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TITLE PAGE

TRANSCRIPTIONAL REPRESSION OF *O*^{6}-METHYLGUANINE DNA METHYLTRANSFERASE GENE RENDERING CELLS HYPERSENSITIVE TO *N,N'*-BIS(2-CHLOROETHYL)-*N*-NITROSUREA IN CAMPTOTHECIN-RESISTANT CELLS

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RUNNING TITLE PAGE

Running Title: Transcriptional repression of MGMT in CPT-resistant cells

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Abbreviations: 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, di-methylation of lysine 4 on histone H3; H3K9me2, di-methylation of lysine 9 on histone H3; H4Ac, acetylation of lysine residues on histone H4; IC_{50}, the concentration of drug that inhibited 50% of cell growth; MDB, methyl-CpG binding domain; MeCP2, methyl-CpG-binding protein 2; MGMT, O^6-Methylguanine-DNA methyltransferase; MSP, methylation-specific PCR; PBMC, peripheral blood mononuclear cell; PLDBs, protein-linked DNA breaks; Q-PCR, Real-time quantitative PCR; SDS, sodium dodecyl sulfate; Top, topoisomerase; TSA, trichostatin A (TSA)

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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 di-methylation of H3K9 in the promoter region were found in CPT30 and KB100 cells. Furthermore, increased MeCP2 binding on MGMT promoter were also found to be corrected with MGMT gene silencing in short-term CPT treatment, thus, enhanced BCNU sensitivity in CPT-treated cells. Taken together, we suggest that CPT is able to suppress the transcription of MGMT gene through recruiting of MeCP2 and H3K9 di-methylation thus cause synergistic interaction with BCNU. These findings provide a possible explanation regarding why the combination of CPT and BCNU results in better objective response than single use alone. In addition, this study also suggest a new indication to treat patients whom refractory CPT derivatives with BCNU.

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INTRODUCTION

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 (Wang, 1996;Lee et al., 1993). 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 (Kaufmann et al., 1991;Hsiang et al., 1989). Despite Top I is the primary target of CPT derivatives (CPTs), however, no direct correlation between the levels of Top I/DNA cleavable complex and CPTs cytotoxicity has been observed (Goldwasser et al., 1996). The efficacy of CPTs for cancer therapy is explained only in part by their ability to damage DNA. Therefore, the response of individual genes to CPTs 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: (a) pre-target events such as uptake and remove of CPT; (b) drug-target events such as altered Top I level, activity, and Top I mutation; and (c) post-target events such as cell proliferation and DNA repair/recombination. Recently, post-target events have been shown to play

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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, hypersensitive to CPTs were showed, despite CPTs produced equal amounts of protein-linked DNA breaks (PLDBs) when compared to normal strains (Eng et al., 1988). Over-expression of X-ray repair crosscomplementing gene 1 (XRCC-1) has been found to play a role in the development of CPT-resistance in cancer cells (Park et al., 2002). Recently, we and others proposed that DNA repair protein O^6-Methylguanine DNA methyltransferase (MGMT) is at least in part 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 transfer the alkyl group from the DNA to an internal cysteine residue in the MGMT, thus restoring the integrity of DNA (Pegg, 1990). This action utilizes one MGMT molecule for each lesion repaired and makes MGMT as 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 anti-tumor 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 mono-functional triazenes (*e.g.*, dacarbazine) (Shiraishi et al., 2000). Increased expression of MGMT is associated with resistance of tumor cells to these drugs (Kokkinakis et al., 1997;Pegg, 1990;Brent et al., 1985). Conversely, MGMT-deficient cells showed hyper-sensitivity towards O^6-alkylating agents (Dolan et al., 1991;Day, III et al., 1980;Pegg, 1990). Thus, finding ways of controlling MGMT expression, which could enhance cytotoxicity of O^6-alkylating agents toward cancer cells, are of significant clinical

interest.

We have previously established 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 is 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 formation of PLDBs (Beidler et al., 1996). It is interesting to observe that these two CPT resistant cell lines showed hyper-sensitivity towards BCNU, compared to their parental cells due to down-regulation of MGMT gene. The aim of the present study was to elucidate the responsive factors of MGMT inactivation in these two CPT-resistant cells.

MATERIALS & 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 units/ml penicillin, and 100 μg/ml streptomycin. The two CPT-resistant cell lines, CPT30 and KB100 cells were maintained in growth medium supplemented with 30 and 100 nM of CPT (Sigma-Aldrich, St. Louis, MO), respectively. Medium was changed every 3-4 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 generation time. 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 concentration of drug that inhibited 50% of

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cell growth (IC_{50}) 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 previously described (Kuo et al., 2004). In brief, protein extracts were electrophoresed on sodium dodecyl sulfate (SDS) -polyacrylamide gels. After electroblotting to nitrocellulose membranes, the proteins were probed with anti-human MGMT monoclonal antibody (BD PharMigen, San Diego, CA), anti-humanα-tubulin antibody (Sigma-Aldrich), anti-MeCP2 polyclonal antibody (Upstate, Lake Placid, NY) or anti-nucleoporin p62 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Detection of immunoreactive signal was accomplished with Western Blot Chemoilluminescent Reagent Plus (PerkinElmer, Boston, 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). Briefly, the primers for PCR are 5'-AAGGATCCCCGTTTGCGACTTGGTACTT-3' (sense) and 5'-CGACGATATCAAGCGGCCGCCCGATGCAGTGTTACACG-3' (anti-sense), respectively. The PCR was performed for 30 cycles using thermal cycler as follows: 30s at 94℃; 60s at 64℃; 60s at 72℃ and final extension of 7 min at 72℃. 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). 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 BamH1 and Not 1.

Northern Blot Analysis. Total RNA was isolated from each cells by using Trizol

reagent (Invitrogen), and analysis of MGMT mRNA levels were performed as described previously (Chang et al., 2002). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as 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 GAPDH band.

Measurement of mRNA Stabilities. An inhibitor analysis of mRNA stability using actinomycin D was performed as previously described (Kroes and Erickson, 1995). Cells were treated with 2 μg/ml of actinomycin D for 3, 6, 9, 12 and 24 hr. Total RNA was isolated from each cell lines by using 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). Briefly, Nuclei were resuspended in nuclear freezing buffer (50mM Tris-HCl pH 8.3, 40% v/v glycerol. 5 mM $MgCl_2$, and 0.1 mM EDTA) and stored at –80^{o}C until used. Plasmid DNA containing MGMT and GAPDH were first cleaved with restriction enzyme, BamHI and EcoRI (New England Biolabs), respectively, to linearize the double-stranded DNA. The DNA samples were then denatured with 3M 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 (5mM 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/mmole, Amersham, Bucks, UK) and incubated at 37^{o}C for 30 min. The RNA was isolated with Trizol

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reagent (Invitrogen), according to the manufacturer's instructions. The RNA pellets were resuspended with DEPC-treated H_2O and then hybridized in a hybridization buffer containing 250 mM Na_2HPO_4 and 0.7% SDS for 48 hr at 65^oC with membrane loaded with 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 were digested to completion with EcoRI, HindIII, 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 previously described by using methylation-specific PCR (MSP) (Herman et al., 1996). Primer sets of MGMT promoter were for the unmethylated reaction 5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3' (sense) and 5'-AACTCCACACTCTTCCAAAAACAAAACA-3' (anti-sense) and for the methylated reaction 5'-TTTCGACGTTCGTAGGTTTTCGC-3' (sense) and 5'-GCACTCTTCCGAAAACGAAACG-3' (anti-sense). DNA from peripheral blood mononuclear cell (PBMC) was used as a control for unmethylated alleles of MGMT. Briefly, 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: 30s at 94^oC, 30s at 59^oC, 45s at 72^oC and a final extension of 2 min at 72^oC. The PCR products were resolved with 6% nondenatured polyacrylamide gels,

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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 are as followed: 5'-AGGTGTGTTAGGATCCTGCT-3' (sense); 5'-TGATTCAGTCTGCGCATCCT-3' (anti-sense). The PCR was performed for 30 cycles using thermal cycler as follows: 30s at 94^{o}C; 30s at 62^{o}C; 1min at 72^{o}C and final extension of 7 min at 72^{o}C. The PCR product was cloned into a plasmid by using TA Cloning Kit (Invitrogen). The probe for hybridization was digested with BamHI and NarI (New England Biolabs, Beverly, MA). The size of this probe is 558 bp.

Southern Bolt Analysis of MGMT Promoter Methylation. To analyze the methylation status of MGMT promoter, ten micrograms of genomic DNA was first digested to completion with Sac I 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 on 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 (ChIP) Assays. ChIP analyses were performed basically using EZ ChIP Assay Kit following the instructions of the manufacturer (Upstate Biotechnology). Briefly, protein extract from 1×10^{6} cells was cross-linked to DNA by 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

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suspended in 1 mL of ice-cold PBS 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 ^{o}C for 15 minutes. Supernatants were diluted 5-fold in ChIP dilution buffer (provide in kit). An aliquot (100 μL) of the chromatin preparation was set aside and designated as 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 ^{o}C with agitation. The cleared chromatin was immunoprecipitated with 5 μg of either anti-RNA pol II (for the positive control; Upstate), normal mouse IgG (for the negative control; Upstate), anti-acetyl-histone H3 (H3Ac; Upstate), anti-acetyl-histone H4 (H4Ac; Upstate), anti-dimethyl-histone H3 (Lys4) (H3K4me2; Upstate), anti- dimethyl-histone H3 (Lys9) (H3K9me2; Upstate), or anti-methyl-CpG-binding protein 2 (MeCP2) (Upstate) antibody and incubated overnight at 4^{o}C with rotation. Later, salmon sperm DNA/Protein G agarose beads was added to these samples and rocked for 1 hour at 4 ^{o}C. Protein A immune complexes were collected by centrifugation and washed with the recommended buffers for 5 minutes each. Immune complexes were eluted twice with 250 μL of elution buffer for 15 minutes at room temperature. Twenty microliters of 5 M NaCl were added to the combined eluents, and the samples were incubated at 65 ^{o}C for 4 hours; 10 mM EDTA, 40 mM Tris-HCl (pH 6.5), and 20 μg proteinase K were then added to the samples and incubated at 45 ^{o}C for 2 hours. 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

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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; 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C to amplify GAPDH. All 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 (Ct) method, as described in the ABI 7000 user guide and by Litt *et al.* (Litt et al., 2001) and Oshiro *et al.* (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: $2^{[(Ct\ \mathrm{GAPDH}-Ct\ \mathrm{Input})-(Ct\ \mathrm{MGMT}-Ct\ \mathrm{Input})]}$, 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 (Chou and Talalay,

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1984), based on the multiple drug effect equation. This analysis requires: (a) that each drug alone has a dose-effect relationship, and (b) 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}s. The advantage to this method is the automatic construction of a fraction affected-CI table, graph, and classic isobologram by the software. CIs of < 1 indicate greater than additive effects (synergism; the smaller the value, the greater the degree of synergy), CIs equal to 1 indicate additivity, and CIs > 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 hyper-sensitivity to BCNU. The sensitivities of parental and CPT-resistant cells to BCNU were presented in Figure 1A. The IC_{50} for HONE-1, CPT30, KB, and KB100 cells was 52±6, 14±3, 22±3, 8±2 μM, respectively. Accordingly, two CPT-resistant cells, CPT30 and KB100, were about 3.7 and 2.8-times more sensitive to BCNU than their parental cells. As MGMT is a primary determinant for BCNU cytotoxicity, we therefore examined the expression of MGMT in two pairs of cell lines. As shown in Figure 1B, the MGMT protein levels in CPT30 and KB100 cells were about 38% and 65%, respectively, compared with those in parental cells. In addition, the MGMT mRNA expression in CPT-resistant is consistent with the findings of MGMT protein analysis (Figure 1C) and showed by Northern blot analysis.

