving refractory CPT derivatives with BCNU.

Topoisomerase I (Top I) is an essential enzyme in higher eukaryotic cells. It regulates DNA topology during crucial processes such as replication, transcription, chromosome condensation, and segregation during mitosis (Lee et al., 1993; Wang, 1996). Top I introduces transient single-strand DNA breaks in one of the phosphodiester backbone of the duplex DNA and results in a reversible Top I/DNA covalent complex (Champoux, 1976). Under normal conditions, the religation step of the DNA cleavage/religation equilibrium is favored, and only a small fraction of the DNA is cleaved at any given time. Top I inhibitors such as camptothecin (CPT) and its derivative stabilize (trap) the cleavage complexes by inhibiting the religation step of the equilibrium reaction. Trapping of cleavage complexes by CPT generates Top I-mediated DNA damage, leads to collision of the replication forks, and induces cell death subsequently (Hsiang et al., 1989; Kaufmann et al., 1991). Despite that Top I is the primary target of CPT derivatives, however, no direct correlation between the levels of Top I/DNA-cleavable complex and CPT derivative cytotoxicity has been observed (Goldwasser et al., 1996). The efficacy of CPT derivatives for cancer therapy is explained only in part by their ability to damage

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ABBREVIATIONS: Top, topoisomerase; 5-aza-dC, 5-aza-2′-deoxycytidine; BCNU, *N,N′*-bis(2-chloroethyl)-*N*-nitrosurea; ChIP, chromatin immunoprecipitation; CI, combination index; CPT, camptothecin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H3Ac, acetylation of lysine residues on histone H3; H3K4me2, dimethylation of lysine 4 on histone H3; H3K9me2, dimethylation of lysine 9 on histone H3; H4Ac, acetylation of lysine residues on histone H4; MBD, methyl-CpG binding domain; MeCP2, methyl-CpG-binding protein 2; MGMT, O^6-methylguanine-DNA methyltransferase; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; Q-PCR, real-time quantitative polymerase chain reaction; SDS, sodium dodecyl sulfate; TSA, trichostatin A; bp, base pair; nt, nucleotide; SN38, 7-ethyl-10-hydroxycamptothecin.

DNA. Therefore, the response of individual genes to CPT derivatives may result directly from enzyme inhibition or may arise through secondary mechanisms.

Intrinsic or acquired tumor-mediated drug resistance is the major obstacle that can result in the lack of tumor responsiveness in patients undergoing therapy. The mechanisms of CPT resistance have been divided into three categories: 1) pretarget events, such as uptake and removal of CPT; 2) drug-target events, such as altered Top I level, activity, and Top I mutation; and 3) post-target events, such as cell proliferation and DNA repair/recombination. Post-target events have been shown to play an important role in sensitivity of Top I poison (Beidler et al., 1996; Larsen and Skladanowski, 1998). In DNA-repair gene *RAD52*-deficient yeast cell lines, hypersensitivity to CPT derivatives was shown, despite that CPT derivatives produced equal amounts of protein-linked DNA breaks compared with normal strains (Eng et al., 1988). Overexpression of X-ray repair cross-complementing gene 1 has been found to play a role in the development of CPT-resistance in cancer cells (Park et al., 2002). We and others have proposed that DNA repair protein O^6-methylguanine DNA methyltransferase (MGMT) is at least partly responsible for the sensitivity of CPT (Okamoto et al., 2002; Kuo et al., 2006).

MGMT is a DNA repair protein that removes alkyl-adducts from the O^6-position of guanine in a reaction that transfers the alkyl group from the DNA to an internal cysteine residue in the MGMT, thus restoring the integrity of DNA (Pegg, 1990). This action uses one MGMT molecule for each lesion repaired and makes MGMT a suicide protein, because alkylated-MGMT will be degraded via ubiquitin/proteasomal pathway (Srivenugopal et al., 1996). The alkylated base adduct can be generated endogenously or through exposure to alkylating carcinogens and antitumor drugs with methylating/chloroethylating properties, such as chemotherapeutic 2-chloroethyl-*N*-nitrosourea derivatives [e.g., *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosurea (BCNU)] (Pegg, 1990; Tano et al., 1997) and monofunctional triazenes (e.g., dacarbazine) (Shiraishi et al., 2000). Increased expression of MGMT is associated with the resistance of tumor cells to these drugs (Brent et al., 1985; Pegg, 1990; Kokkinakis et al., 1997). On the other hand, MGMT-deficient cells showed hypersensitivity toward O^6-alkylating agents (Day et al., 1980; Pegg, 1990; Dolan et al., 1991). Thus, finding ways of controlling MGMT expression, which could enhance the cytotoxicity of O^6-alkylating agents toward cancer cells, is of significant clinical interest.

We have established previously two CPT-resistant cell lines, CPT30 and KB100, from human nasopharyngeal carcinoma HONE-1 and oral epidermoid carcinoma KB cell lines, respectively. A single amino acid mutation in E418K causes the quantitative and 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. It involves steps subsequent to the formation of protein-linked DNA breaks-(Beidler et al., 1996). It is interesting to observe that these two CPT-resistant cell lines showed hypersensitivity toward BCNU, compared with their parental cells, caused by the down-regulation of the MGMT gene. The aim of the present study was to elucidate the responsive factors of MGMT inactivation in these two CPT-resistant cells.

Materials and Methods

Cell Lines. The established human cancer cell lines, including nasopharyngeal carcinoma HONE-1, oral epidermoid carcinoma KB, and colorectal carcinoma HT-29 cells, were routinely maintained in RPMI 1640 medium supplied with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The two CPT-resistant cell lines, CPT30 and KB100, were maintained in growth medium supplemented with 30 and 100 nM CPT (Sigma-Aldrich, St. Louis, MO), respectively. Medium was changed every 3 to 4 days.

Growth Inhibition Assay. Cells in logarithmic growth phase were cultured at a density of 1×10^4 cells/ml/well in a 24-well plate and then exposed to various concentrations of tested drugs for three generations. The methylene blue dye assay was used to evaluate the effects of the drugs on cell growth, as described previously (Finlay et al., 1984), and the IC_{50} value was determined.

Western Blot Analysis. Cell nuclear protein was isolated by Nuclear Protein extraction kit (Pierce Biotechnology, Rockford, IL). Crude cellular extracts and Western blot analysis was performed as described previously (Kuo et al., 2004). In brief, protein extracts were electrophoresed on SDS-polyacrylamide gels. After electroblotting to nitrocellulose membranes, the proteins were probed with anti-human MGMT monoclonal antibody (BD PharMingen, San Diego, CA), anti-human α-tubulin antibody (Sigma-Aldrich), anti-MeCP2 polyclonal antibody (Millipore, Billerica, MA), or anti-nucleoporin p62 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Detection of immunoreactive signal was accomplished with Western Blot Chemoluminescent Reagent Plus (PerkinElmer, Waltham, MA).

