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O^6-Methylguanine-DNA Methyltransferase (MGMT) as a Determinant of Resistance to Camptothecin Derivatives

Ryo Okamoto,[1] Hiroshi Takano,[1] Tatsunori Okamura,[2] Ji-Seon Park,[1] Keiji Tanimoto,[1] Takashi Sekikawa,[3] Wataru Yamamoto,[3] Alex Sparreboom,[4] Jaap Verweij[4] and Masahiko Nishiyama[1, 5]

[1]Department of Biochemistry and Biophysics, Research Institute for Radiation Biology and Medicine, [2]Department of Neurosurgery, School of Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, [3]Department of Internal Medicine, Toyosu Hospital, Showa University, 4-1-18 Toyosu, Koto-ku, Tokyo 135-8577 and [4]Department of Medical Oncology, Rotterdam Cancer Institute, Daniel den Hoed Kliniek and University Hospital Rotterdam, Groene Hilledijk 301, 3008 AE Rotterdam, The Netherlands

The precise mechanisms of resistance to camptothecin (CPT)-derived DNA topoisomerase (topo I) inhibitors and the determinants remain unclear. We found that a DNA repair protein, O^6-methylguanine-DNA methyltransferase (MGMT), participated in resistance to irinotecan hydrochloride (CPT-11), its active metabolite SN-38, and a novel CPT derivative, DX-8951f. In 17 human cancer cell lines, *MGMT* gene expression level closely correlated with sensitivity to the CPT derivatives, and inhibition of MGMT activity by nontoxic 5 μM O^6-benzylguanine augmented the drug activity in relation to the MGMT expression levels in 8 cell lines examined. Transfection of pCR/MGMT-sense into U-251MG and pCR/MGMT-antisense into T98G and HEC-46 cells revealed that increased MGMT expression decreased the sensitivity to CPT-11, SN-38, and DX-8951f, whereas repressed MGMT expression sensitized cells to the drugs. Western analysis revealed that treatment of *MGMT*-expressing T98G cells with the drugs caused a decrease of both MGMT and topo I in a dose-dependent manner. Although, in the transfectants, *MGMT* expression did not so closely correlate with the sensitivity to drugs as to nimustine hydrochloride (ACNU), MGMT is probably an important resistance determinant to CPT derivatives, and may play some role in the topo I-mediated DNA damage and/or the repair process.

Key words: MGMT — CPT-11 — DX-8951f — Drug resistance

Camptothecin (CPT) derivatives are among the most promising anticancer agents with a unique mechanism of action to inhibit DNA topoisomerase (topo) I activity. Their clinical importance has stimulated detailed research on mechanisms of resistance and their determinants to develop more effective chemotherapy. Several mechanisms of resistance to CPT derivatives have been reported: a point mutation of the *topo I* gene, a low *topo I* expression level, reduced drug accumulation, a decrease of drug activation for prodrug-type derivatives such as irinotecan hydrochloride (CPT-11), and an increase of drug inactivation by glucuronidation or oxidative metabolism by cytochrome P450 3A.[1, 2] The mechanisms of resistance to CPT derivatives, however, remain controversial.[3–6]

CPT derivatives bind to topo I-DNA complex, stabilizing the enzyme on DNA and preventing topo I from repairing the DNA strand prior to division and replication.[7] Recently, the repair of topo I-mediated DNA damage was suggested to be a key to resistance to CPT derivatives. It has been demonstrated that a wide range of base lesions, such as mismatches and alkylation, can poison topo I and DNA single-strand breaks occurring in close proximity to topo I complexes into irreversible topo I-DNA.[8] Further, among such lesions, alkyl groups at position 6 of guanines (O^6MG) influence cellular sensitivity to CPT derivatives.[9] O^6MG in DNA can enhance CPT-induced topo I cleavage complexes through its trapping action on the nuclear enzyme. In addition, our previous studies have suggested that O^6-methylguanine-DNA methyltransferase (MGMT), a DNA repair protein that removes alkyl adducts from O^6MG, may correlate with cytotoxicity induced by topo I inhibitors.[10, 11] Those findings led us to hypothesize that O^6MG-mediated topo I lesions and repair probably play an important role in resistance to CPT derivatives, and MGMT may be an additional important determinant of resistance to the drugs.

The purposes of this study are to establish whether MGMT participates in resistance to CPT derivatives, and to clarify the extent to which MGMT accounts for the resistance. Using 17 human cancer cell lines, we attempted to determine properties that correlate with observed sensitivity to the drugs to understand the importance of MGMT as a resistance determinant. We then clarified the functional significance of MGMT in the resistance mainly through gene transfection experiments. We investigated

[5] To whom correspondence should be addressed.
E-mail: yamacho@hiroshima-u.ac.jp

here CPT-11, its active form SN-38, and a novel CPT derivative DX-8951f, (1*S*,9*S*)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano-[3′,4′:6,7]indolizino[1,2*b*]quinoline-10,13(9*H*,15*H*)dione-monomethane-sulfate dihydrate.[5)]

MATERIALS AND METHODS

Cells Twenty human cancer cell lines were kindly provided and used as follows: a myelogenous leukemia cell line, K562, and its multidrug-resistant cell variant, K562/DOX (Dr. T. Tsuruo, Tokyo University, Tokyo); an oral epidermoid carcinoma line KB (Dr. S. Akiyama, Kagoshima University, Kagoshima); gastric adenocarcinoma HSC-42, esophageal squamous cell carcinoma HEC-46, colon adenocarcinoma cell lines HCC-48 and HCC-50 (Dr. K. Yanagihara, National Cancer Center, Tokyo); glioblastoma cell lines T98G, U-373MG, and U-251MG (Dr. S. Hama, Hiroshima University, Hiroshima); an oat-cell lung cancer cell line PC-6 and its SN-38- and CPT-11-resistant cell variants, PC-6/SN2-2 and PC-6/CPT2-2 (Dr. A. Tohgo, Daiichi Pharmaceutical Co., Ltd., Tokyo), small-cell lung cancer PC-9, non small-cell lung cancer PC-14 (Dr. K. Nishio, National Cancer Center, Tokyo); and squamous cell lung carcinoma LC-S and non small-cell lung cancer A549 cell lines (Dr. Y. Yamaguchi, Hiroshima University, Hiroshima). Colon adenocarcinoma lines, COLO201 and COLO320DM, and gastric adenocarcinoma line MKN45 were obtained from the Japanese Cancer Research Resources Bank. K562 and K562/DOX were non-adherent cells, while the other 17 cell lines were adherent ones. All cultured cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and maintained in continuous exponential growth by passage every 3 days.