Reduced MGMT Gene Expression Was Regulated at the Transcriptional Level.

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Down-regulation of mRNA expression may result from a decrease in messenger RNA stability and/or a reduction in transcriptional activity of its gene. To address the question of whether CPT-resistant cells had shorter messenger RNA half-life of MGMT, we performed a time course experiment to measure the messenger RNA stability of MGMT in KB and KB100 cells. We treated cells with 2 μM of RNA polymerase inhibitor, actinomycin-D, to inhibit transcription and harvested cellular RNA at time points indicated on Figure 2A. Northern blot analysis was used to measure the rate of MGMT mRNA degradation. No significance difference in messenger RNA stability was showed in KB100 cells compare to the parental KB cells. In fact, the half-life of MGMT mRNA in KB cells (7.7±1 hr) was similar to that in KB100 cells (8.8±0.5 hr). We were unable to compare the half-life of MGMT mRNA between NONE-1 and CPT30 cells due to 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 if decreases in mRNA levels found in the CPT-resistant cells were due to a lower transcription rate. As shown in Figure 2B, the transcriptional rate in CPT30 and KB100 cells is respectively about 38±5% and 62±8% of their parental cells 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 of MGMT genomic sequence could affect the MGMT transcripts, Southern blot analysis was performed to detect any gross changes of 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 Figure 3, banding patterns created by all three restriction enzymes were identical between parental and CPT-resistant cells (HONE-1 *vs.* CPT30, KB *vs.* 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. Initially, methylation of MGMT promoter was studied using MSP of DNA obtained from two-paired 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 Figure 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 Figure 4B. Extensive methylation at the BssHII (nt –386, -44, -23), SacII (nt –329), EagI (nt –230) and NaeI (nt –163 and –171) sites were observed in both HONE-1 and CPT30 cells and showed by Figure 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

EagI site in KB100 cells is noted. Furthermore, no methylation at the SmaI (nt –69) is observed in all tested cells.

Pharmacologic manipulation of MGMT expression with DNA methylation inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), in CPT30 and KB100 cells have been performed in this study, however, addition of 5-aza-dC to CPT-resistant cells did not increase MGMT expression (Figure 4D). Taken together, these observations indicate the hypermethylation at the promoter region of 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 indicated that histone modifications and MDB proteins binding also play critical roles in epigenetic silencing. As shown in Figure 5A, the expression level of MeCP2 protein was significantly increased in both CPT-resistant cell lines. To investigate whether histone modification and MDB proteins binding at the MGMT promoter area are associated with MGMT expression in developing CPT-resistance, ChIP-Q-PCR assay was performed to analysis histone modification and MeCP2 binding between parental and resistant cells. Acetylation of lysine residues on histone H3, H4 (H3Ac and H4Ac), and di-methylation of lysine 4 on histone H3 (H3K4me2) are believed to be associated with open chromatin and active transcription, whereas di-methylation 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 Figure 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 compare to related-parental cells. MeCP2 bound to MGMT promoter also exhibited an apparently high level in resistant cells compared with

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parental cells.

Because histone deacetylase inhibitor, trichostatin A (TSA), could relieve the TRD-mediated repression induced by MeCP2, therefore, pharmacologic manipulation of MGMT expression with TSA in CPT-resistant lines were performed in this study. As shown in Figure 5C, MGMT protein was re-expressed with TAS treatment in time-dependent manner. Taken together, these observations suggested that high level 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.

As the two CPT-resistant cell lines, CPT30 and KB100, were established by continuous exposure to stepwise increasing concentration of CPT, we further investigated the short term effect of CPT on MGMT-proficient HT-29 cells. As shown in Fiugre 6A, expression of MGMT gene was decreased in a concentration- and time-dependent manner in CPT-treated HT-29 cells. Consistently, decrease in MGMT transcripts was also found in CPT derivatives－topotecan and 7-ethyl-10-hydroxycamptothecin (SN38, the active metabolite of irinotecan)-treated cells (Supplemental Figure 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 hr post-treatment of CPT, while the amount of MeCP2 was significantly increased ($P < 0.05$) (Fiugre 6B).

CPT Is Synergistic with BCNU in MGMT-expressing Human Cancer Cells.

To further clarify downregulation of MGMT gene by short term exposure to CPT whether enhanced BCNU sensitivity in human cancer cells, we used CalcuSyn analysis to evaluate if inactivation of MGMT by CPT impacts the cellular sensitivity

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to BCNU. Two MGMT-expressing human cancer cell lines were chosen in this study. As shown in Figure 7A and 7B, 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 were summarized in Figure 7C.

DISCUSSION

CPTs have been demonstrated to be effective against a broad spectrum of tumors (Hsiang and Liu, 1988). Due to clinical importance of CPTs, resistance mechanisms of CPTs have been studied extensively in various CPT-resistant cell lines. We and others have demonstrated that decreased-expression or alteration to Top I, reduced intracellular accumulation of CPT and alteration in DNA repairing machinery are responsible for CPT resistance (Beidler et al., 1996;Chang et al., 2002;Fujimori et al., 1996;Urasaki et al., 2001;Chang et al., 1992). Other than mechanisms of CPT resistance, numerous studies have investigated the relationship between CPT-resistant cells and other chemotherapeutic drugs. Interestingly, several evidences demonstrated that cells resistant to Top I-directed agents are, in some cases at least, hypersensitive to certain Top II-directed agents (Urasaki et al., 2001;Chang et al., 1992;Sugimoto et al., 1990). Therefore, it was suggested that co-administration 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 in 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 line, KB100, with its parental KB, and found similar result.

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Several evidences have showed 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 when compared to their parental lines. Furthermore, our study also demonstrated that down-regulation of MGMT protein was the result of decreased level of corresponding mRNA, suggested that reduction of MGMT expression in CPT-resistant lines were 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 when compared to their parental cell lines. These data indicated that reduced MGMT expression in CPT-resistant cells was regulated at transcriptional level through blocking of MGMT gene transcription, and independent from messenger RNA stability.

Genetic and epigenetic alterations have been identified that lead to transcriptional dysregulation. Our result 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 indicated that the reduction of MGMT gene was not due to gene abnormalities. Thus, we proposed that epigenetic modification may involve in MGMT silencing in CPT-resistant cells.

Two major epigentic changes include 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 is account 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

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promoter between parental and CPT-resistant cells, treatment with 5-aza-dC could not restored expression of MGMT in CPT-resistant cells, suggesting that MGMT gene maybe silenced in CPT-resistant cells via methylation-independent mechanisms.

Recently, the histone modifications and MDB proteins binding have been demonstrated to play critical roles in epigenetic silencing (Peters and Schubeler, 2005;Wade, 2001). H3K9 di-methylation is largerly associated with gene silencing and heterochromatin formation among the various sites of histone modification (Zhang and Reinberg, 2001). In this study, high level of H3K9me2 through the CpG-island was also an important epigenetic factor for MGMT gene silencing in CPT-resistant cells. MeCP2, which function as transcriptional repressor, contains a central transcriptional repression domain that can interact with various co-repressor complexes. MeCP2 recruits histone deacetylase repressive machinery, which removes acetyl groups from histones and resulting in gene silencing (Nan et al., 1998;Nan et al., 1997). It is generally known that MeCP2 bind 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 showed that recruitment of MeCP2 is involved in both CPT-resistant cells. Our result also indicated that treatment with TSA restored expression of MGMT in CPT-resistant cells suggested that TSA functioned on epigenetic reactivation of silenced MGMT gene maybe through relieve transcriptional repression by MeCP2. These results are consistent with the recent findings that both of 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

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factors might participate in MGMT down-regulation as the changes of MeCP2 and H3K9me2 seems not 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 (REST) and Rest-corepressor-1 (RCOR1) 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, SCN2A gene), recruits MeCP2 and inactivates transcription through H3K9 methylation, which is carried out by the histone lysine methyltransferase suppressor of variegation 3-9 homologue 1 (SUV39H1) (Fuks et al., 2003a). Our study showed 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 di-methylation is another important repressive mechanism for MeCP2 to silence gene transcription aside from recruitment of histone deacetylase. In addition, the phenomena of H3K9 di-methylation 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 two conditions. These data revealed that MeCP2(s) recruitment might be prior to H3K9 di-methylation and further support the MeCP2-associated H3K9 methylation. Altogether, this study indicated that MeCP2 contributes to the methylation of H3K9, and highlight the potential involvement of these MeCP2-related epigenetic modifications in transcriptional repression of MGMT gene in our CPT-resistant cells.

Documents 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 (Castellino et al., 2000;Coggins et al., 1998). This combination therapy has been undergone a formal Phase II trial (Friedman et al., 2003), although the mechanism of the synergistic irinotecan/BCNU-induced anti-tumor effect was still unclear. In this study, our data showed that down-regulation of MGMT gene could be achieved by a continuous exposure of CPT in cancer cells during CPT resistance development. In addition, decreased 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 MGMT gene.

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

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FOOTNOTES

[a] Li-Chen Ma and Ching-Chuan Kuo contributed equally and may be cited in either order.