Generation of Probes for Northern/Southern Blot Analysis. The probe for MGMT gene was obtained by using the cDNA of HT29 cells as a template as described previously (Kuo et al., 2006). In brief, the primers for PCR are 5′-AAGGATCCCCGTTTGCGACTTGGTACTT-3′ (sense) and 5′-CGACGATATCAAGCGGCCGCCCGATGCAGTGTTACACG-3′ (antisense). The PCR was performed for 30 cycles using thermal cycler as follows: 30 s at 94°C, 60 s at 64°C, 60 s at 72°C, and final extension of 7 min at 72°C. The length of PCR product is 704 bp, and the product has been verified by sequencing. The PCR product was cloned into a plasmid by using TA Cloning Kit (Invitrogen, Carlsbad, CA). The MGMT sequence in a recombinant plasmid showing the correct orientation was then verified by sequencing. The probe for hybridization was digested the plasmid with BamHI and NotI.

Northern Blot Analysis. Total RNA was isolated from each cells by using TRIzol reagent (Invitrogen), and analysis of MGMT mRNA levels was performed as described previously (Chang et al., 2002). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as the internal control. The filter was scanned, and band-specific intensity was quantitated with an AlphaImager 2000 system (Alpha Innotech, San Leandro, CA). Expression level of MGMT mRNA was calculated as the ratio of the radioactivity in the MGMT band relative to that of the GAPDH band.

Measurement of mRNA Stabilities. An inhibitor analysis of mRNA stability using actinomycin D was performed as described previously (Kroes and Erickson, 1995). Cells were treated with 2 μg/ml actinomycin D for 3, 6, 9, 12, and 24 h. Total RNA was isolated from each cell lines by using the TRIzol reagent (Invitrogen) and then subjected to Northern blot analysis. The expression level of MGMT was adjusted to the level of GAPDH. Regression analysis was performed on the quantified levels of mRNA to determine the half-life of parental and CPT-resistant cells.

Nuclear Run-On. Nuclear isolation and run-on were performed as described previously (Chan et al., 1992). In brief, Nuclei were resuspended in nuclear freezing buffer (50 mM Tris-HCl, pH 8.3, 40% v/v glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA) and stored at −80°C until use. Plasmid DNA containing MGMT and GAPDH were first cleaved with a restriction enzyme, BamHI and EcoRI (New England Biolabs, Danvers, MA), respectively, to linearize the double-stranded DNA. The DNA samples were then denatured with 3 M

NaOH, incubated for 30 min at room temperature, and followed by applying to a Hybond-N^+ nylon membrane on a slot-blot apparatus. The frozen nuclei were thawed on ice and resuspended in a run-on buffer (5 mM Tris-HCl, pH 8, 2.5 mM Mg_2Cl, 150 mM KCl, and 0.25 mM ATP, GTP, and CTP, respectively). The reaction mixtures were then added with 150 μCi of [α-^{32}P]UTP (3000 Ci/mmol; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and incubated at 37°C for 30 min. The RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA pellets were resuspended with diethyl pyrocarbonate-treated H_2O and then hybridized in a hybridization buffer containing 250 mM Na_2HPO_4 and 0.7% SDS for 48 h at 65°C with membrane loaded with the DNA of MGMT and GAPDH, respectively. The membranes were washed and analyzed by autoradiography analysis.

Southern Blot Analysis of MGMT Gene. Genomic DNA was isolated from HONE-1, CPT30, KB, and KB100 cells. Five micrograms of genomic DNA was digested to completion with EcoRI, HindIII, and BamHI (New England Biolabs). Digested DNAs were separated on 1% agarose gel and then transferred to nylon membranes. The membranes were probed with a ^{32}P-labeled human MGMT cDNA fragment.

Methylation-Specific Polymerase Chain Reaction. The method of measurement of MGMT promoter methylation was described previously by using methylation-specific PCR (Herman et al., 1996). Primer sets of MGMT promoter were, for the unmethylated reaction, 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′ (sense) and 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′ (antisense), and for the methylated reaction, 5′-TTTCGACGTTCGTAGGTTTTCGC-3′ (sense) and 5′-GCACTCTTCCGAAAACGAAACG-3′ (antisense). DNA from peripheral blood mononuclear cell (PBMC) was used as a control for unmethylated alleles of MGMT. In brief, genomic DNA was denatured with NaOH and modified by sodium bisulfide. DNA samples were then purified using Wizard DNA purification resin (Promega, San Luis Obispo, CA), again treated with NaOH, precipitated with ethanol, and resuspended in water. The PCR was then carried out for 35 cycles using thermal cycler as follows: 30 s at 94°C, 30 s at 59°C, 45 s at 72°C, and a final extension of 2 min at 72°C. The PCR products were resolved with 6% nondenatured polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Generation of Probes for Southern Bolt Analysis of MGMT Promoter Methylation. The probe for MGMT promoter region was obtained by using genomic DNA of PBMC as a template. The primers for PCR were 5′-AGGTGTGTTAGGATCCTGCT-3′ (sense) and 5′-TGATTCAGTCTGCGCATCCT-3′ (anti-sense). The PCR was performed for 30 cycles using thermal cycler as follows: 30 s at 94°C, 30 s at 62°C, 1 min at 72°C, and final extension of 7 min at 72°C. The PCR product was cloned into a plasmid by using the TA Cloning Kit (Invitrogen). The probe for hybridization was digested with BamHI and NarI (New England Biolabs). The size of this probe was 558 bp.

Southern Bolt Analysis of MGMT Promoter Methylation. To analyze the methylation status of MGMT promoter, 10 μg of genomic DNA was first digested to completion with SacI enzyme (New England Biolabs). The DNA was ethanol-precipitated, and a second complete digest was performed with one of the methylation-sensitive restriction enzymes BssHII, EagI, NaeI, SacII, and SmaI (New England Biolabs). The digestion products were size-separated onto 1% agarose gel, denatured by NaOH, and transferred to nylon membranes (Herfarth et al., 1999). The membranes were probed with a ^{32}P-labeled human MGMT promoter fragment.

Chromatin Immunoprecipitation Assays. Chromatin immunoprecipitation (ChIP) analyses were performed basically using the EZ ChIP Assay Kit by following the instructions of the manufacturer (Millipore). In brief, protein extract from 1×10^6 cells was cross-linked to DNA by the addition of formaldehyde directly to the culture medium to a final concentration of 1% for 10 min at room temperature. The cross-linking reaction was quenched by adding glycine solution to a final concentration of 0.125 M for 5 min at room temperature. The medium was then removed, and cells were collected and suspended in 1 ml of ice-cold phosphate-buffered saline containing protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A). Cells were pelleted, resuspended in 0.2 ml of SDS lysis buffer, and sonicated to yield fragments of 500-bp average size of DNA. Sonicated lysates were centrifuged at 14,000 rpm at 4°C for 15 min. Supernatants were diluted 5-fold in ChIP dilution buffer (provided in kit). An aliquot (100 μl) of the chromatin preparation was set aside and designated as the input fraction for input normalization. To reduce the nonspecific background, the chromatin solution was precleared with 60 μl of salmon sperm DNA/Protein G agarose beads for 1 h at 4°C with agitation. The cleared chromatin was immunoprecipitated with 5 μg of either anti-RNA pol II (for the positive control; Millipore), normal mouse IgG (for the negative control; Millipore), anti-acetyl-histone H3 (H3Ac; Millipore), anti-acetyl-histone H4 (H4Ac; Millipore), anti-dimethyl-histone H3 (Lys4) (H3K4me2; Millipore), anti-dimethyl-histone H3 (Lys9) (H3K9me2; Millipore), or anti-methyl-CpG-binding protein 2 (MeCP2) (Millipore) antibody and incubated overnight at 4°C with rotation. Later, salmon sperm DNA/Protein G agarose beads was added to these samples and rocked for 1 h at 4°C. Protein A immune complexes were collected by centrifugation and washed with the recommended buffers for 5 min each. Immune complexes were eluted twice with 250 μl of elution buffer for 15 min at room temperature. Twenty microliters of 5 M NaCl was added to the combined eluents, and the samples were incubated at 65°C for 4 h; 10 mM EDTA, 40 mM Tris-HCl, pH 6.5, and 20 μg of proteinase K were then added to the samples and incubated at 45°C for 2 h. DNA (both from immunoprecipitation samples and input) was recovered and purified with QIAquick PCR purification kit (Qiagen, Valencia, CA).