Drugs and chemicals All chemicals were of analytical grade. CPT-11 and its active compound SN-38 and DX-8951f were generously provided by Yakult Honsha Co., Ltd. (Tokyo) and Daiichi Pharmaceutical Co., Ltd. (Tokyo).

Evaluation of drug efficacy Exponentially growing cultured cells were concentrated to 2.5×10^4/ml and exposed to the indicated drug concentrations in RPMI 1640 with 10% fetal bovine serum, seeded in 24-well plates, and incubated for 72 h at 37°C. After the incubation, cells were trypsinized and surviving cells were counted with a Coulter counter (Hialeah, FL) and by means of the trypan blue exclusion test.

Biochemical quantification assays Enzymatic activity of NADPH/cytochrome P450 reductase (P450R), NADPH/quinone oxidoreductase (NQO), and glutathione S-transferase (GST), and total cellular glutathione (GSH) content were measured as described previously.[12)]

RNA preparation and northern analysis Total RNA isolation and northern analysis were performed as described previously.[10, 12–15)] The probes used for hybridization were a 3.6-kb *Topo I* cDNA derived from HEC-46 cells (position 1–3645); a 0.8-kb *Topo IIα* cDNA derived from KB cells (position 1346–4377); a 0.8-kb cDNA fragment of human MGMT (position 1–829); a 0.8-kb fragment of human *P450R* cDNA derived from HCC-48 cells (position 44–853); a 1.1-kb fragment of human *NQO1* cDNA derived from K562 cells (position 7–1091); a 0.75-kb *Eco*RI cDNA insert of human *GSTπ* clone pGPi2 (position 1–714); a 0.88-kb fragment of the heavy subunit of human γ-glutamyl cysteine synthetase (*γ-GCS*) cDNA from KB cells (position 169–2634); a 0.7-kb fragment of human 170 kD GP-170 cDNA (*MDR1*) derived from K562 cells (position 3488–4192); a 0.9-kb *Eco*RI cDNA fragment of the human *MRP1* gene (position 4076–5011); a 0.5-kb *cMOAT* cDNA (canalicular multispecific organic anion transporter) from K562 cells (position 1056–1567); a 2.1-kb *Eco*RI cDNA fragment of the human *ABCP*/*BCRP* gene (position 232–2316); a 1.7-kb *Hind*III+*Xba*I cDNA fragment of the human *p53* clone pKS53-SN3 (position 1–1760); a 2.1-kb *Not*I cDNA insert of the human *p21* clone p-WAF1-S (position 1–2121), a 0.7-kb *Eco*RI cDNA fragment of the human *Bcl-2* clone Bluescript hu-bcl-2 cDNA 58 cDNA (position 1459–2179); a 0.6-kb *Eco*RI cDNA insert of the human *Bax* clone (position 1–579); and a 0.85-kb fragment (containing exons 4, 5, 6, and 7 of the human glyceraldehyde-3-phosphate dehydrogenase gene [*GAPDH*]) (position 131–940).

Electrophoresis and western blot analysis Protein fractions were separated by sodium dodecyl sulfate-polyacrylamide electrophoresis on 7% to 13.5% polyacrylamide gels, followed by transfer to polyvinylidene difluoride membranes for 30 min using a semidry blotting apparatus. After incubation with antibody for 1 h at room temperature, the blots were incubated with secondary antibody and visualized by the Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products, Boston, MA). Antibodies to MGMT and human DNA topo I were purchased from PharMingen (San Diego, CA) and Sigma-Genosys, Ltd. (Cambridge, UK), respectively.

Transfection of *MGMT* expression plasmids *MGMT* cDNA was prepared from human normal colon cDNA library (Invitrogen, Carlsbad, CA) by polymerase chain reaction (PCR) and then cloned into the eukaryotic expression vector pCR 3.1 (Invitrogen). The resulting plasmids were designated as pCR/MGMT-S for the sense direction and pCR/MGMT-AS for the antisense direction. The primers were 5′-ATGGACAAGGATTGTGAAATGAA-AC-3′ (nucleotides 97–121) and 5′-GTGTCGCTCAAA-CATCCATCCTACT-3′ (nucleotides 753–729).[16)] U-251MG cells were transfected with pCR/MGMT-S, whereas pCR/MGMT-AS was introduced into T98G cells, using Superfect reagent (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Cells were selected

by growing them for 2 weeks in medium containing 600 μg/ml of geneticin (Sigma, St. Louis, MO) and then cloned.

MGMT activity assay The assay for MGMT activity was described previously[17, 18] and carried out with some modification. Cells were suspended at a concentration of 1×10^7 cells/ml of assay buffer (50 m*M* Tris-HCl (pH 8.0), 1 m*M* dithiothreitol, 1 m*M* EDTA, 5% glycerol), then sonicated, and centrifuged at 16 000*g* for 10 min at 4°C. The 18-base-pair oligomer containing an O^6MG lesion was radiolabeled by filling in the 3′-recessed end with [α-^{32}P]TTP (Amersham Pharmacia Biotech UK, Buckinghamshire, England). A 20 p*M* probe was incubated with 10 U of Stoffel *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT), 1.5 m*M* $MgCl_2$, and 1× *Taq* buffer at 42°C for 1 h. Various amounts of cell extracts were incubated with radiolabeled oligomer in a total volume of 20 μl at 37°C for 2 h and digested with 40 μg of proteinase K in the presence of 1% sodium dodecyl sulfate (SDS) at 45°C for 1 h. After phenol:chloroform:isoamyl alcohol extraction, the radiolabeled oligomer was digested with *Pvu*II (Toyobo Co., Tokyo) and then electrophoresed on a 20% denaturing polyacrylamide gel. The extent of cleavage of the radiolabeled 18mer to an 8mer by *Pvu*II is directly proportional to MGMT activity. Oligomers were purchased from Hokkaido System Science Co. (Sapporo).

p53 status *p53* cDNA reverse transcriptase-PCR (RT-PCR) products from each cell line were subcloned into T Easy vector (Promega, Madison, WI) and sequenced with an automated sequencer 373 (PE Applied Biosystems, Foster City, FL).