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FIGURE LEGENDS

Figure 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 time. Growth inhibition of cells was determined by methylene blue dye assay. *Left panel*, effect of BCNU on the HONE-1 series cell line; *right panel*, Effect of BCNU on the KB series cell line. Each data point represents the mean ± SD 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 has been used as 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. Result shown is a representative data from three independent experiments.

Figure 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 the representative of three independent experiments in which similar results were obtained. Band density was quantified with scanning densitometer. Amount of MGMT mRNA was expressed as a

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relative percentage prior to the addition of actinomycin-D, and then the half-life of MGMT mRNA has been 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 in *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 (upper bands) and GAPDH (lower bands) fragments immobilized using a slot blot apparatus. The autoradiograms above are the representative of three independent assays in which similar results were obtained and expressed as the means ± SD.

Figure 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 the representative of three independent experiments in which similar results were obtained.

Figure 4. Methylation status of MGMT gene in two sets of parental and CPT-resistant cells.

(A) Methylation specific PCR anaylsis of MGMT promoter CpG methylation. Genomic DNA from HONE-1, CPT30, KB, and KB100 were modified with bisulfite as described under "*Material and Method*", and analyzed for methylated CpG sites using PCR primers, which distinguish unmethylated (U) and methylated (M)

sequences. DNA from peripheral blood mononuclear cell (PBMC) was used as a control for unmethylated alleles of MGMT. (B) Schematic representation of the 5'-portion of the MGMT loucs. 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 Sac I, 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 following exposure to 5-aza-dC in CPT-resistant cells measured by Western blot analysis. Cells were treated with maximum non-toxic dose of 5-aza-dC for the indicated times (0.4 μM and 1 μM for CPT30 and KB100 cells, respectively). α–Tubulin has been used as internal control in this study.

<u>Figure 5.</u> 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 manufacture's instructions with a Nuclear Protein extraction kit (Pierce Biotechnology, Rockford, IL), 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 bound to the endogenous MGMT promoter in two sets of parental and CPT-resistant cell lines. *Left panel*: Quantification of the ChIP results in HONE-1 and CPT30 cells; *right panel*: Quantification of the ChIP results in KB and KB100 cells. Experiments were repeated

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three times independently. The results are expressed as the means ± SD, * $P < 0.05$ compared to parental cell lines. (C) MGMT protein expression following 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 has been used as internal control in this study.

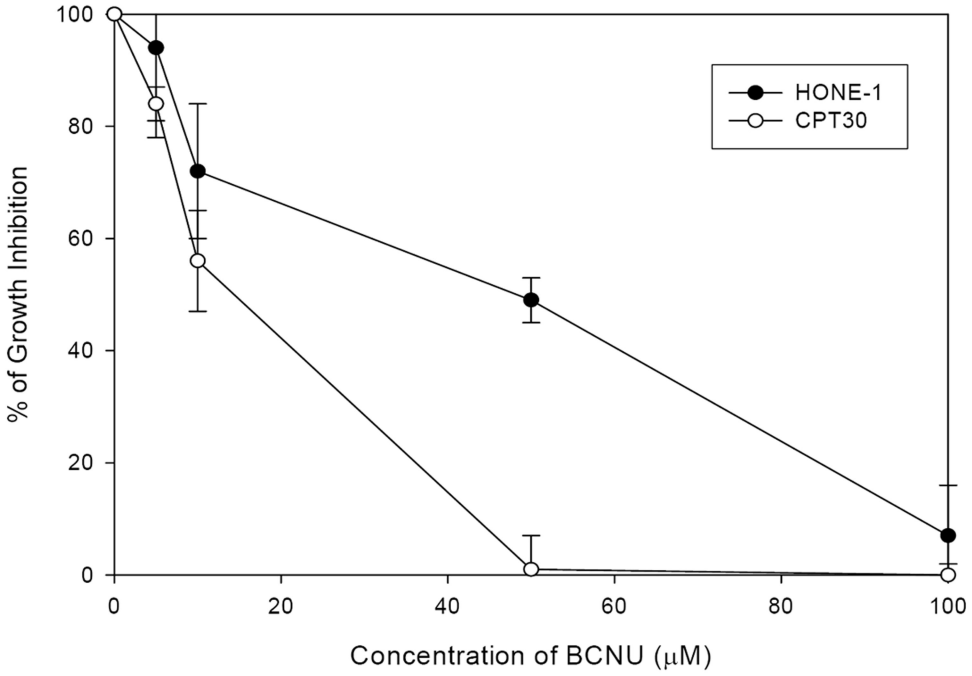
Figure 6. Short term effect of CPT on MGMT transcripts, H3K9 di-methylation, MeCP2 binding in MGMT-proficient HT-29 cells.

(A) Changes in MGMT mRNA levels in response to CPT in HT-29 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 panel*: Time course of the response to CPT. Cells were stimulated with 200nM CPT after plating and harvested at the indicated times (0, 3, 6, 9, 12, 24 hr). *Right panels*: Dosage effect of the response to CPT. Cells were stimulated with increased concentration of CPT (0, 50, 100, 200, 400, 600 nM) after plating and harvested at the 24hr. Result shown is a representative data from three independent experiments. (B) Evaluation of the status of H3K9 di-methylation and MeCP2 binding in CPT-treated cells. HT-29 cells were short term exposure to CPT at concentration of 100 and 200 nM for 24h, and analyzed by ChIP-Q-PCR. Experiments were repeated three times independently. The results are expressed as the means ± SD, * $P < 0.05$ compared to untreated cells.

Figure 7. Combinaiton index 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 3 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 *dashed line*) reflects additive effects, whereas values above and below 1.0 indicate antagonism and synergy, respectively. (C) Summary of combination index at 50, 75, and 90% fraction affected.