Real-Time PCR Quantification Analysis of Immunoprecipitated DNA. To allow accurate measurement of the amount of DNA precipitated, quantitative PCR was performed in this study. The input and immunoprecipitated DNA were amplified across the MGMT promoter region using the primers 5′-GCCCCGGATATGCTGGGACA-3′ and 5′-GGGCAACACCTGGGAGGCAC-3′ as described previously (Zhao et al., 2005). The internal control primers used were as GAPDH 1107 5′-TCTCTTCCTCTTGTGCTCTTG-3′ and GAPDH 943 5′-ACCCACTCCTCCACCTTTGACG-3′. For real-time PCR analysis, a standard curve was prepared from each purified input DNA sample with serial dilutions as 1/4, 1/16, 1/64, 1/256, and 1/1024 for each primer set. Real-time PCR was performed with SYBR Green Reagent Mix using a 5700 Real-Time PCR System (Applied Biosystems, Foster City, CA). Cycling parameters are as follows: 15 s at 95°C, 30 s at 66°C, and 30 s at 72°C for the MGMT; and 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C to amplify GAPDH. All of the PCR reactions were at least 40 cycles long and were performed in triplicate with negative controls (DNA template-negative) included. The level of ChIP DNA was normalized with that of input DNA. In each experiment, samples were analyzed in triplicate. Quantitation of PCR products was determined by applying the comparative threshold cycle number (C_t) method, as described in the ABI 7000 user guide and by Litt et al. (2001) and Oshiro et al. (2003). The -fold changes of histone modification and methyl-CpG binding domain (MBD) protein on the MGMT promoters were determined by the following equation: $2^{[(C_t\ \mathrm{GAPDH} - C_t\ \mathrm{Input}) - (C_t\ \mathrm{MGMT} - C_t\ \mathrm{Input})]}$, where C_t is the threshold cycle number. Statistical significance of results was determined by the Student's t test ($P < 0.05$).

Median Effect Analysis. The nature of the interaction observed between CPT and BCNU was analyzed using the software CalcuSyn (Biosoft, Ferguson, MO), which uses the combination index (CI) method of Chou and Talalay (1984), based on the multiple drug effect equation. This analysis requires 1) that each drug alone has a dose-effect relationship and 2) that at least three or more data points for each single drug are available in each experiment. The constant ratio combination design was chosen to assess the combination effect of both drugs, in which dose-response curves were determined with both drugs in combination, at a fixed ratio equivalent to the ratio of their IC_{50} values. The advantage to this method is the automatic

construction of a fraction-affected CI table, graph, and classic isobologram by the software. CI values of < 1 indicate greater than additive effects (synergism; the smaller the value, the greater the degree of synergy); CI values equal to 1 indicate additivity; and CI values >1 indicate antagonism. Each CI ratio represented here is the mean value derived from at least three independent experiments.

Results

Down-Regulation of MGMT Expression in CPT-Resistant Cells Induced Hypersensitivity to BCNU. The sensitivities of parental and CPT-resistant cells to BCNU are presented in Fig. 1A. The IC_{50} value for HONE-1, CPT30, KB, and KB100 cells was 52 ± 6, 14 ± 3, 22 ± 3, and 8 ± 2 μM, respectively. Therefore, two CPT-resistant cells, CPT30 and KB100, were approximately 3.7 and 2.8 times more sensitive to BCNU than their parental cells. Because MGMT is a primary determinant for BCNU cytotoxicity, we therefore examined the expression of MGMT in two pairs of cell lines. As shown in Fig. 1B, the MGMT protein levels in CPT30 and KB100 cells were approximately 38 and 65%, respectively, compared with those in parental cells. In addition, the MGMT mRNA expression in CPT-resistant cells is consistent with the findings of MGMT protein analysis (Fig. 1C) and is shown by Northern blot analysis.

Reduced MGMT Gene Expression Was Regulated at the Transcriptional Level. Down-regulation of mRNA expression may result from a decrease in mRNA stability and/or a reduction in transcriptional activity of its gene. To address the question of whether CPT-resistant cells had a shorter mRNA half-life of MGMT, we performed a time course experiment to measure the mRNA stability of MGMT in KB and KB100 cells. We treated cells with 2 μM RNA polymerase inhibitor, actinomycin D, to inhibit transcription and harvested cellular RNA at time points indicated on Fig. 2A. Northern blot analysis was used to measure the rate of MGMT mRNA degradation. No significant difference in mRNA stability was showed in KB100 cells compared with the parental KB cells. In fact, the half-life of MGMT mRNA in KB cells (7.7 ± 1 h) was similar to that in KB100 cells (8.8 ± 0.5 h). We were unable to compare the half-life of MGMT mRNA between NONE-1 and CPT30 cells because of a very low level of MGMT expression in CPT30 cells.

To further evaluate the basal transcriptional rate of parental and CPT-resistant cells, we used nuclear run-on analysis to determine whether decreases in mRNA levels found in the CPT-resistant cells were caused by a lower transcription rate. As shown in Fig. 2B, the transcriptional rate in CPT30 and KB100 cells is approximately 38 ± 5 and 62 ± 8% of their parental cells, respectively, and just in accordance with the MGMT mRNA expression.

No Gross Deletion, Rearrangement, or Amplification of the *MGMT* Gene Was Detected in CPT-Resistant Cell Lines. To elucidate whether deletions and/or rearrangements