Measurement of accumulated CPT-11, SN-38 and DX-8951f Exponentially growing cells (5×10^6) were incubated with 250 μ*M* CPT-11, 5 μ*M* SN-38, and 5 μ*M* DX-8951f for 1 h at 37°C, then the accumulated amount was measured by high-performance liquid chromatography (HPLC) as described previously.[5, 19]

Data analysis Student's *t* test and linear regression analysis were used as appropriate.

RESULTS

Possible factors determining cellular resistance to CPT-11, SN-38 and DX-8951f To obtain more insight into the factors that account for differential sensitivity or resistance to CPT derivatives, we investigated here prodrug-type

Table I. Correlation of Various Factors with Observed Sensitivity to CPT-11, SN-38 and DX-8951f

	CPT-11		SN-38		DX-8951f	
	r [a]	P [b]	r	P	r	P
mRNA expression						
Topo I	0.309	0.232	0.109	0.682	−0.023	0.933
Topo IIα	0.019	0.942	0.189	0.475	0.296	0.254
P450R	0.120	0.452	0.181	0.492	0.171	0.519
NQO1	0.655	0.003	0.475	0.053	0.074	0.781
GSTπ	0.421	0.093	0.533	0.026	0.427	0.088
γ-GCS	0.798	<0.001	0.821	<0.001	0.677	0.002
MDR1	0.497	0.041	0.398	0.115	0.094	0.723
MRP1	0.741	<0.001	0.614	0.008	0.218	0.407
MRP2 (*cMOAT*)	−0.036	0.894	0.207	0.432	0.271	0.298
ABCP/BCRP	0.447	0.072	0.773	<0.001	0.553	0.020
MGMT	0.518	0.032	0.543	0.023	0.496	0.042
P21	0.400	0.160	0.668	0.008	0.584	0.027
BAX	0.318	0.275	0.518	0.057	0.355	0.219
Enzymatic activity or concentration						
P450R	0.063	0.812	0.267	0.306	0.354	0.167
NQO	0.518	0.032	0.397	0.116	0.092	0.730
GST	0.423	0.091	0.394	0.119	0.291	0.262
GSH	0.532	0.027	0.605	0.009	0.556	0.019

mRNA levels for each gene are expressed relative to those of *GAPDH* expression using northern blots, and IC_{50} was determined after 72-h incubation of cells (2.5×10^4/ml) in the presence of the indicated drug. Correlation analysis was done from the mean values for 17 cell lines, which were obtained in three individual experiments.

a) Correlation coefficient.

b) *P* value.

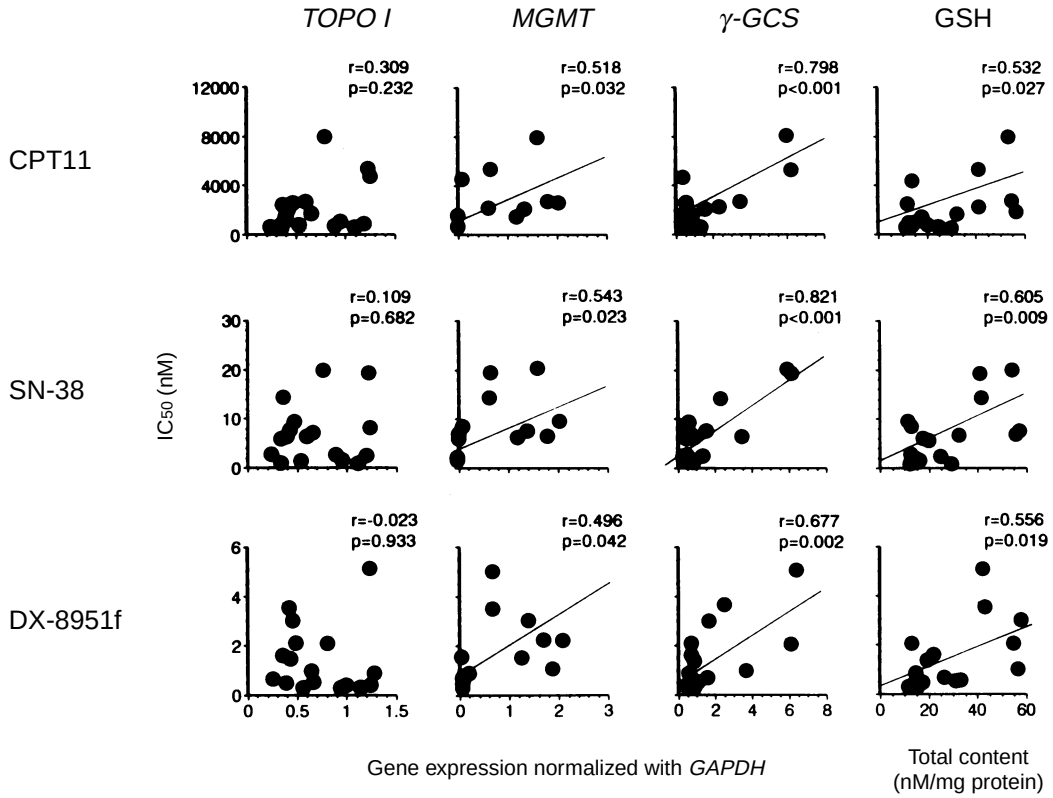

Fig. 1. Correlation of various factors with sensitivity to CPT-11, SN-38, and DX-8951f. mRNA levels for each gene are expressed relative to those of *GAPDH* using northern blots. GSH content was measured from the protein-free lysate using an enzyme recycling assay. IC_{50} was determined after 72-h incubation of exponentially growing cells (2.5×10^4/ml) in complete medium in the presence of the indicated drug. Each data point represents the mean values for three individual experiments in each cell line, and correlation analysis was done using the mean values for 17 cell lines.