Figure 1A



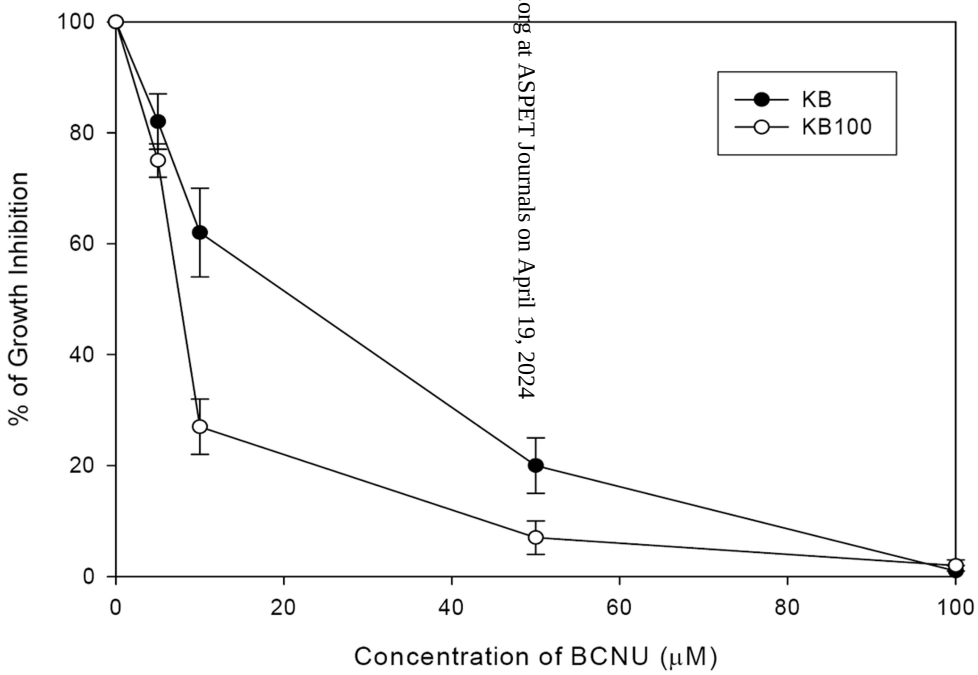


Figure 1B

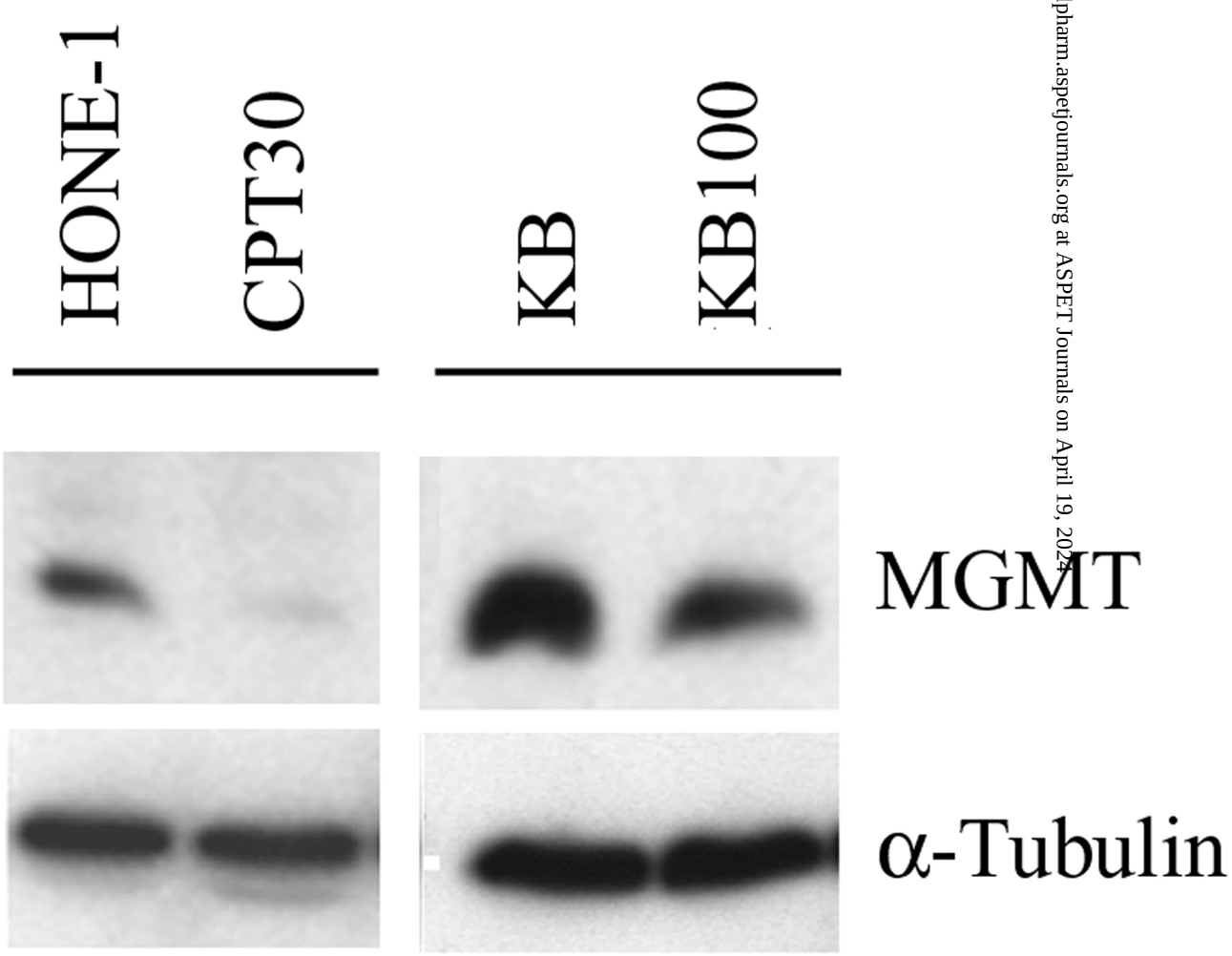


Figure 1C

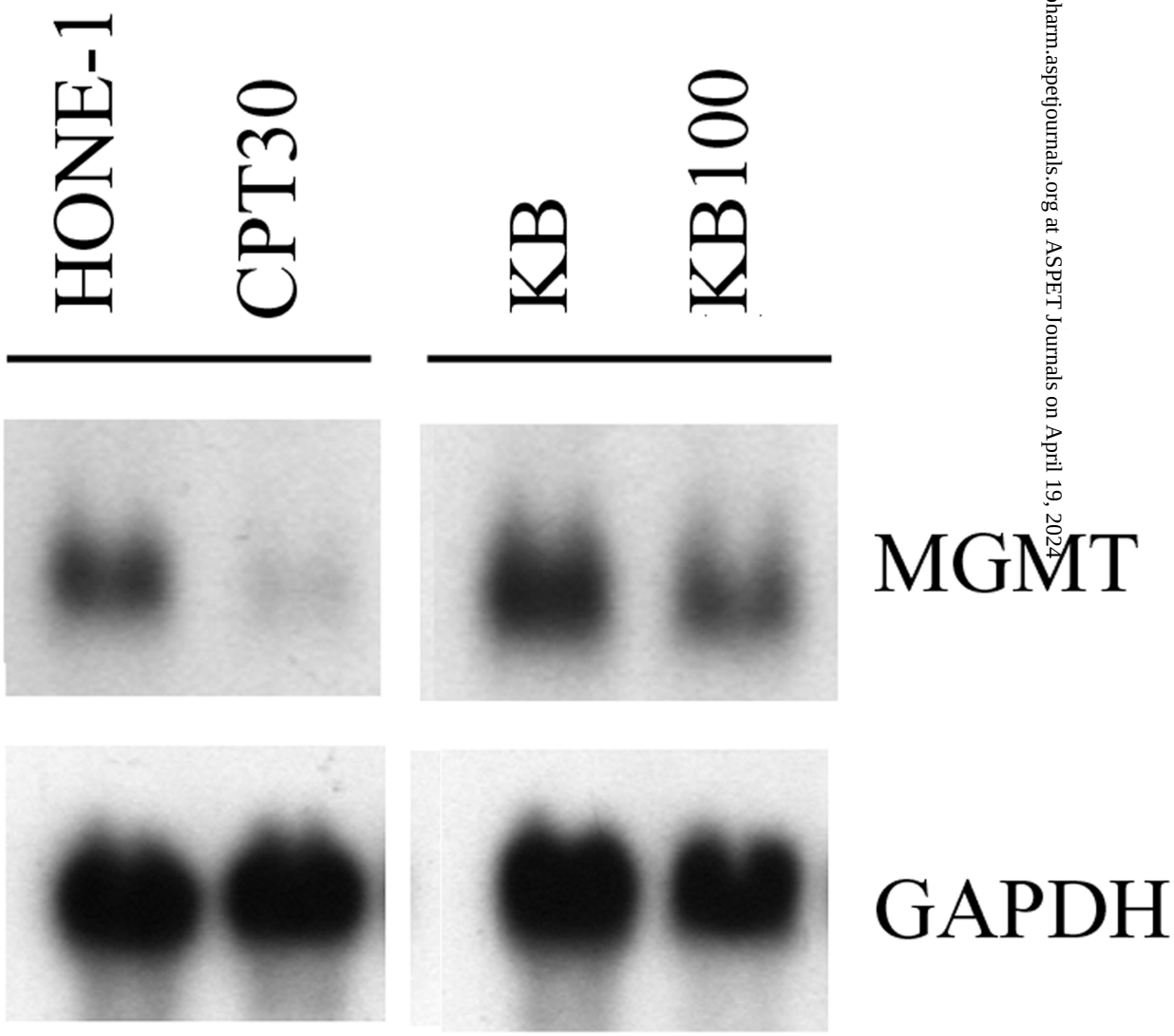


Figure 2A

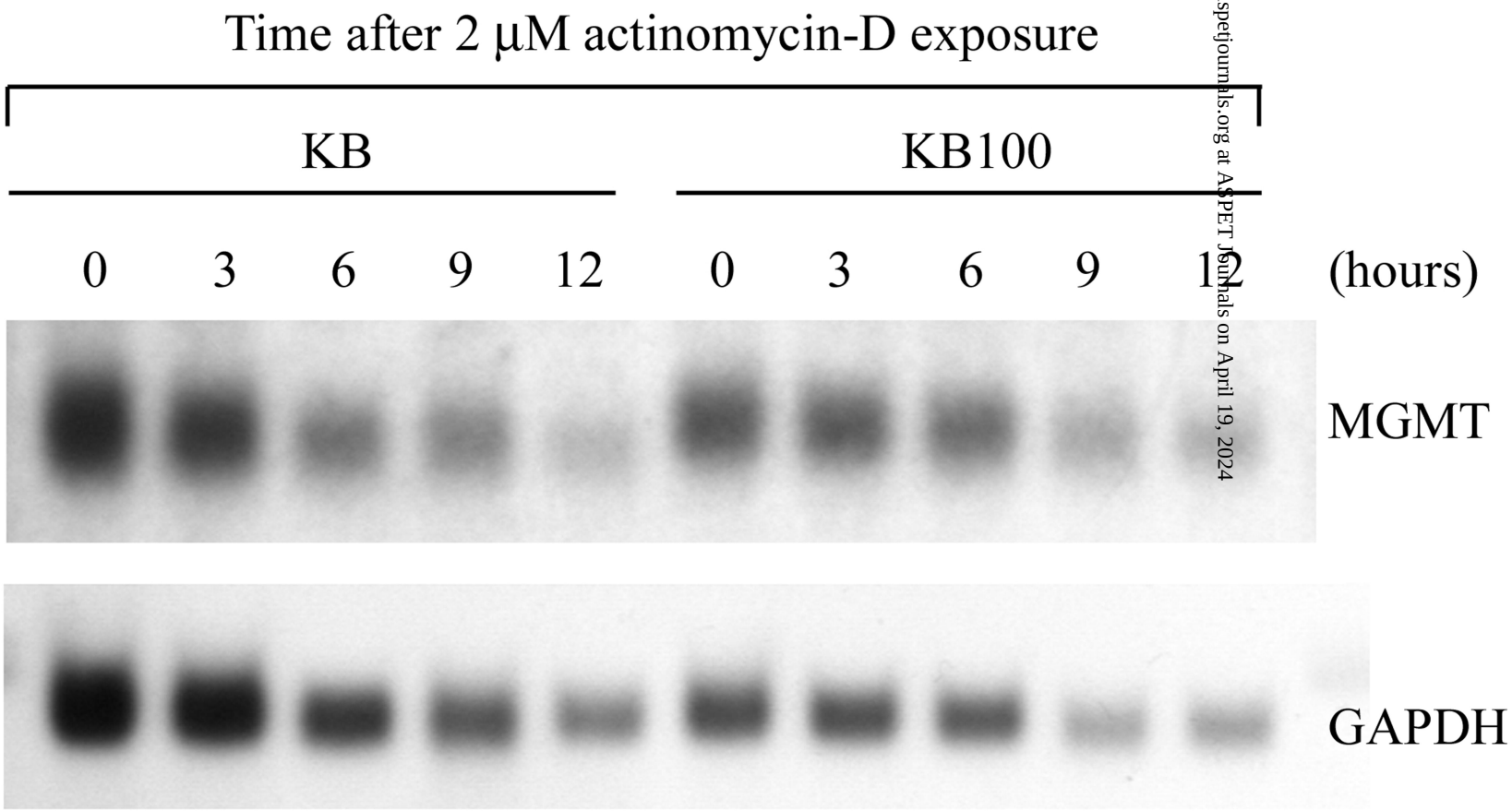