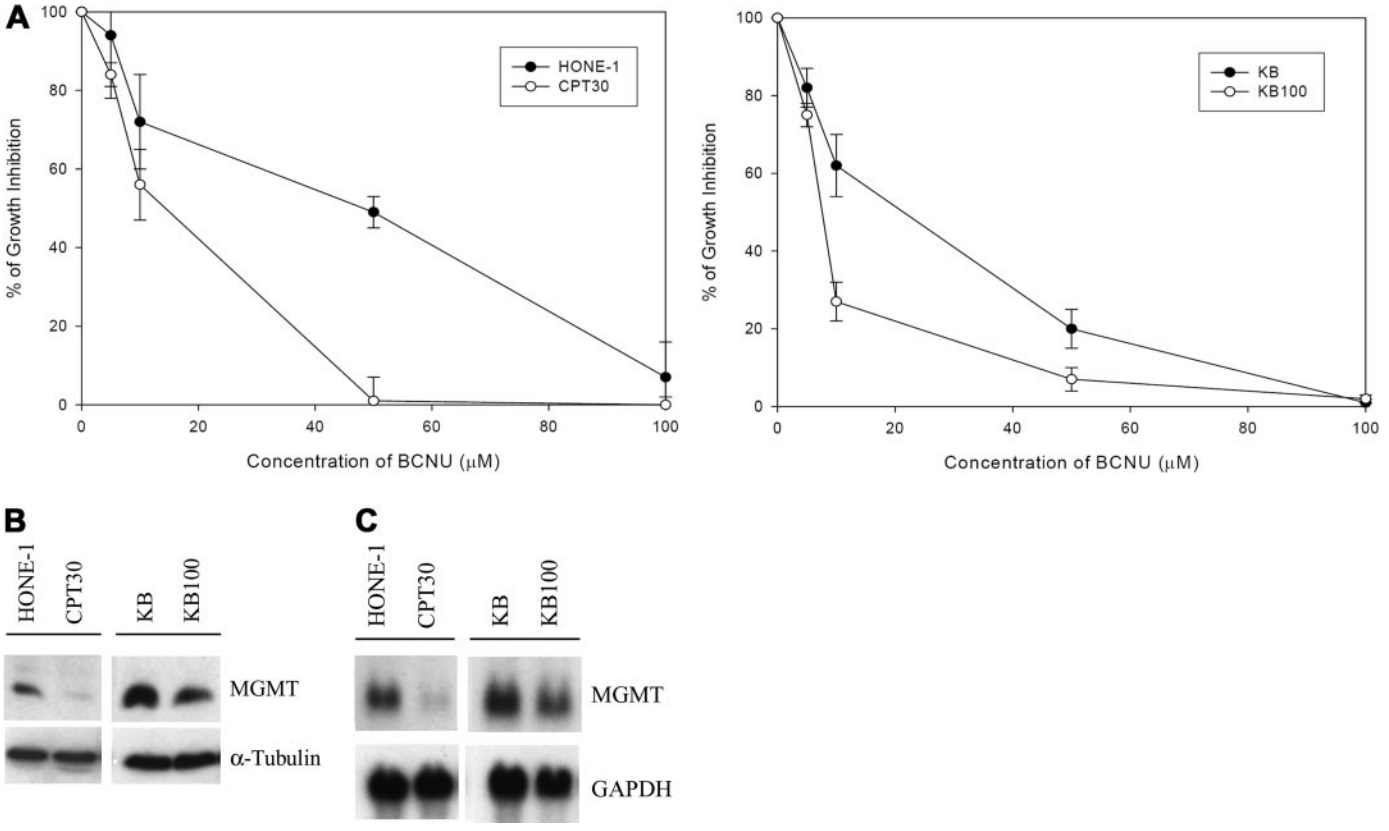


Fig. 1. Evaluation of MGMT expression and BCNU sensitivity in two sets of parental and CPT-resistant cell lines. A, growth inhibition rates of two sets of parental and CPT-resistant cell lines against BCNU treatment. Cells were treated with various concentrations of BCNU for three doubling times. Growth inhibition of cells was determined by methylene blue dye assay. Left, the effect of BCNU on the HONE-1 series cell line; right, the effect of BCNU on the KB series cell line. Each data point represents the mean $\pm$ S.D. of three independent experiments. B, measurement of the expression level of MGMT protein in two sets of parental and CPT-resistant cells. Crude cellular proteins were extracted, and an aliquot of protein (50 μg) from each sample was subjected to Western blot analysis. α-Tubulin was used as an internal control in this study. C, Northern blot analysis of MGMT transcripts in two sets of parental and CPT-resistant cells. Total RNA was isolated from each cell and probed with ^{32}P-labeled MGMT cDNA. GAPDH hybridization was used as a loading control. The results shown are representative data from three independent experiments.

of the MGMT genomic sequence could affect the MGMT transcripts, Southern blot analysis was performed to detect any gross changes of the MGMT gene that might have occurred in CPT-resistant cells. Genomic DNA from HONE-1, CPT30, KB, and KB100 cells were digested with three unique restriction enzymes, BamHI, EcoRI, or HindIII, and electrophoresed on a 1% agarose gel. The blot was probed with a 704-bp full-length human MGMT cDNA probe to provide a complete coverage of the MGMT gene. As shown in Fig. 3, banding patterns created by all three restriction enzymes were identical between parental and CPT-resistant cells (HONE-1 versus CPT30, KB versus KB100). No obvious deletion, rearrangement, amplification, or loss of the MGMT gene has occurred in CPT-resistant cell lines.

MGMT Promoter Methylation Is Not Associated with Reduced Expression of MGMT in CPT-Resistant Cells. Several studies have reported that aberrant hypermethylation of the cytosine CpG island in the promoter region of MGMT is responsible for silencing of the MGMT gene. To analyze and determine any hypermethylation of MGMT promotor in CPT-resistant cells, two independent assays were performed. At first, methylation of the MGMT promoter was studied using methylation-specific PCR of DNA obtained from two pairs of cells. The region chosen for MGMT spans the area of greatest CpG density immediately 5′ to the transcriptional start site. Representative results are shown in Fig. 4A. The methylated allele was detected in both HONE-1 and CPT30 cells, whereas no methylated allele was observed in KB and KB100 cells.

In addition, methylation patterns of MGMT promoter in parental and CPT-resistant cells were also analyzed by using methylation-sensitive restriction enzymes. The position of the restriction sites in MGMT promoter, relative to the transcription start site, is shown in Fig. 4B. Extensive methylation at the BssHII (nt −386, −44, and −23), SacII (nt −329), EagI (nt −230), and NaeI (nt −163 and −171) sites were observed in both HONE-1 and CPT30 cells and shown in Fig. 4C. The ratio of methylation to unmethylation at the SacII, EagI, and NaeI sites is similar between HONE-1 and CPT30 cells. Furthermore, methylation is also demonstrated at the BssHII, SacII, and NaeI sites in both KB and KB100 cells, whereas no methylation at the EagI site in KB100 cells is noted. Furthermore, no methylation at the SmaI site (nt −69) is observed in all tested cells.

Pharmacological manipulation of MGMT expression with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-aza-dC) in CPT30 and KB100 cells has been performed in this study; however, the addition of 5-aza-dC to CPT-resistant cells did not increase MGMT expression (Fig. 4D). Taken together, these observations indicate the hypermethylation at the promoter region of the MGMT gene might not be associated with the down-regulation of MGMT expression in these two CPT-resistant cells.

Alteration of Histone Modification and MeCP2 Binding in CPT-Resistant Cells. Recent studies indicate that histone modifications and MBD protein binding also play critical roles in epigenetic silencing. As shown in Fig. 5A, the expression level of MeCP2 protein was significantly increased in both CPT-resistant cell lines. To investigate whether histone modification and MBD protein binding at