CPT-11, its active form SN-38, and a novel non-prodrug-type CPT derivative, DX-8951f. The activation enzymes such as carboxylesterase strongly influence the efficacy of CPT-11, but not that of SN-38 or DX-8951f.[1, 5] Glucuronidation also may not play a significant role in the inactivation of DX-8951f due to the lack of a hydroxyphenol moiety.[4] In 17 human cancer cell lines except 3 cell variants with acquired drug-resistance to DOX, CPT-11 and SN-38, we analyzed gene expressions, enzymatic activity, and properties relevant to drug activity in relation to cytotoxicity induced by the drugs.

When normalized relative to glyceraldehyde 3′-phosphate dehydrogenase (GAPDH), the basal expression level of the *MGMT* gene was correlated with the IC_{50} value for all drugs (Table I and Fig. 1). Also, *γ-GCS* expression and cellular GSH, the biosynthetic pathway of which is regulated by the first enzyme, γ-GCS, were correlated closely with resistance to the CPT derivatives in the 17 cell lines (Fig. 1). Expression of *Bcl-2* was almost undetectable in all 17 cell lines, and expression of *Topo I*, *Topo IIα*, *P450R*, *GSTπ*, *MRP2* (*cMOAT*), and *BAX* did not correlate at all to the IC_{50} values for CPT-11, SN-38, and DX-8951f. Enzymatic activity of P450R and GST also did not correlate to cellular sensitivity to the topo I inhibitors. Genes such as *NQO1*, *MDR1*, *MRP1*, *ABCP/BDRP*, and *P21* correlated in part with sensitivity to CPT derivatives, while NQO activity was related to the IC_{50} value only for CPT-11.

MGMT as a determinant of resistance common to CPT-11, SN-38 and DX-8951f MGMT may participate in resistance to CPT-11, SN-38, and DX-8951f, but γ-GCS and GSH are also suggested to play some roles in the resistance. The *γ-GCS* expression and GSH content in cells with acquired resistance to CPT-11 (PC-6/CPT2-2) and to SN-38 (PC-6/SN2-5), however, did not differ from those in the parental cells (PC-6) (Fig. 2A). Further, inhibition of γ-GCS activity by 5 *μM* buthionine sulfoximine (BSO) for 72 h did not increase the cellular sensitivity to CPT-11, SN-38, and DX-8951f in A549 and T98G cells, although BSO treatment caused a significant depletion of GSH content (Fig. 2B).

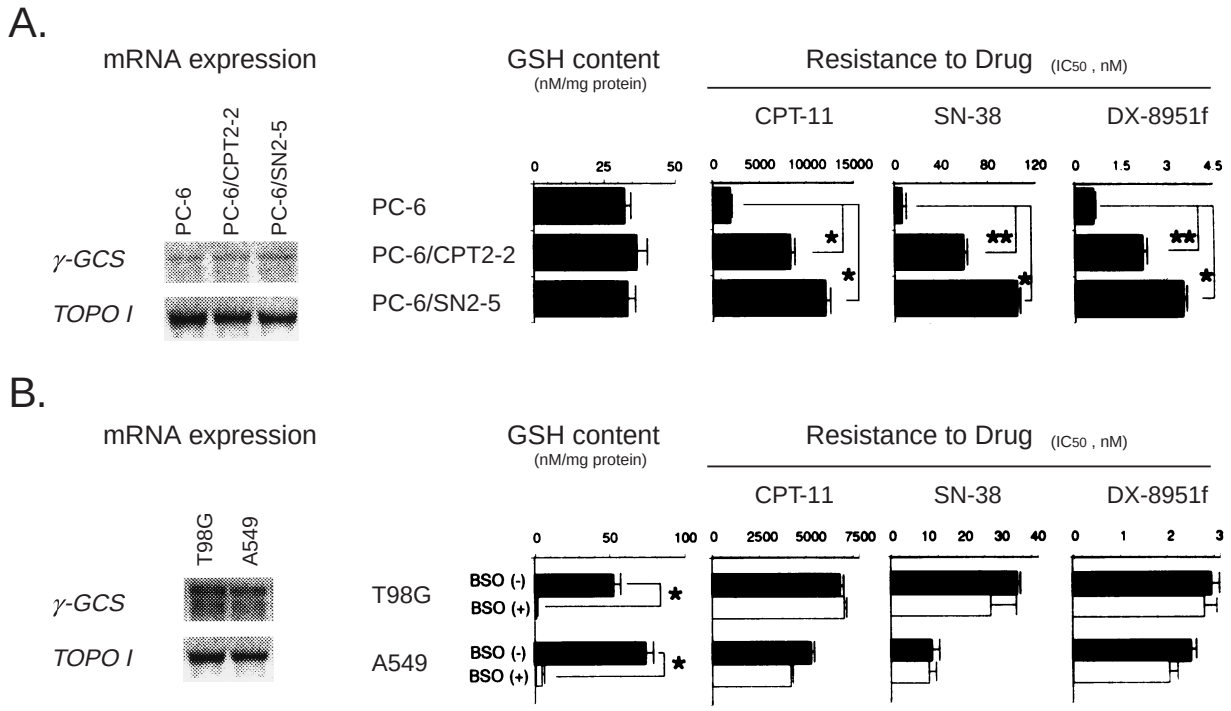

Fig. 2. *γ-GCS* gene expression, GSH content, and sensitivity to CPT-11, SN-38, and DX-8951f. A. *γ-GCS* gene expression and GSH content in CPT-11- (PC-6/CPT2-2) and SN-38- (PC-6/SN2-5) resistant cell lines. CPT-11- and SN-38-resistant cell lines were selected after long-term exposure of cells to stepwise increasing drug concentrations, established using the limiting dilution technique, and kindly provided by Dr. A. Tohgo. B. Inhibition analysis of γ-GCS (depletion assay of GSH) in T98G and A549 cells. Total cellular GSH content was measured with (open bar) and without (closed bar) γ-GCS activity inhibition (GSH depletion assay). In the GSH depletion assay, exponentially growing cells (2.5×10^4/ml) were exposed to 5 μM buthionine sulfoximine (BSO) for 72 h, then divided into two parts, one for the measurement of total GSH content and the other for cytotoxicity assay. Total RNA was isolated from each cell line and subjected to northern blotting. The total GSH content was measured from the protein-free lysate using an enzyme recycling assay, while cytotoxicity assay was performed as described in "Materials and Methods." Experiments were performed three times independently. Data represent the mean±SD for three individual experiments (∗ $P<0.01$, ∗∗ $P<0.05$).