Figure 2B

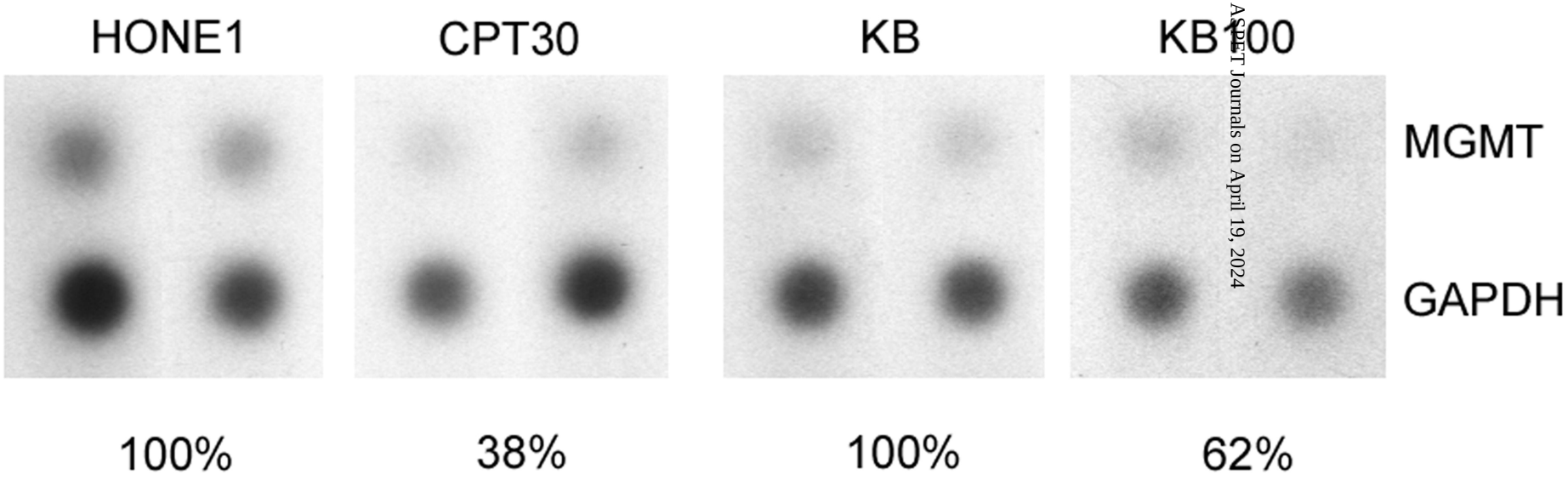


Figure 3

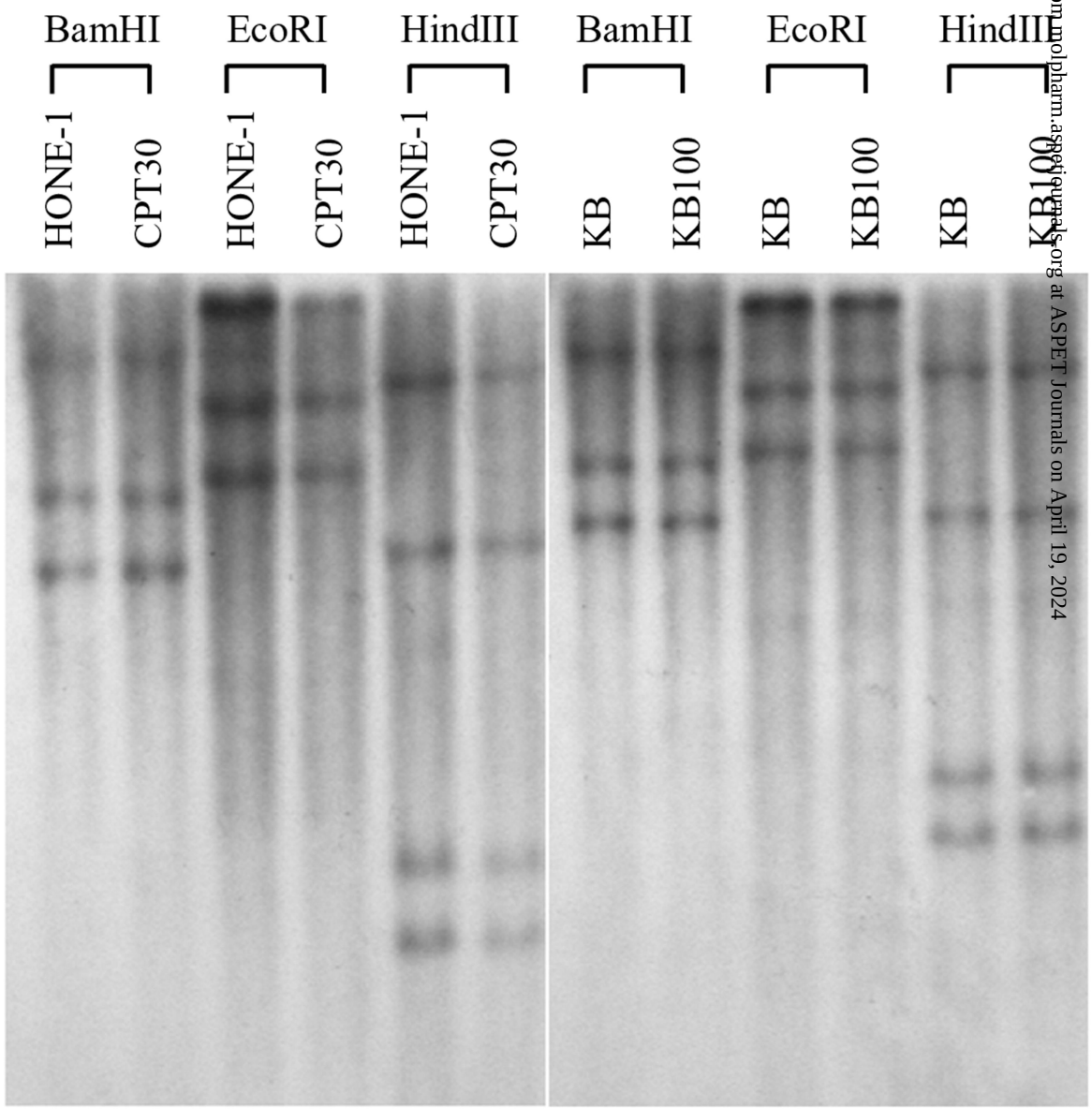


Figure 4A

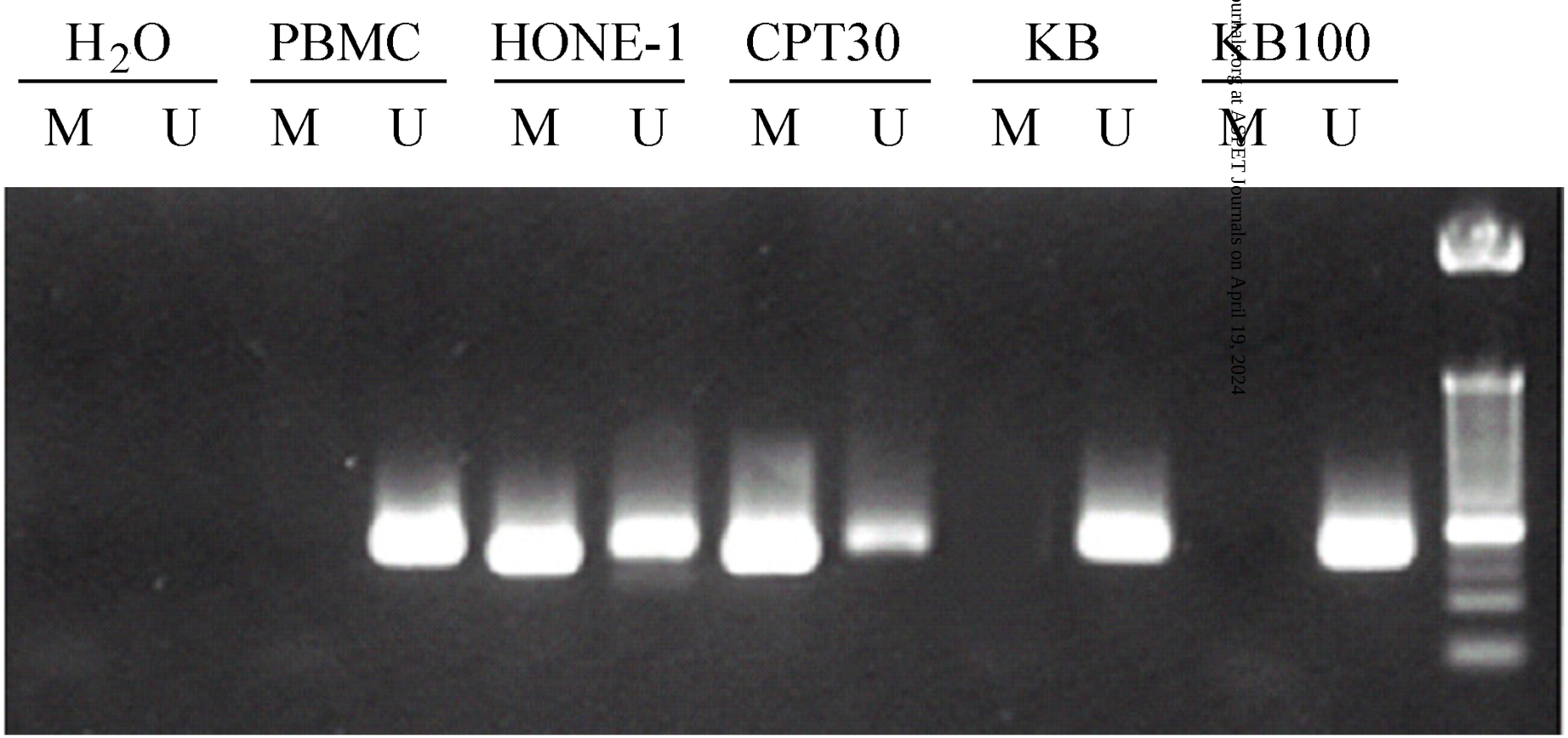


Figure 4B