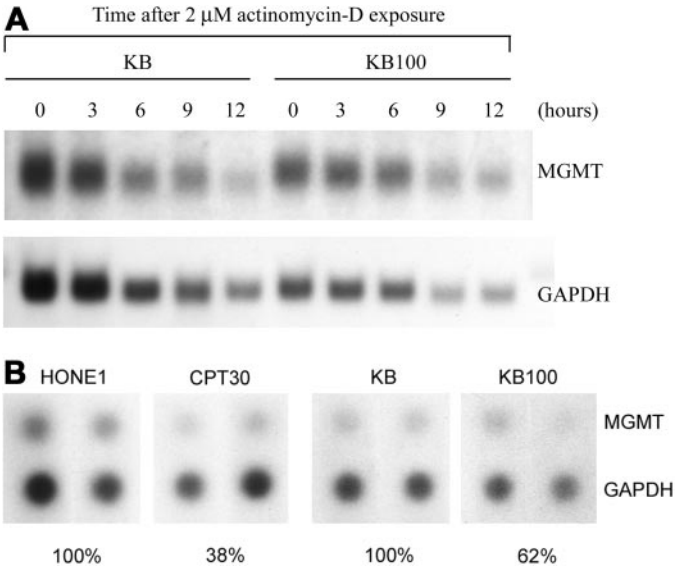


Fig. 2. MGMT message stabilities and transcription rate in parental and CPT-resistant cells. A, MGMT message stabilities in KB and KB100 cells. Cells were treated with 2 μM actinomycin-D for the indicated times. Total cellular RNA was isolated, and the decay of MGMT mRNA was analyzed with Northern blot analysis. GAPDH cDNA was used as internal control. The autoradiographs above are representative of three independent experiments in which similar results were obtained. Band density was quantified with a scanning densitometer. The amount of MGMT mRNA was expressed as a relative percentage before the addition of actinomycin-D, and then the half-life of MGMT mRNA was calculated. B, analysis of the transcriptional activity of the MGMT gene in two sets of parental and CPT-resistant cell lines. Nuclei were isolated from cells and used in nuclear run-on transcription assays as described under *Materials and Methods*. Equal counts of [α-^{32}P]UTP-labeled nuclear run-on transcripts were hybridized to Gene Screen membranes containing full-length MGMT cDNA (top bands) and GAPDH (bottom bands) fragments immobilized using a slot-blot apparatus. The autoradiograms above are representative of three independent assays in which similar results were obtained and expressed as the means ± S.D.

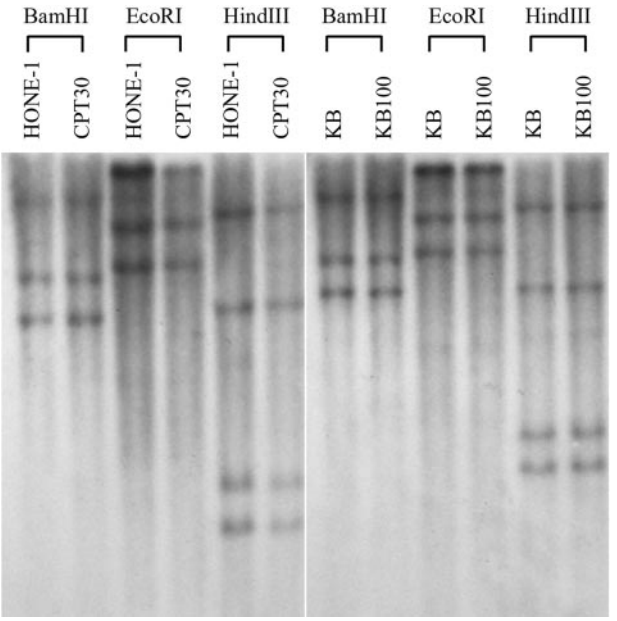


Fig. 3. Southern blot analysis of the MGMT gene in two sets of parental and CPT-resistant cells. Genomic DNA was isolated from the HONE-1, CPT30, KB, and KB100 cells and digested with the appropriate restriction enzyme (BamHI, EcoRI, or HindIII). Restriction products were electrophoresed in 1% DNA agarose gel, blotted to nylon membrane, and probed with ^{32}P-labeled MGMT cDNA. The autoradiographs above are representative of three independent experiments in which similar results were obtained.

the MGMT promoter area are associated with MGMT expression in developing CPT resistance, ChIP-Q-PCR assay was performed to analyze histone modification and MeCP2 binding between parental and resistant cells. Acetylation of lysine residues on histone H3, H4 (H3Ac and H4Ac) and dimethylation of lysine 4 on histone H3 (H3K4me2) are believed to be associated with open chromatin and active transcription, whereas dimethylation of lysine 9 on histone H3 (H3K9me2) serves as a marker of condensed and inactive chromatin. Here we examined H3Ac, H4Ac, H3K4me2, and H3K9me2 in CpG island promoter of MGMT gene. As shown in Fig. 5B, there was no significant difference in H3Ac, H4Ac, and H3K4me2 between the two sets of parental and CPT-resistant cells lines. However, H3K9me2 was significantly higher in both CPT-resistant cells compared with related parental cells. MeCP2 bound to MGMT promoter also exhibited an apparently high level in resistant cells compared with parental cells.

Because the histone deacetylase inhibitor trichostatin A (TSA) could relieve the TRD-mediated repression induced by MeCP2, pharmacological manipulation of MGMT expression with TSA in CPT-resistant lines was performed in this study. As shown in Fig. 5C, MGMT protein was re-expressed with TSA treatment in time-dependent manner. Taken together, these observations suggest that high levels of H3K9me2 and MeCP2 binding through the CpG island were important epigenetic factors for MGMT gene down-regulation in CPT-resistant cells.

Short-Term Treatment of CPT Induces MGMT Gene Silencing in HT-29 Cells. Because the two CPT-resistant cell lines, CPT30 and KB100, were established by continuous exposure to stepwise increasing concentrations of CPT, we further investigated the short-term effect of CPT on MGMT-proficient HT-29 cells. As shown in Fig. 6A, the expression of MGMT gene was decreased in a concentration- and time-dependent manner in CPT-treated HT-29 cells. A consistent decrease in MGMT transcripts was also found in cells treated with the CPT derivatives topotecan and SN38 (the active metabolite of irinotecan) (Supplemental Figs. 1 and 2). Next, we performed ChIP-Q-PCR analysis to assess the effect of CPT-induced MGMT down-regulation on the levels of H3meK9 and MeCP2 in HT-29 cells. The amount of H3K9me2 associated with the MGMT promoter was not changed after 24 h post-