Contrary to the findings in the case of γ-GCS, inhibition of MGMT activity by nontoxic 5 μM O^6-benzylguanine (O^6-BG) for 2 h caused a significant increase of cytotoxicity induced by drugs to which *MGMT* expression is relevant, in 8 randomly selected cell lines (Fig. 3A). After the inhibition of MGMT activity, the sensitivity to CPT-11, SN-38 and DX-8951f was increased in *MGMT*-overexpressing T98G, HEC-46 and KB cells, whereas the drug sensitivity was not influenced at all in *MGMT*-nonexpressing cells such as U-373MG, MKN-45, K562, HSC-42, and U-251-MG. Additionally, we found that treatment with CPT-11, SN-38, and DX-8951f decreased MGMT protein in cells, associated with a decrease of the drug action target, topo I protein, in a dose-dependent manner (Fig. 3B). Exposure of T98G cells to the drugs for 24 h at the IC_{50} (75 μM CPT-11, 250 n*M* SN-38, and 150 n*M* DX-8951f) caused a decrease of both MGMT and topo I protein, although the decrease of the MGMT protein was relatively less than that of topo I. Such associated decreases of MGMT and topo I were also observed after treatment with nimustine hydrochloride (ACNU), but cisplatin (CDDP) had no influence at all. However, ACNU-induced protein reduction was more significant in the case of MGMT than topo I, contrary to the findings for CPT derivatives.

p53 is suggested to be involved in regulation of DNA repair genes.[20] However, in the 17 cell lines, MGMT expression was not related to *p53* expression or status. Sequence study of the cDNA subcloned from each cell line revealed that p53 status was different in the 17 cell lines: KB, PC-6, and A549 cells had wild-type p53, K562 and K562/DOX cells did not express p53, and the other 13 cell lines had mutant-type p53. MGMT appears to be a common factor related to resistance to CPT-11, SN-38, and DX-8951f among a total of 14 properties, which have been linked to drug activity, and which might play some role in topo I-mediated DNA damage and/or the repair process.

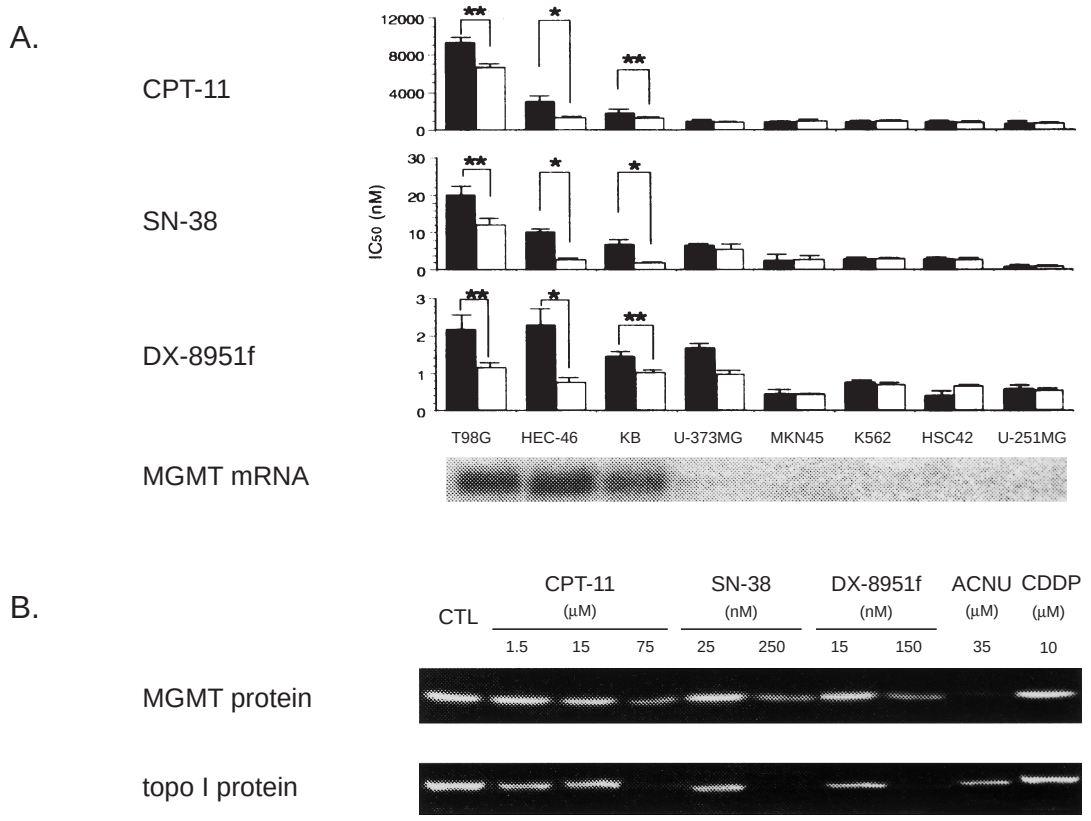

Fig. 3. O^6-Methylguanine-DNA methyltransferase (MGMT) and action of CPT-11, SN-38, and DX-8951f. A. Effect of inhibition of MGMT on cytotoxicity induced by CPT-11, SN-38, and DX-8951f. Exponentially growing cells (2.5×10^4/ml) were seeded in 24-well plates and exposed to the indicated drug with (cross-hatched bar) or without pre-depletion (closed bar) of MGMT activity by nontoxic 5 μM O^6-benzylguanine (O^6-BG) for 2 h. After 72-h incubation, surviving cells were counted and IC_{50}s were calculated. Total RNA was isolated from 8 cell lines and subjected to northern blotting. Experiments were performed in triplicate. Data represent the mean±SD for three individual experiments (* $P<0.01$, ** $P<0.05$). B. Effect of CPT derivatives on MGMT and topo I protein in T98G cells. Exponentially growing T98G cells (5×10^6/ml) were seeded in 6-cm diameter dishes and incubated for 24 h. After changing the medium, cells were exposed to the indicated drug for 24 h, then the whole cell lysate (WCL) was prepared and subjected to immunoblotting.