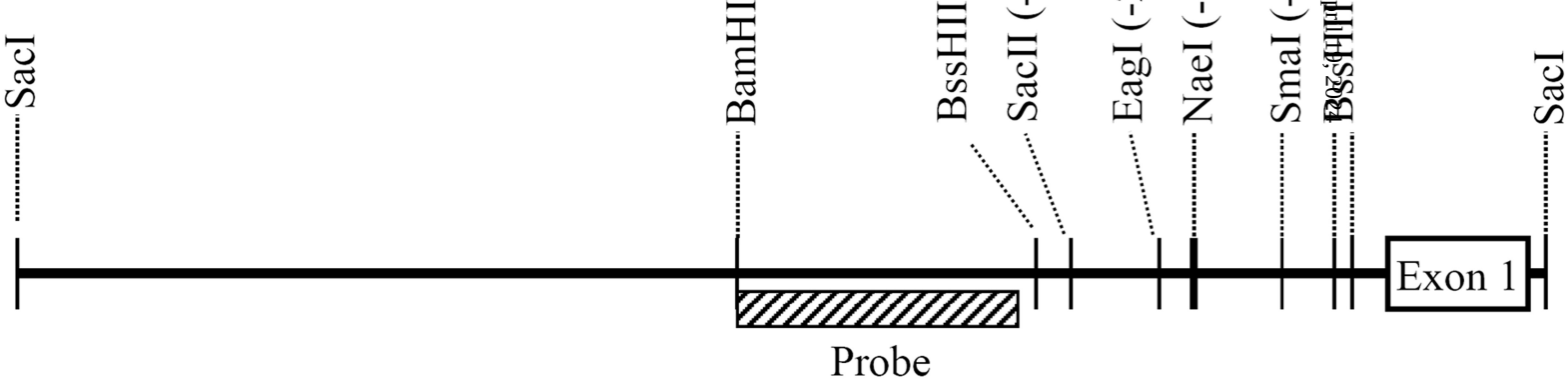


Figure 4C

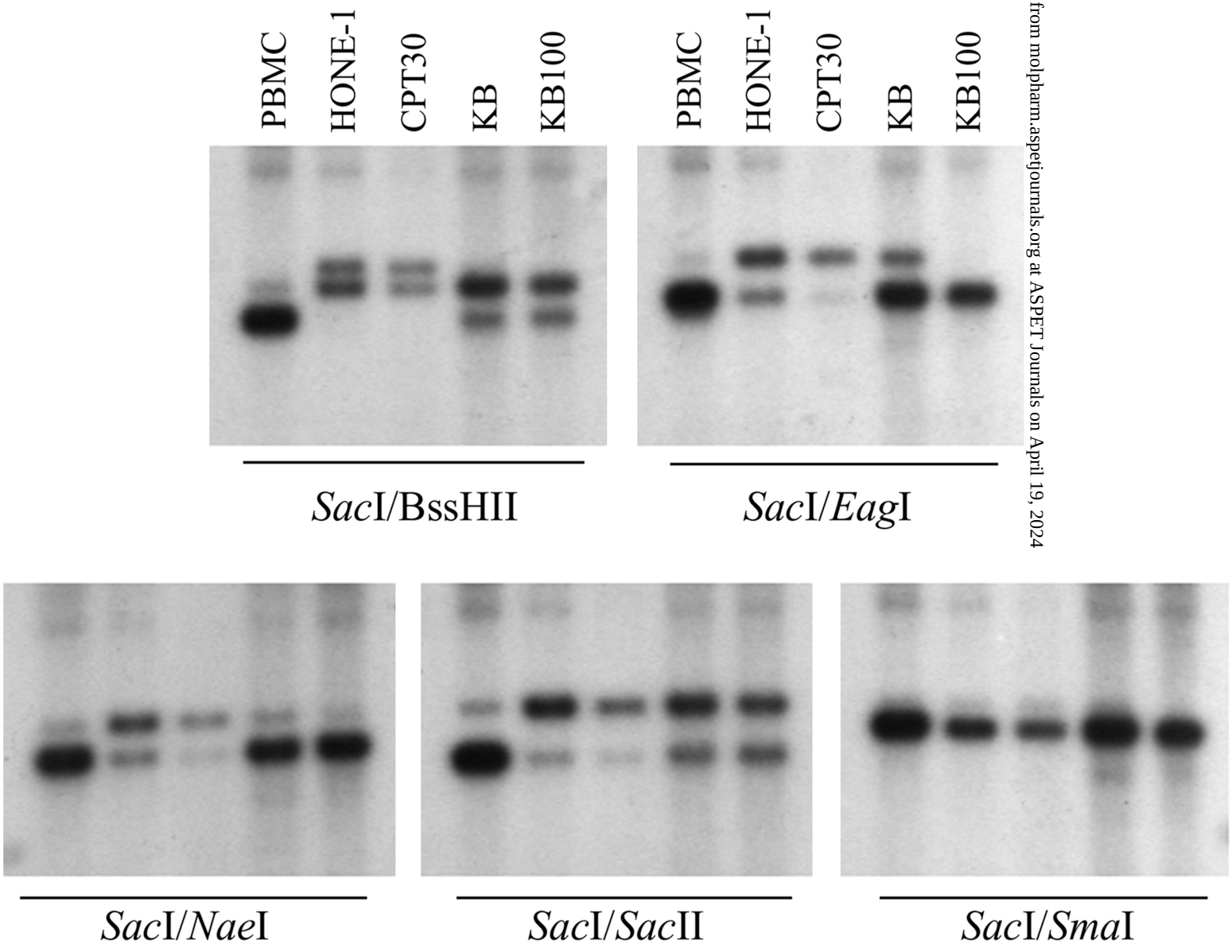


Figure 4D

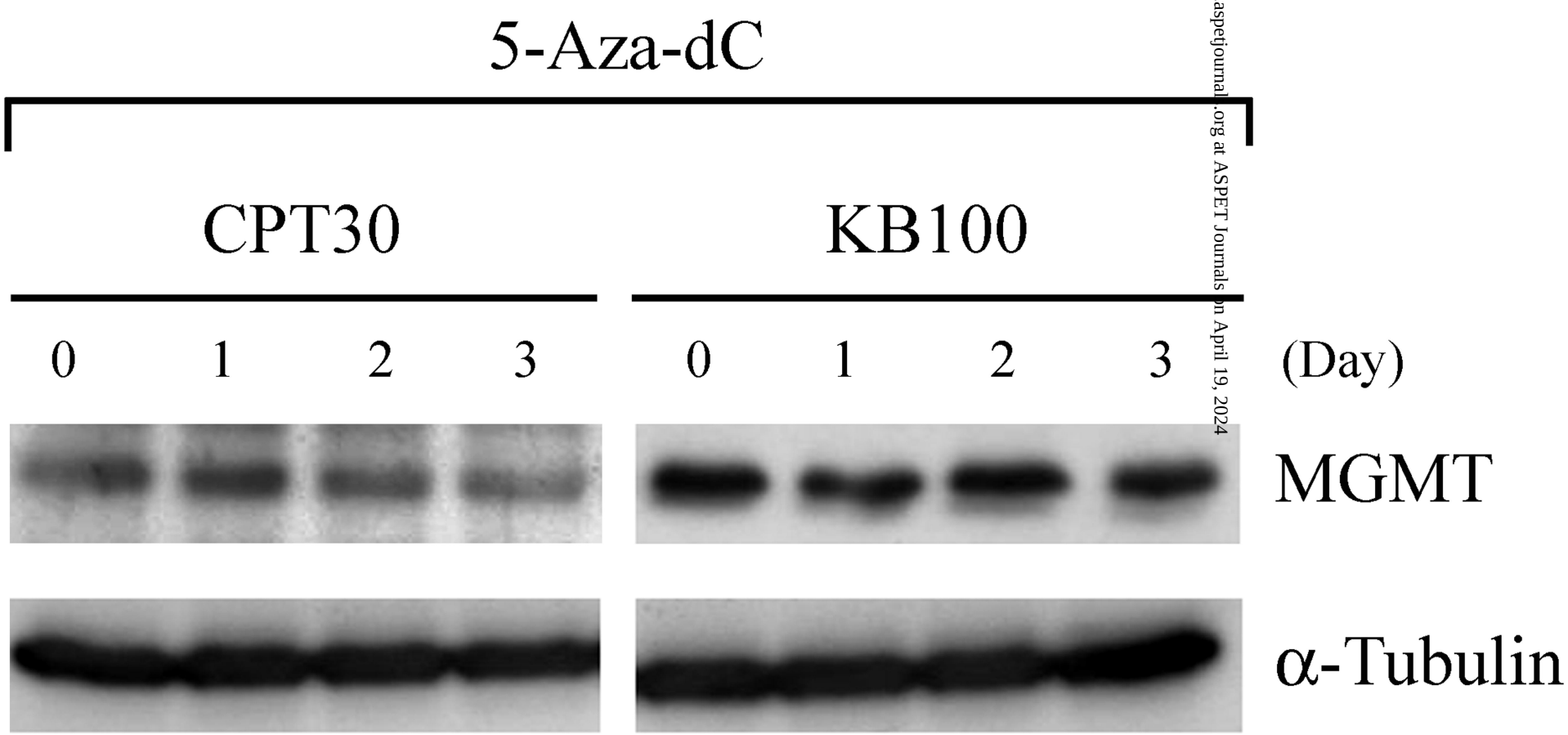


Figure 5A

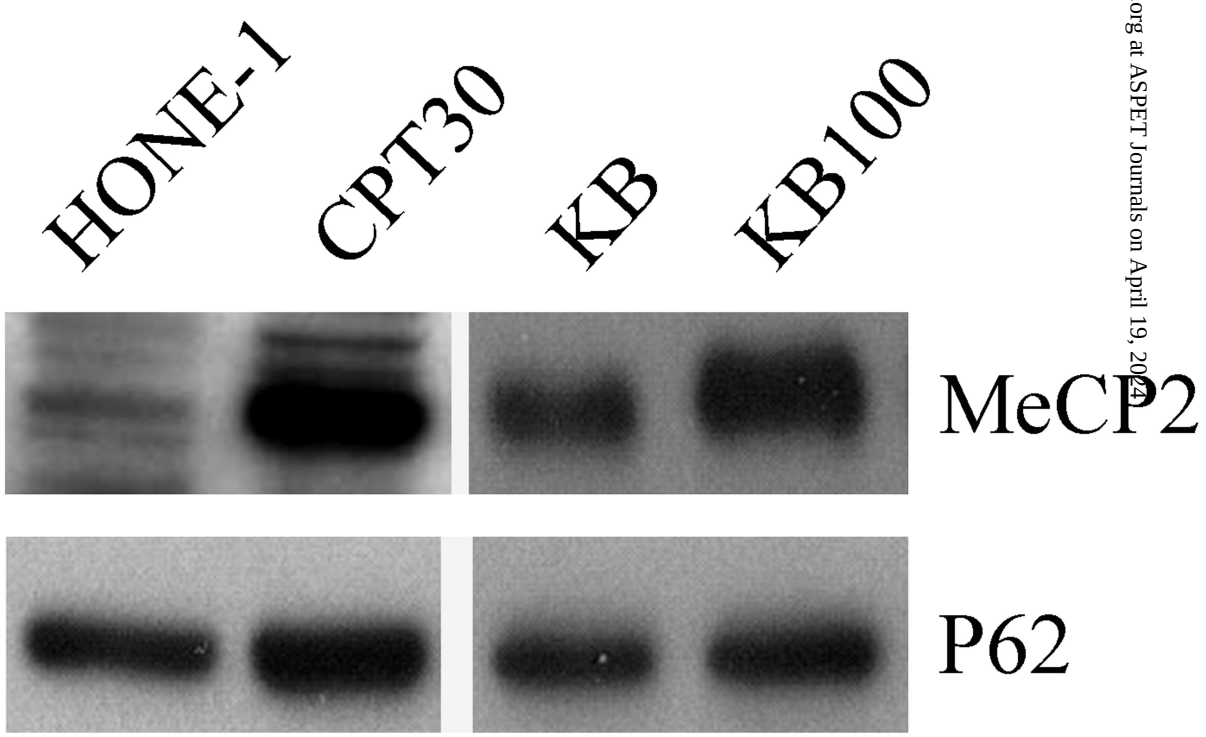