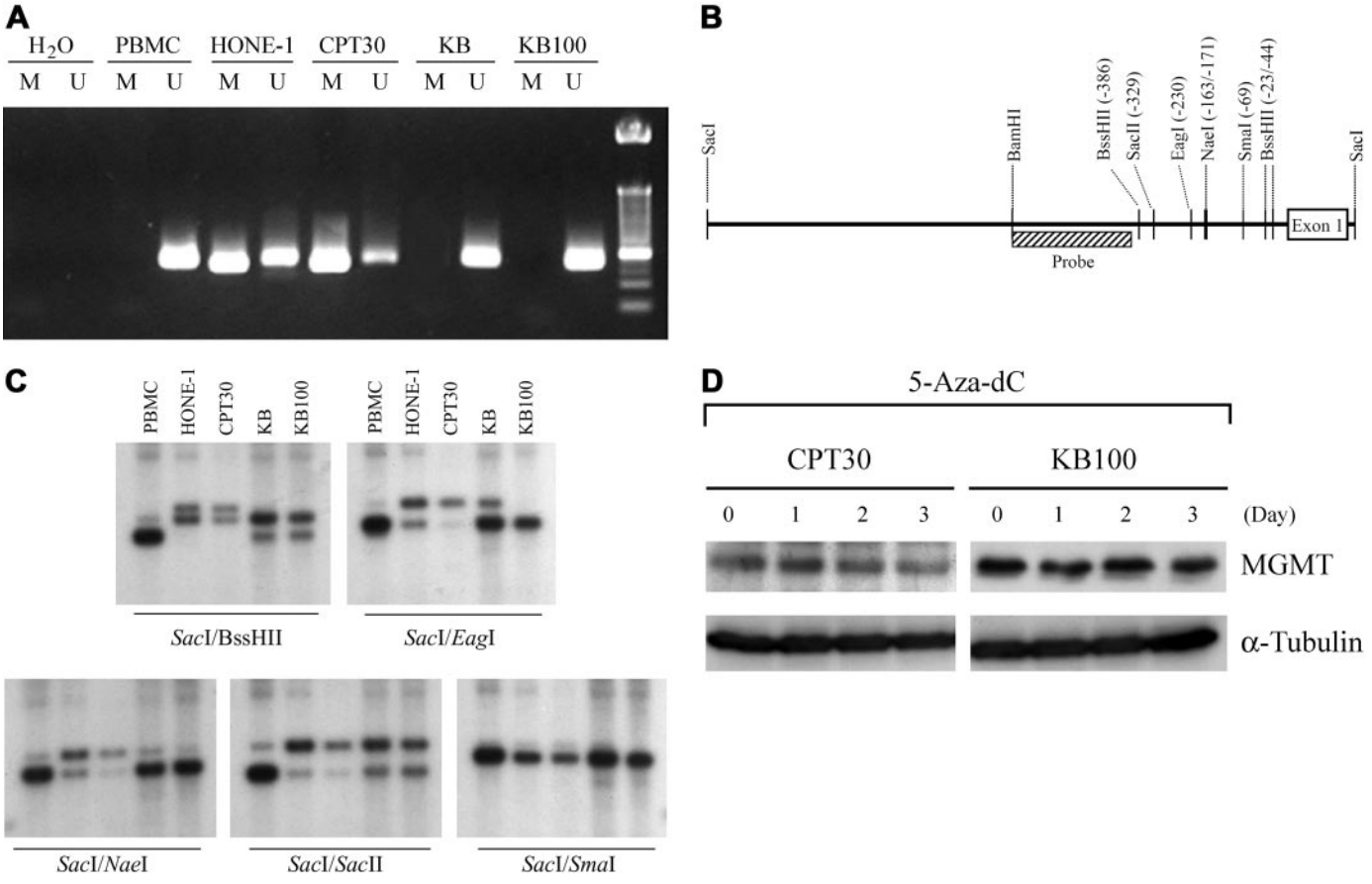


Fig. 4. Methylation status of MGMT gene in two sets of parental and CPT-resistant cells. A, methylation-specific PCR analysis of MGMT promoter CpG methylation. Genomic DNA from HONE-1, CPT30, KB, and KB100 cells were modified with bisulfite as described under *Materials and Methods* and analyzed for methylated CpG sites using PCR primers, which distinguish unmethylated (U) and methylated (M) sequences. DNA from PBMCs was used as a control for unmethylated alleles of MGMT. B, schematic representation of the 5′-portion of the MGMT locus. The positions of the sites recognized by methylation-sensitive restriction enzymes, relative to the start of transcription, are indicated in base pairs above the horizontal line. C, Southern blot analysis of methylation of restriction enzyme sites in the MGMT promoter. Genomic DNA from two-paired of parental and CPT-resistant cells was first digested with SacI, and then DNA was subsequently incubated with BssHII, EagI, NaeI, SacII, or SmaI. Equal amounts of DNA were subjected to Southern blot analysis. D, MGMT protein expression after exposure to 5-aza-dC in CPT-resistant cells measured by Western blot analysis. Cells were treated with a maximum nontoxic dose of 5-aza-dC for the indicated times (0.4 and 1 μM for CPT30 and KB100 cells, respectively). α-Tubulin was used as an internal control in this study.

treatment of CPT, whereas the amount of MeCP2 was significantly increased ($P < 0.05$) (Fig. 6B).

CPT Is Synergistic with BCNU in MGMT-Expressing Human Cancer Cells. To further clarify whether the down-regulation of MGMT gene by short-term exposure to CPT enhanced BCNU sensitivity in human cancer cells, we used CalcuSyn analysis to evaluate whether inactivation of MGMT by CPT affects the cellular sensitivity to BCNU. Two MGMT-expressing human cancer cell lines were chosen in this study. As shown in Fig. 7, A and B, HT-29 and HONE-1 cells were simultaneously exposed to CPT and BCNU at equipotent molar ratios for three generation times to obtain CI plats. The curves demonstrate synergy between both drugs in both cell lines. The CI values are summarized in Fig. 7C.

Discussion

CPT derivatives have been demonstrated to be effective against a broad spectrum of tumors (Hsiang and Liu, 1988). Because of the clinical importance of CPT derivatives, resistance mechanisms of CPT derivatives have been studied extensively in various CPT-resistant cell lines. We and others have demonstrated that decreased expression or alteration of Top I, reduced intracellular accumulation of CPT, and alteration in DNA-repairing machinery are responsible for CPT resistance (Chang et al., 1992, 2002; Beidler et al., 1996; Fujimori et al., 1996; Urasaki et al., 2001). Other than mechanisms of CPT resistance, numerous studies have investigated the relationship between CPT-resistant cells and other chemotherapeutic drugs. It is interesting that several studies demonstrated that cells resistant to Top I-directed agents are, in some cases, at least, hypersensitive to certain Top II-directed agents (Sugimoto et al., 1990; Chang et al., 1992; Urasaki et al., 2001). Therefore, it was suggested that coadministration of agents trapping both Top I and Top II could effectively prevent the development of resistance to these agents and cause complete cell killing (Chang et al., 1992). Recently, our study demonstrated that increasing drug sensitivity toward BCNU is observed in a CPT-resistant cell line, CPT30 (Chang et al., 2002). We further examined the BCNU sensitivity in another set of CPT-resistant lines, KB100, with its parental KB, and found a similar result.

Several studies have shown that MGMT is a primary determinant for BCNU cytotoxicity; we therefore investigated the MGMT expression in two pairs of cell lines. The result showed that the expression level of MGMT protein was decreased in both CPT-resistant cells compared with their parental lines. Furthermore, our study also demonstrated that down-regulation of MGMT protein was the result of a decreased level of corresponding mRNA, suggesting that the reduction of MGMT expression in CPT-resistant lines was controlled at the RNA level. The half life of MGMT mRNA was similar between parental and CPT-resistant cells, but transcription activities were decreased in CPT30 and KB100 compared with their parental cell lines. These data indicated that reduced MGMT expression in CPT-resistant cells was