Transfection analysis of *MGMT* into HEC-46 and T-98G cells To directly elucidate the role of MGMT in resistance to CPT-11, SN-38, and DX-8951f, we introduced *MGMT-sense* (*S*) into *MGMT*-nonexpressing U-251MG cells and *MGMT-antisense* (*AS*) into *MGMT*-overexpressing T98G and HEC-46 cells (Fig. 4). Two clones of each were obtained, in which the growth did not vary more than 10% from that of each parent cell line. The cloned transfectants with the *MGMT* gene, U-251MG/MGMT-S1 and -S2, exhibited different levels of increase of MGMT at the mRNA, protein, and activity levels. U-251MG/MGMT-S2 clone had higher expression and enzymatic activity of MGMT than U-251MG/MGMT-S2 clone. Conversely, the levels of mRNA, protein, and activity of MGMT were reduced in the transfectants with *MGMT-AS*. The expression level and activity of MGMT in the T98G transfectants was reduced equivalently to below 35% of those in the parent and vector control clones. HEC-46/MGMT-AS1 had lower (approximately 70% of control) and -AS2 had the lowest expression and enzymatic activity of MGMT (approximately 50% of control). The expression levels and enzymatic activity of topo I in such clones did not differ from those of the parent and vector control cells.

The *MGMT-S* transfection decreased the cellular sensitivity of U-251MG to CPT-11, SN-38, DX-8951f, whereas *MGMT* transfection in an antisense orientation sensitized both T98G and HEC-46 cells to CPT derivatives, indicating the participation of MGMT in the CPT derivatives-induced cytotoxicity (Fig. 5). However, the expression level of *MGMT* in the transfectants did not always closely correlate with sensitivity to topo I derivatives. In T98G/

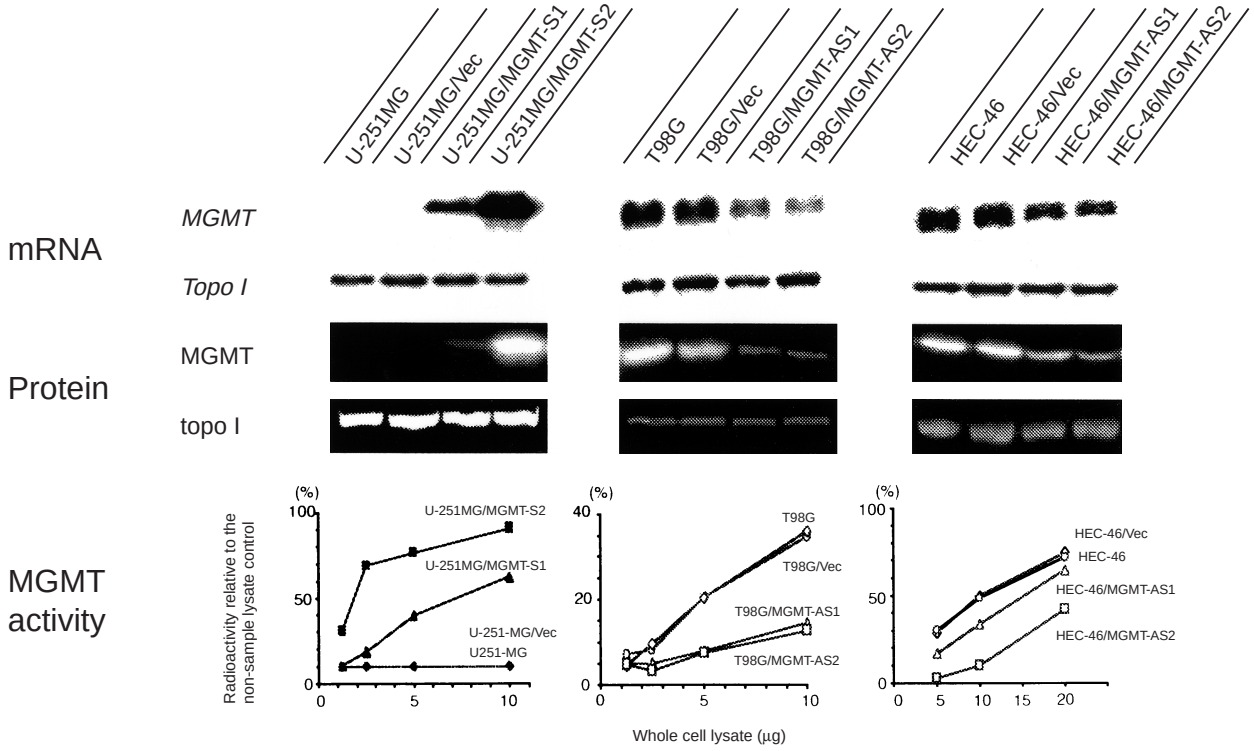

Fig. 4. Northern blot, immunoblot, and activity of MGMT in U-251MG clones transfected with *MGMT-sense* (*S*) and T98G and HEC-46 clones with *MGMT-antisense* (*AS*). Total RNA and cell lysates from 12 clones were subjected to northern blot and immunoblot analyses. MGMT activity was measured as described in "Materials and Methods."

MGMT-AS1 and -AS2, the IC_{50} values for CPT-11, SN-38, and DX-8951f correlated with the expression level of *MGMT*, but *MGMT* expression in the U-251MG and HEC-46 transfectants did not closely correlate with sensitivity to the CPT derivatives as compared to the sensitivity to ACNU. In U-251MG cells, transfection of *MGMT-S* significantly increased the *MGMT* expression and the resistance to ACNU, but the increase of resistance to CPT derivatives was not so marked as to ACNU. HEC-46/MGMT-AS2 expressed a more reduced level of MGMT, but the IC_{50} values for CPT-11, SN-38 and DX-8951f were less than those of HEC-46/MGMT-AS1. Nonetheless, no factors, other than MGMT, related to the sensitivity to the drugs were identified among the genes and properties investigated in this study (Fig. 6). HPLC analysis also revealed that altered drug accumulation did not play any role in the sensitivity of the transfectants (data not shown).