Figure 5B

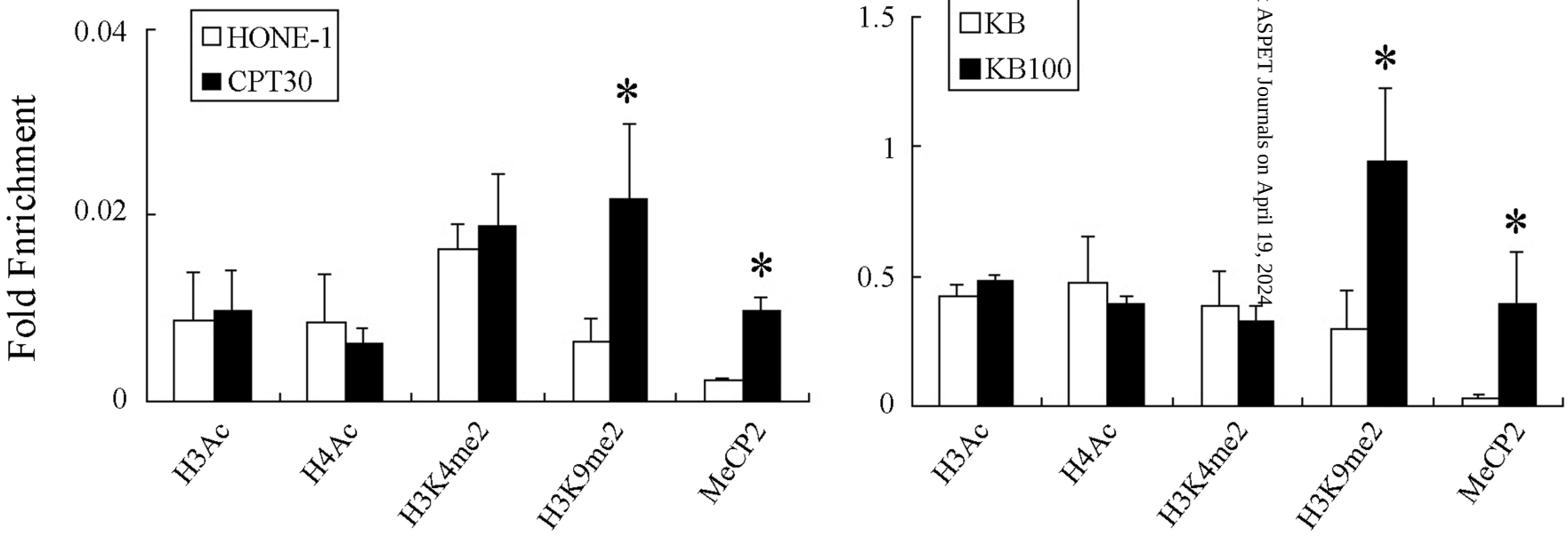


Figure 5C

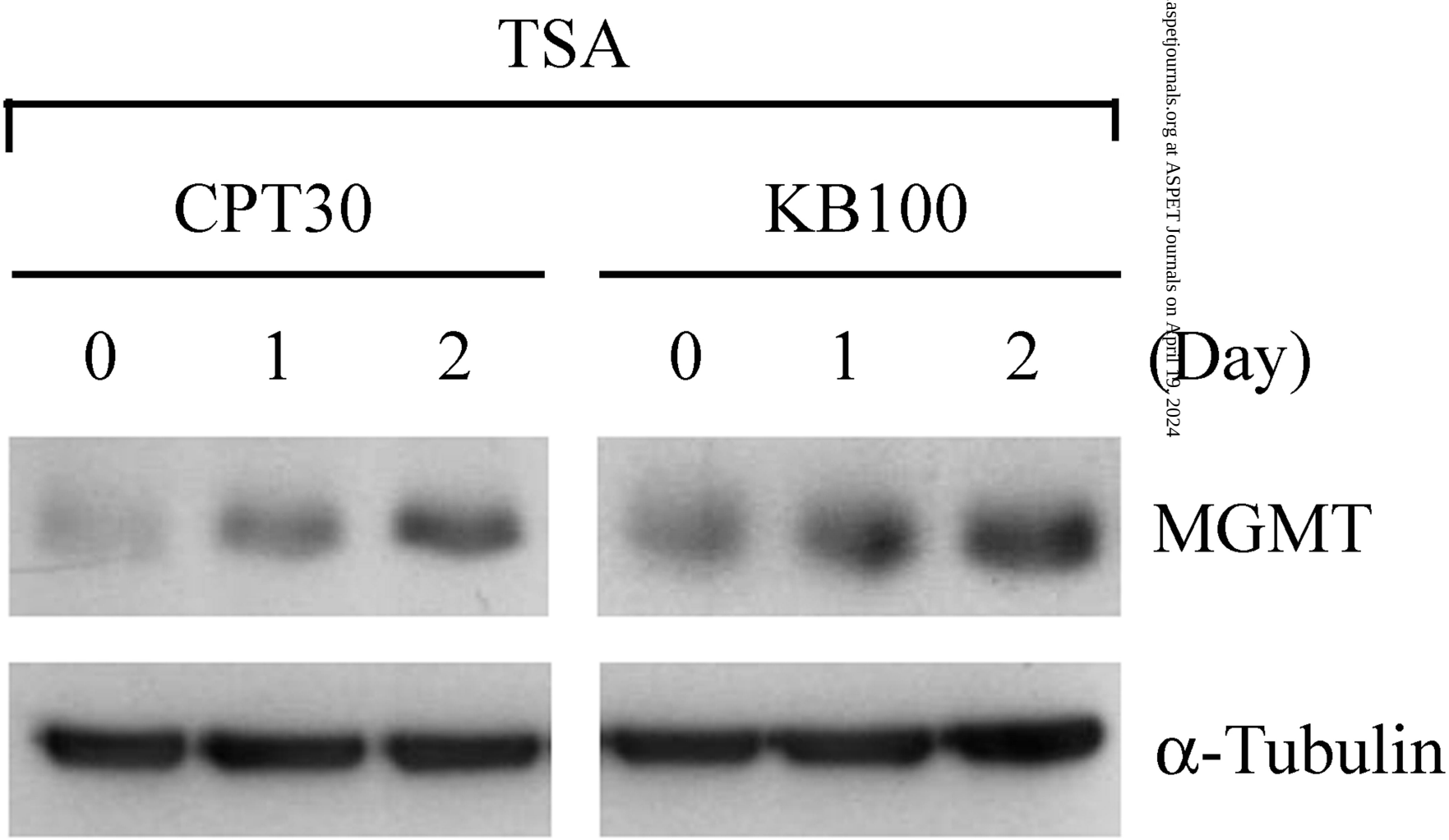


Figure 6A

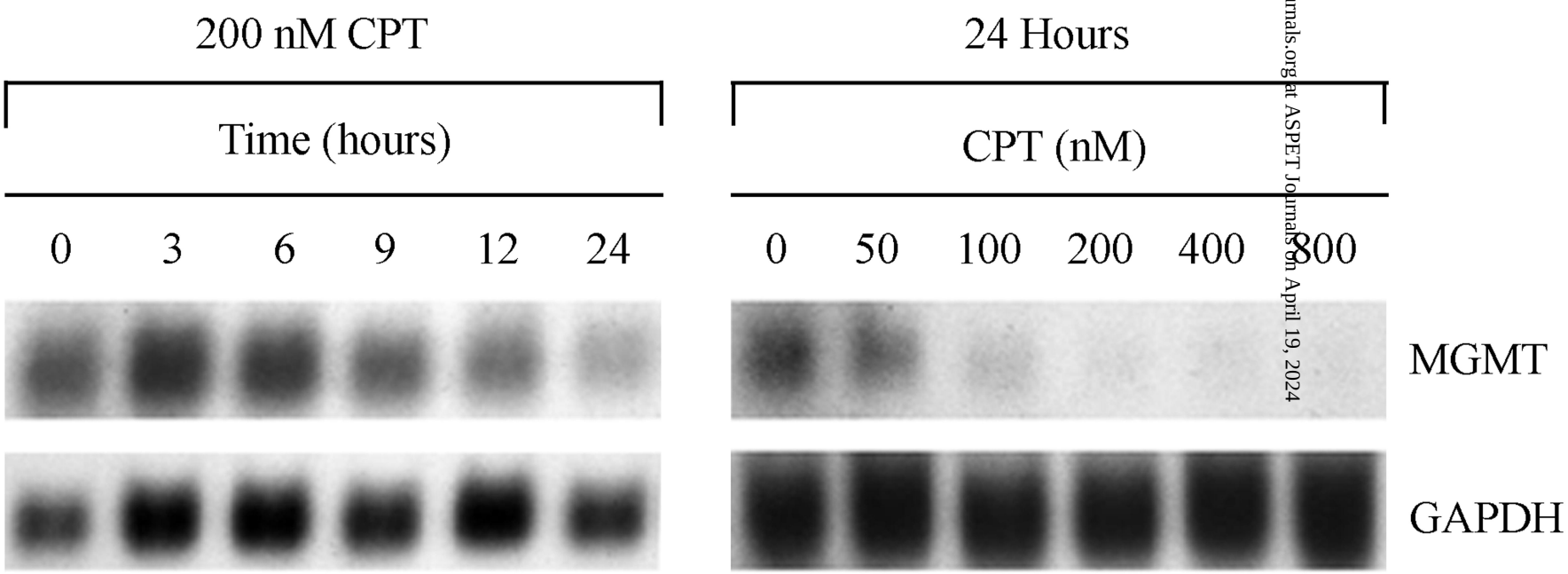


Figure 6B