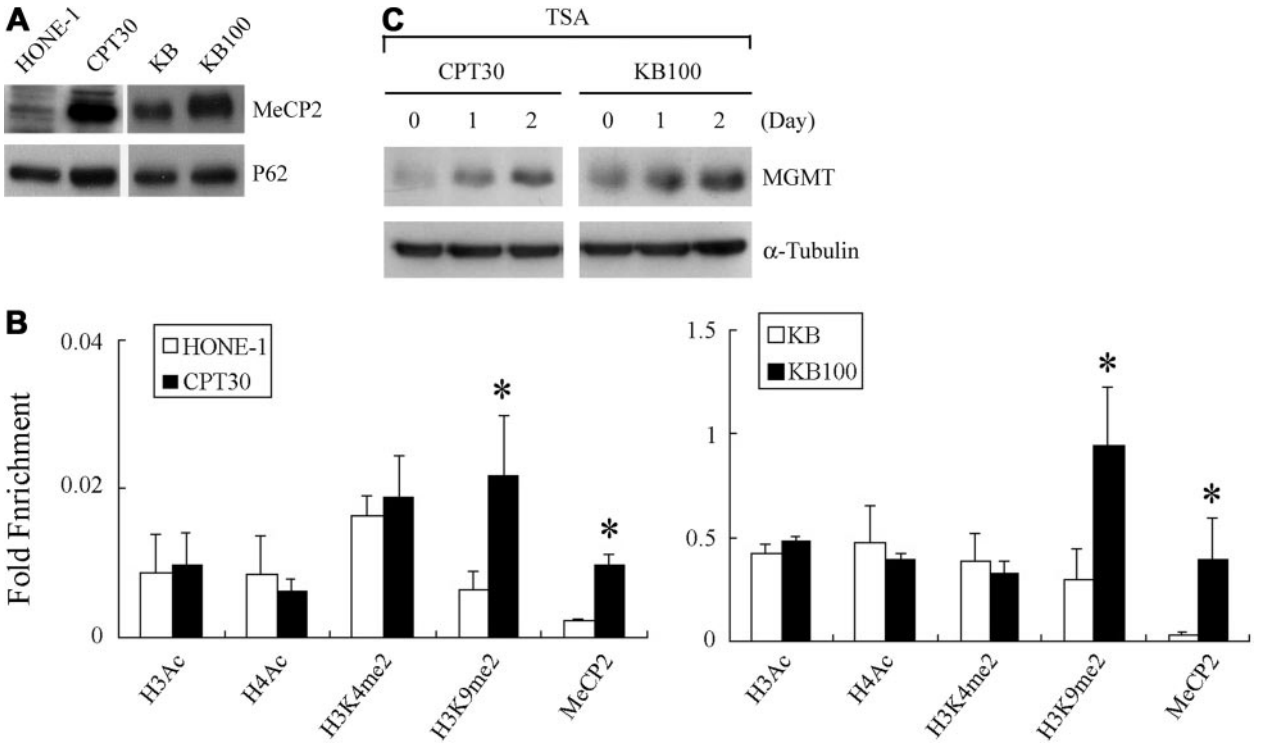


Fig. 5. Status of histone modification and MeCP2 binding in two sets of parental and CPT-resistant cell lines. A, measurement of the expression level of MeCP2 protein in two sets of parental and CPT-resistant cells. Cell nuclear proteins were extracted according to manufacturer's instructions with a Nuclear Protein extraction kit (Pierce), and an aliquot of protein (50 μg) from each sample was subjected to Western blot analysis. Nucleoporin p62 was used as the loading control of nuclear protein. B, ChIP-Q-PCR assay of H3Ac, H4Ac, H3K4me2, H3K9me2, and MeCP2 was bound to the endogenous MGMT promoter in two sets of parental and CPT-resistant cell lines. Left, quantification of the ChIP results in HONE-1 and CPT30 cells; right, quantification of the ChIP results in KB and KB100 cells. Experiments were repeated three times independently. The results are expressed as the means ± S.D.; *, $P < 0.05$ compared with parental cell lines. C, MGMT protein expression after exposure to TSA in CPT-resistant cells measured by Western blot analysis. Cells were treated with 0.5 μM TSA for the indicated times. α-Tubulin was used as an internal control in this study.

regulated at the transcriptional level through the blocking of MGMT gene transcription and independent from mRNA stability.

Genetic and epigenetic alterations have been identified that lead to transcriptional dysregulation. Our results demonstrated that reduction of MGMT transcripts is not associated with any gross changes of MGMT gene, because no gross deletions, rearrangements, or amplification had taken place in genomic sequence encoding for the MGMT gene in CPT-resistant cells. This result indicates that the reduction of MGMT gene was not due to gene abnormalities. Thus, we proposed that epigenetic modification may involve MGMT silencing in CPT-resistant cells.

Two major epigentic changes, including aberrant DNA methylation and alterations of histone modifications in chromatin, have been found to play an important role in epigenetic dysregulation of gene expression (Feinberg and Tycko, 2004). Aberrant hypermethylation of the cytosine of CpG island in the promoter region accounts for the silencing of MGMT gene (Watts et al., 1997; Esteller et al., 1999). In contrast, our data indicated that there is no significant difference in CpG methylation of MGMT promoter between parental and CPT-resistant cells, and treatment with 5-aza-dC could not restore the expression of MGMT in CPT-resistant cells, suggesting that MGMT gene maybe silenced in CPT-resistant cells via methylation-independent mechanisms.

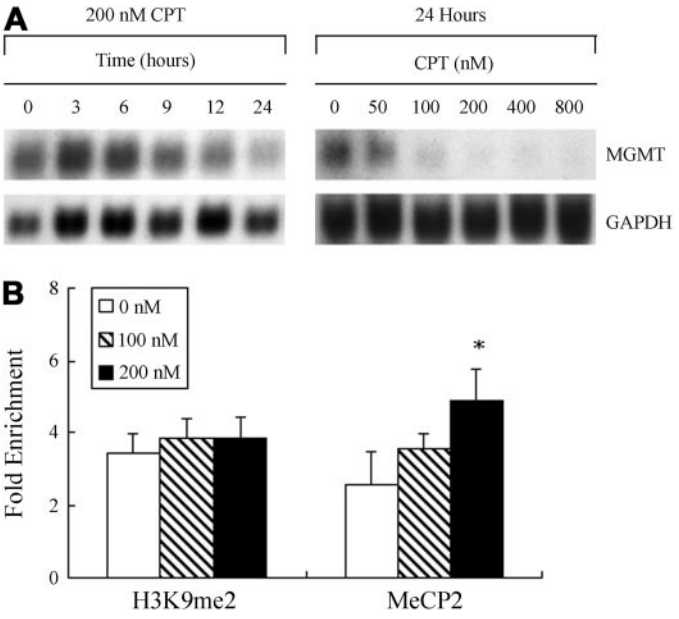
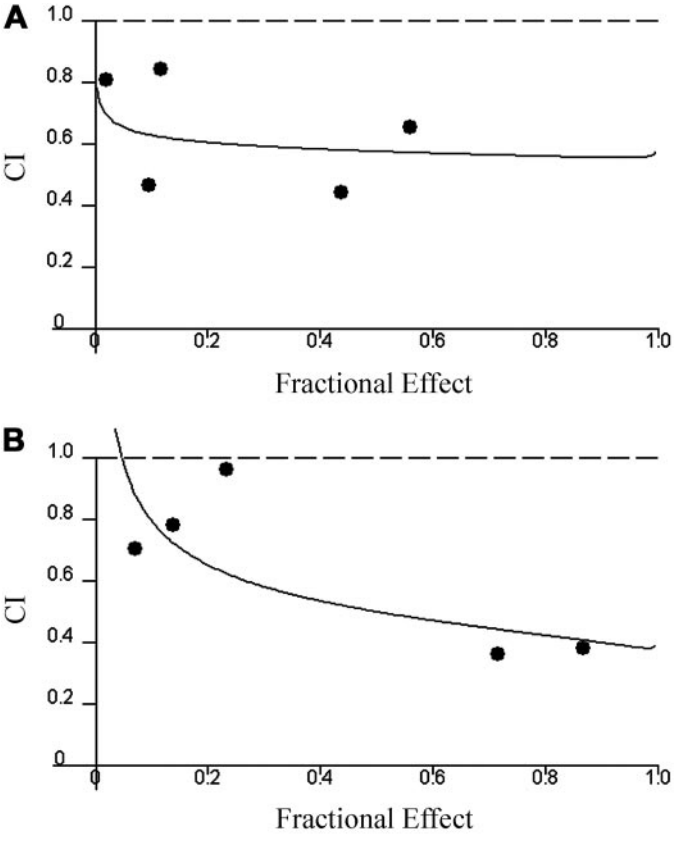