DISCUSSION

The present study demonstrates that MGMT correlates with resistance to CPT-11, SN-38, and DX-8951f in 17 human cancer cell lines. The basal expression level of the *MGMT* gene was correlated with the IC_{50} values for the CPT derivatives, and inhibition of the MGMT activity augmented the cytotoxicity of the drugs in 8 randomly selected cell lines. Further, gene transfection of *MGMT-S* caused an increase of the resistance of U251-MG cells to the drugs, whereas *MGMT-AS* transfection sensitized both T98G and HEC-46 cells to the CPT derivatives. Among a total of 14 properties, which have been linked to drug activity, *γ-GCS* gene expression and GSH content also correlated with IC_{50}s for CPT-11, SN-38 and DX-8951f. Increased levels of γ-GCS and GSH, however, were not observed in CPT-11-resistant PC-6/CPT2-2 and SN-38-resistant PC6/SN2-5 cells, and inhibition of γ-GCS (depletion of GSH) did not sensitize T98G and A549 cells to the CPT derivatives. MGMT appeared to be the most important factor determining cellular sensitivity to CPT-11, SN-38, and DX-8951f in the 17 cell lines.

Several putative mechanisms of resistance to CPT derivatives have been identified.[1, 2] Either decreased expression or mutation causes a decrease of the cleavable complexes, thereby conferring resistance. In a variety of model systems, it has been clearly demonstrated that resistance to CPT derivatives is conferred by reduced drug accumula-

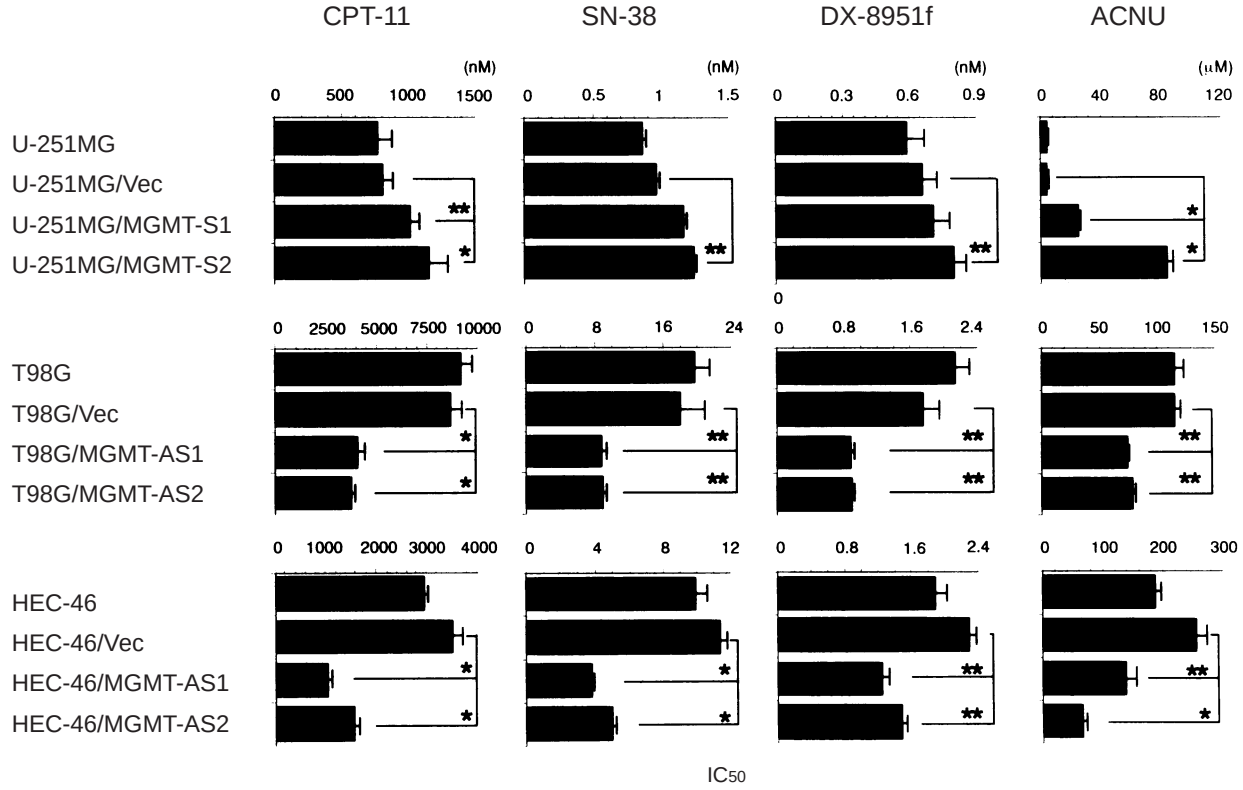

Fig. 5. Altered drug sensitivity in *MGMT-S* and *MGMT-AS* transfectants. Exponentially growing cells (2.5×10^4/ml) were seeded in 24-well plates, and incubated in the presence of the indicated drug for 72 h, then surviving cells were counted to calculate IC_{50}s. Experiments were performed in triplicate. Data represent the mean±SD for three individual experiments (* $P<0.01$, ** $P<0.05$).

tion, a decrease of drug activation for the prodrug-type derivative, and an increase of drug inactivation by glucuronidation or oxidative metabolism by cytochrome P4503A. However, the mechanisms of cellular sensitivity to CPT derivatives vary significantly among cell lines and drugs.[3–6] This study also demonstrated that most of the factors examined did not play an important role in the sensitivity of these 17 human cancer cell lines. Expression of topo I did not correlate with resistance to CPT-11, SN-38, and DX-8951f in the 17 human cancer cell lines, and altered drug accumulation did not play any role in sensitivity to the drugs in *MGMT-S* and *-AS* transfectants. Correlation analysis between gene expression and IC_{50} suggested that ABCP/BCRP could play some role in resistance to the drugs, but the correlation was not statistically significant in the 17 cell lines. The cell lines utilized in this study have different levels of an activation enzyme of CPT-11, carboxylesterase. The variation of enzymatic activity in the cell lines might account for the differences in factors correlated with the cytotoxicity of CPT-11 and SN-38 and the increasing effect of O^6-BG on the drug activity, at least in part. Carboxylesterase, however, does not influence the cytotoxicity of SN-38 and DX-8951f. Resistance to CPT derivatives is obviously determined by multiple factors. It appears that MGMT is probably an additional but fairly potent factor conferring resistance to CPT derivatives.