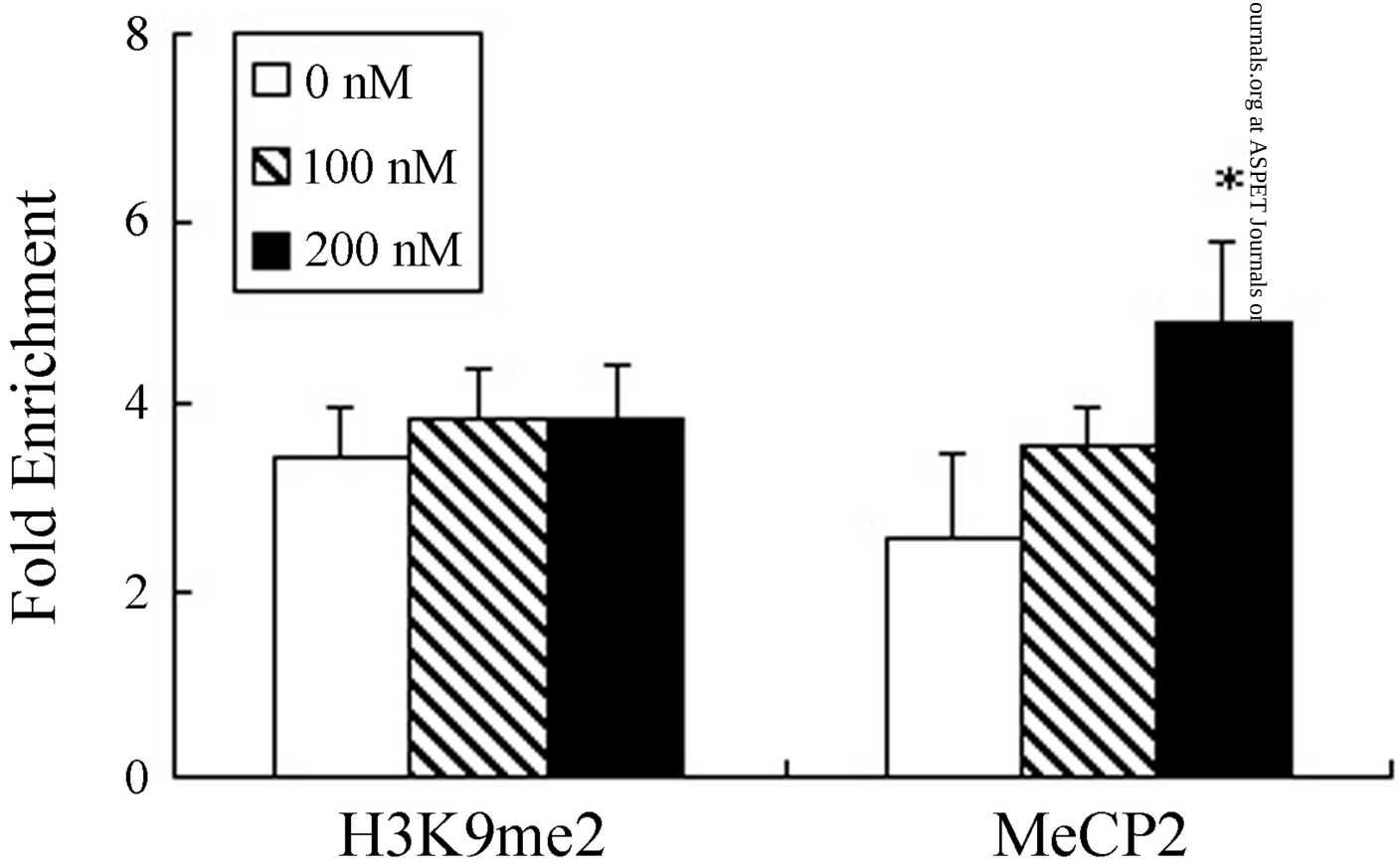


Figure 7A

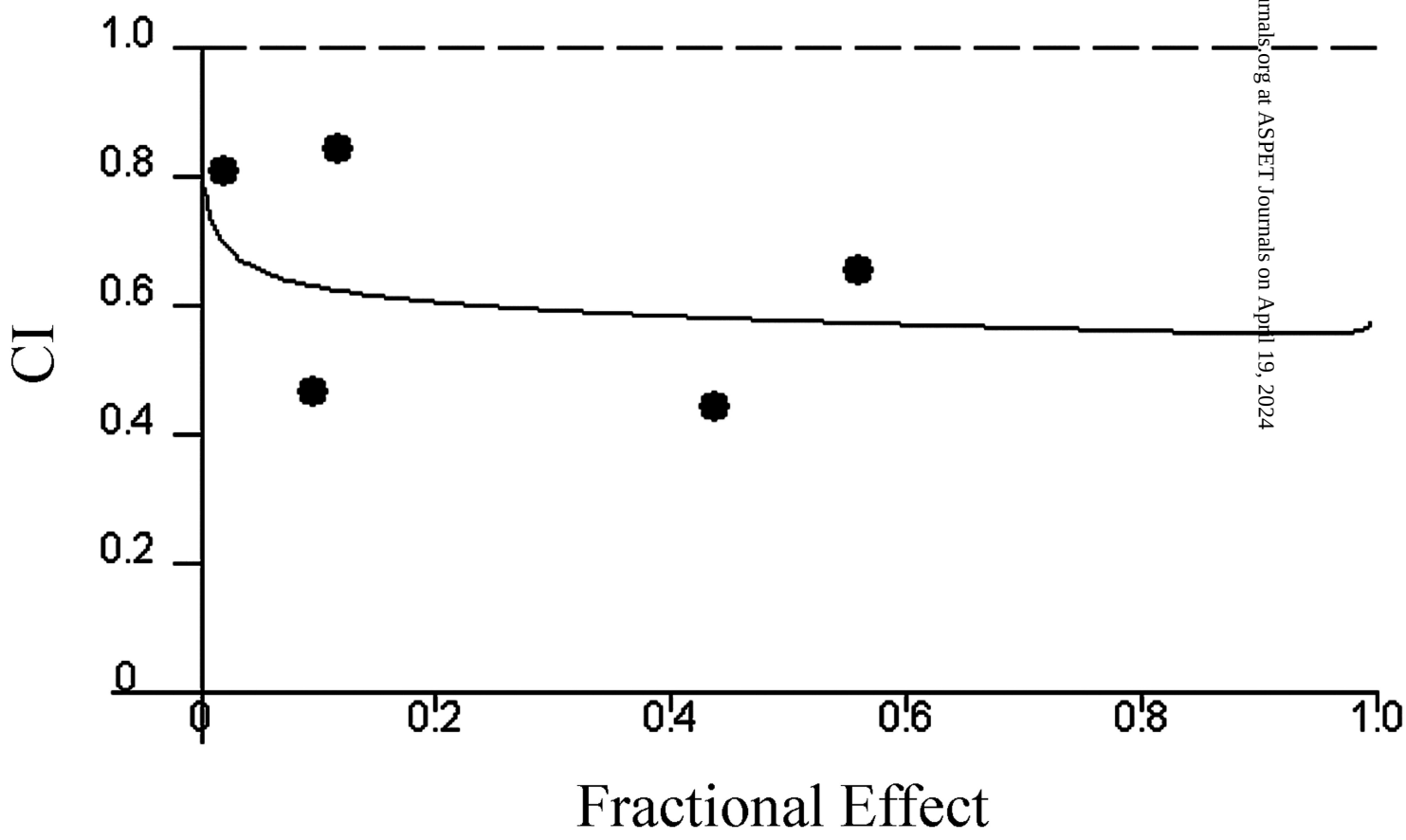


Figure 7B

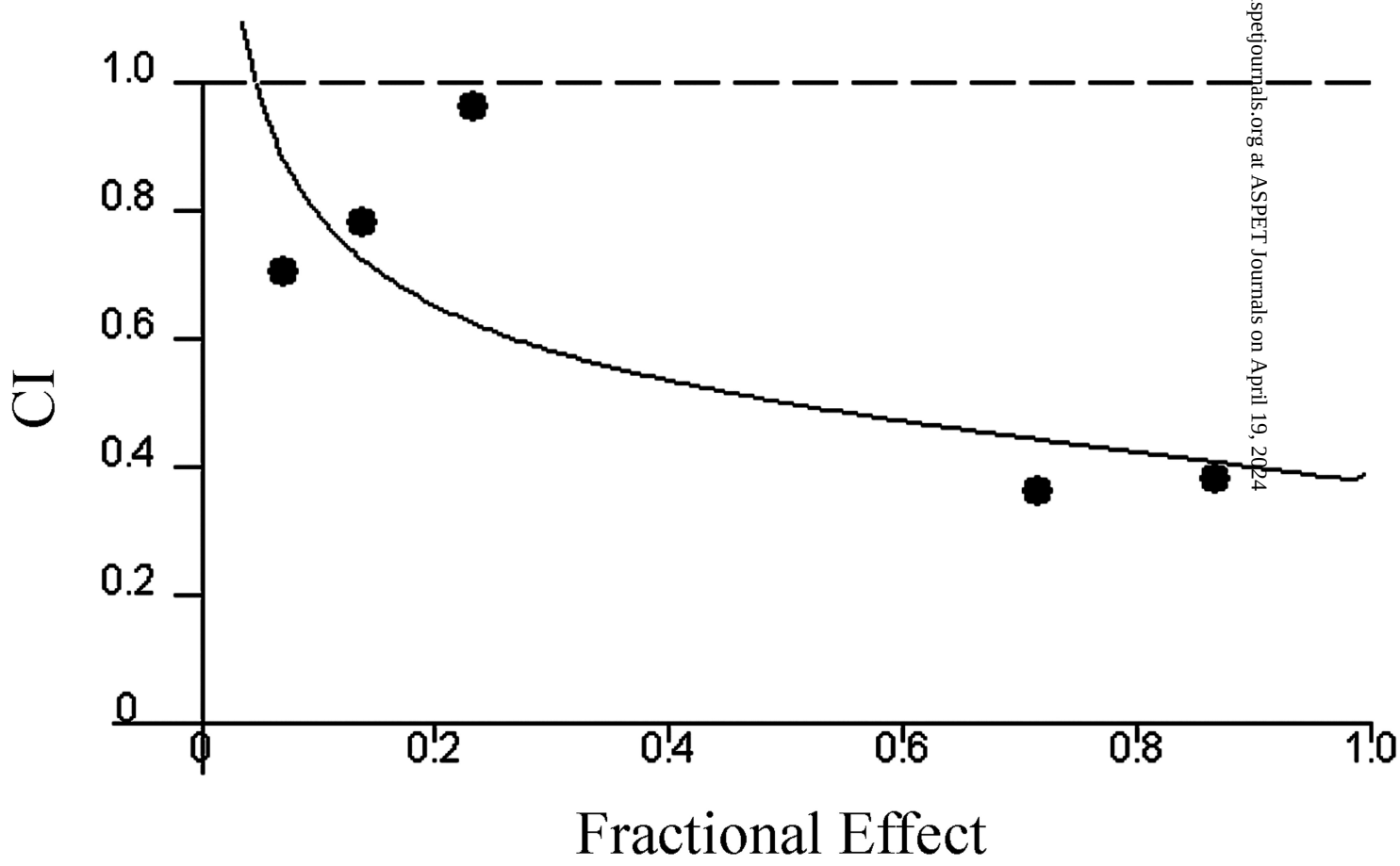


Figure 7C

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