Fig. 6. Short-term effect of CPT on MGMT transcripts, H3K9 dimethylation, and MeCP2 binding in MGMT-proficient HT-29 cells. A, changes in MGMT mRNA levels in response to CPT in HT-29 cells were analyzed by Northern blot. Total RNA was isolated from each cell and probed with ^{32}P-labeled MGMT cDNA. Transcription of GAPDH was used as internal control of this assay. Left, time course of the response to CPT. Cells were stimulated with 200 nM CPT after plating and harvested at the indicated times (0, 3, 6, 9, 12, and 24 h). Right, dosage effect of the response to CPT. Cells were stimulated with increased concentration of CPT (0, 50, 100, 200, 400, and 600 nM) after plating and were harvested at 24 h. Results shown are representative data from three independent experiments. B, evaluation of the status of H3K9 dimethylation and MeCP2 binding in CPT-treated cells. HT-29 cells received short-term exposure to CPT at concentrations of 100 and 200 nM for 24 h and were analyzed by ChIP-Q-PCR. Experiments were repeated three times independently. The results are expressed as means ± S.D.; *, $P < 0.05$ compared with untreated cells.

Recently, the histone modifications and MBD protein binding have been demonstrated to play critical roles in epigenetic silencing (Wade, 2001; Peters and Schubeler, 2005). H3K9 dimethylation is largely associated with gene silencing and heterochromatin formation among the various sites of histone modification (Zhang and Reinberg, 2001). In this study, a high level of H3K9me2 through the CpG island was also an important epigenetic factor for MGMT gene silencing in CPT-resistant cells. MeCP2, which functions as transcriptional repressor, contains a central transcriptional repression domain that can interact with various corepressor complexes.



C

Cells	CI at fraction affected (mean ± SD)		
	50%	75%	90%
HT-29	0.58 ± 0.05	0.57 ± 0.06	0.56 ± 0.08
HONE-1	0.50 ± 0.11	0.44 ± 0.08	0.40 ± 0.09

Fig. 7. Combination index values of sequential exposure to CPT and BCNU in MGMT-expressing cells. Combination index as a function of cell kill in HT-29 cells (A) and HONE-1 cells (B) exposed simultaneously to CPT and BCNU at their equipotent ratios. Cells were treated with five concentrations at a 1:10,000 fixed molar ratio of CPT (0.3125–5 nM) and BCNU (3.125–50 μM) for three generation times and analyzed by methyl blue dye assay as described above. The data represent a combination of three separate experiments, each done in triplicate. The fractional effect of each drug in combination was analyzed by CalcuSyn software with a mutually nonexclusive model. The solid line reflects the combination index plot predicted by the software. A combination index of 1.0 (shown by the broken line) reflects additive effects, whereas values greater than and less than 1.0 indicate antagonism and synergy, respectively. C, summary of combination index at 50, 75, and 90% fraction affected.

MeCP2 recruits histone deacetylase-repressive machinery, which removes acetyl groups from histones and results in gene silencing (Nan et al., 1997, 1998). It is generally known that MeCP2 binds specifically to methylated CpG islands (Nan et al., 1997). However, more recently, MeCP2 has been shown to mediate the assembly of novel chromatin secondary structures independently of its binding to methylated DNA (Georgel et al., 2003). Results from our present studies show that recruitment of MeCP2 is involved in both CPT-resistant cells. Our result also indicate that treatment with TSA restored the expression of MGMT in CPT-resistant cells, suggesting that TSA functions on the epigenetic reactivation of silenced MGMT gene, possibly through reduced transcriptional repression by MeCP2. These results are consistent with the recent findings that both epigenetic factors MeCP2 and H3K9me2 are commonly and completely associated with MGMT silencing in different types of cancer cells, regardless of DNA methylation status and histone deacetylation (Zhao et al., 2005). However, we could not exclude that some other factors might participate in MGMT down-regulation because the changes of MeCP2 and H3K9me2 seem not to be in proportion with the MGMT down-regulation level in CPT30 and KB100 cells.

MeCP2 is involved in histone methylation in vitro and in vivo, and MeCP2-associated methylation is specific for H3K9 at the H19 gene (Fuks et al., 2003b). The repressor-element-1-silencing transcription factor and repressor-element-1-silencing transcription factor-corepressor-1 repressor complex are two key epigenetic factors. This complex binds to repressor elements of target gene promoters (such as that of the sodium channel type II gene), recruits MeCP2, and inactivates transcription through H3K9 methylation, which is carried out by the histone lysine methyltransferase suppressor of variegation 3–9 homolog 1 (Fuks et al., 2003a). Our study shows that there were no significant changes in the acetylation level in H3 and H4 between parental and CPT-resistant cell lines. It supports the above linkage and indicates that MeCP2-mediated H3K9 dimethylation is another important repressive mechanism for MeCP2 to silence gene transcription aside from the recruitment of histone deacetylase. In addition, the phenomenon of H3K9 dimethylation was only increased in the two CPT-resistant cell lines but not in short-term CPT-treated cells; however, the level of MeCP2 increased in both conditions. These data revealed that MeCP2(s) recruitment might occur before H3K9 dimethylation and further support the MeCP2-associated H3K9 methylation. Altogether, this study indicates that MeCP2 contributes to the methylation of H3K9 and highlights the potential involvement of these MeCP2-related epigenetic modifications in transcriptional repression of MGMT gene in our CPT-resistant cells.

Reports have suggested that silencing of MGMT can be a good predictive marker for chemotherapy when O^6-alkylating agents are used. Recent studies showed that synergistic anti-tumor activity arises when irinotecan (CPT-11, a water-soluble chemical derivative of CPT) is given in combination with BCNU (Coggins et al., 1998; Castellino et al., 2000). This combination therapy has undergone a formal phase II trial (Friedman et al., 2003), although the mechanism of the synergistic irinotecan/BCNU-induced antitumor effect is still unclear. In this study, our data showed that down-regulation of the MGMT gene could be achieved by a continuous exposure of CPT in cancer cells during CPT resistance development. In addition, a decrease in MGMT expression was also observed in pulsatile treatment of CPT in HT-29 cells in a concentration- and time-dependent manner. Moreover, strong synergistic interaction between CPT and BCNU was found in both HT-29 and HONE-1 cells. Taken together, the mechanism of synergism between CPT and BCNU might be through transcription repression of the MGMT gene.

In conclusion, our study demonstrated for the first time that CPT is able to suppress the transcription of the MGMT gene through recruiting of MeCP2 and H3K9 dimethylation, thus causing a synergistic interaction with BCNU. These findings provide an explanation regarding why the combination of CPT and BCNU in clinical settings results in better objective response than single-use alone. In addition, this study also supports a new indication for treating patients who are receiving refractory CPT derivatives with BCNU.

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