Nevertheless, the precise mechanism of action of MGMT on the resistance is still unclear. MGMT is well known to be involved in drug resistance to monofunctional alkylating agents such as N-methyl-N-nitrosourea (MNU) and to bifunctional chloroethyl-nitrosoureas (CENUs), such as ACNU.[21, 22] Those alkylating agents covalently link O^6MG in DNA, and MGMT specifically removes the methyl group of O^6MG. The most probable mechanism of MGMT-mediated resistance to CPT-11, SN-38 and DX-8951f, is the reduction of O^6MG adducts that can interact with DNA-topo I cleavable complex. A recent report has shown that O^6MG can trap topo I and increase the topo I cleavable complex, thereby decreasing the topo I-mediated DNA religation.[9] It was demonstrated that the presence of O^6MG also stabilizes CPT-induced topo I cleavage complexes, and so CPT binding might further destabilize the alignment of the 5′-hydroxyl of the cleaved strand down-

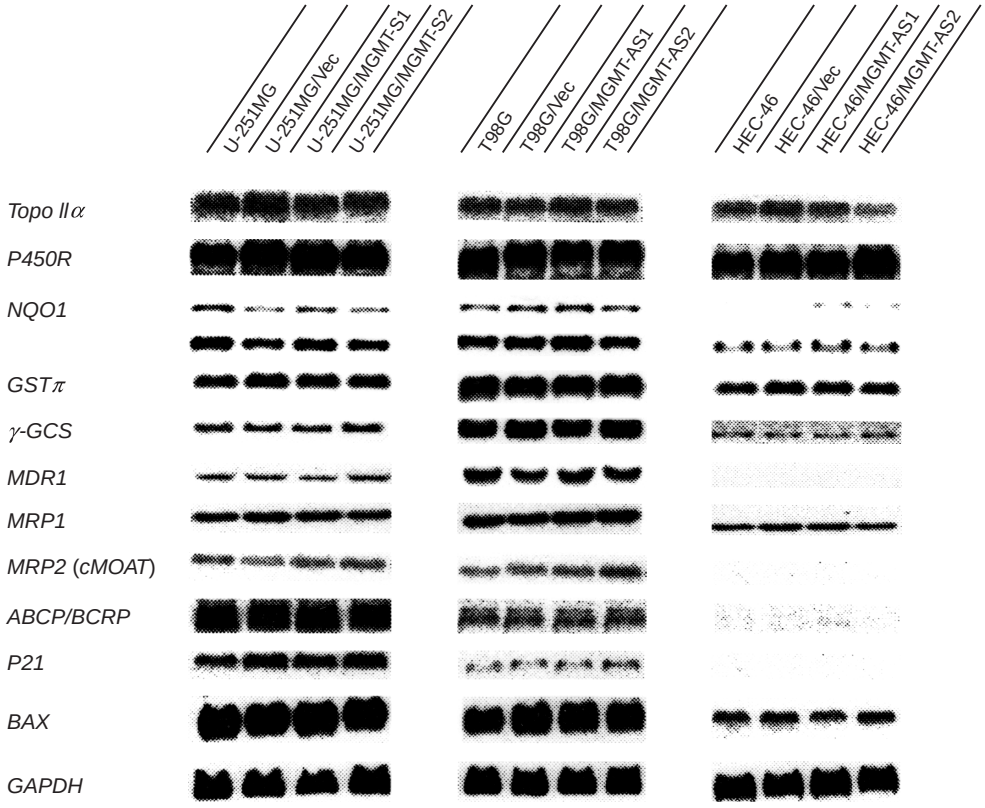

Fig. 6. Expression of genes, which have been linked to drug activity, in *MGMT-S* and *-AS* transfectants. Expression of a total of 12 genes except *MGMT* and *TOPO I* was investigated in U-251MG clones transfected with *MGMT-sense* (*S*) and T98G and HEC-46 clones with *MGMT-antisense* (*AS*). Total RNA from 12 clones was subjected to northern blot analysis.

stream from the cleavage site and render religation even more difficult. MGMT might act on the removal of the methyl group of O^6MG, and increase the topo I-mediated religation as well as the enzyme cleavage step, thereby being related to the resistance to CPT derivatives.

The present study, however, rather suggests that some mechanisms other than the reduction of O^6MG might operate in the MGMT-mediated resistance. MGMT-activity inhibition by O^6-BG and the *MGMT-S* transfection into U-251MG did not cause a remarkable alteration of IC_{50} for the CPT derivatives. Five micromolar O^6-BG for 2 h is reported to inactivate approximately 80% of MGMT of HeLaS3 cell-free extracts,[23] and *MGMT* expression in the transfectants correlated more closely with the sensitivity to ACNU than that to CPT-11, SN-38, and DX-8951f. Further, it remains unclear in this study whether O^6MG can be induced by the CPT derivatives or not. Nonetheless, the expression levels of *MGMT* were closely correlated with the IC_{50} values for the CPT derivatives in the 17 cell lines, while both MGMT inhibition by O^6-BG and *MGMT-AS* transfection sensitized cells to CPT derivatives, and an increase of MGMT by *MGMT-S* transfection reduced cytotoxicity of the drugs. Further, in the U-251MG/*MGMT-S* and HEC-46/*MGMT-AS* transfectants, no factors correlating with the sensitivity to CPT derivatives were identified among the genes and properties investigated in this study. This study also revealed that treatment with CPT-11, SN-38, and DX-8951f for 24 h caused a decrease of cellular protein levels of both MGMT and topo I. The associated decrease was observed after treatment with the CPT derivatives and ACNU, although the effects on the proteins by CPT derivatives appeared to be somewhat different from those by ACNU. CPT derivatives decreased topo I more than MGMT, whereas ACNU reduced MGMT more, suggesting different modes of action of MGMT on the CPT derivatives and ACNU. MGMT might work directly on the DNA-topo I cleavable complex formation or the repair process, or there may exist some intricate network including MGMT to determine the cellular sensitivity to the drugs. To clarify in detail the mechanisms of MGMT-mediated resistance and/or to seek other participants in the mechanisms, *MGMT-S* transfection into cell lines other than U-251MG, transfection experiments to inhibit topo I expression, and comprehensive RNA expression analysis using cDNA microarrays are under study.

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