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Yu Maki, *Okayama University*
Jun Murakami, *Okayama University*
Jun-ichi Asaumi, *Okayama University*
Hidetsugu Tsujigiwa, *Okayama University*
Hitoshi Nagatsuka, *Okayama University*
Susumu Kokeguchi, *Okayama University*
Kazuhiro Fukui, *Okayama University*
Noriko Kawai, *Okayama University*
Yoshinobu Yanagi, *Okayama University*
Masahiro Kuroda, *Okayama University*
Noriaki Tanaka, *Okayama University*
Nagahide Matsubara, *Okayama University*
Kanji Kishi, *Okayama University*

Role of O^6-methylguanine-DNA methyltransferase and effect of O^6-benzylguanine on the anti-tumor activity of *cis*-Diaminedichloroplatinum(II) in oral cancer cell lines

Yu Maki[1†], Jun Murakami[1†]*, Jun-ichi Asaumi[1]*, Hidetsugu Tsujigiwa[2], Hitoshi Nagatsuka[2], Susumu Kokeguchi[3], Kazuhiro Fukui[3], Noriko Kawai[1], Yoshinobu Yanagi[1], Masahiro Kuroda[4], Noriaki Tanaka[5], Nagahide Matsubara[5], Kanji Kishi[1]

[1]Department of Oral and Maxillofacial Radiology, [2]Oral Pathology, [3]Oral Microbiology, [4]Radiology, [5]Department of Surgical Oncology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan

[†]These authors contributed equally to this work.

*Address correspondence to: Jun Murakami and Jun-ichi Asaumi, Department of Oral and Maxillofacial Radiology, Field of Tumor Biology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1, Shikata-cho, Okayama 700-8525, Japan

E-mail: jun-m@md.okayama-u.ac.jp and asaumi@md.okayama-u.ac.jp

Telephone number: +81-86-235-6621

Fax number: +81-86-235-6709

Abbreviated running title: MGMT and O^6-BG on the CDDP sensitivity.

Keywords: MGMT; CDDP; O^6-BG; oral cancer

Abstract *Background:* O^6-methylguanine-DNA methyltransferase (MGMT), DNA repair enzyme, modulates the effectiveness of alkylating agents. However, the relationship between MGMT and the sensitivities to other agents has not been explored. *Experimental design:* In the present study, the association between MGMT expression and the cellular sensitivity to the platinum agent, CDDP, in 4 human oral cancer cell lines, was examined. *Results:* CDDP depleted MGMT protein and mRNA levels in all 4 cell lines. Two cell lines with low MGMT expression were sensitive to an alkylating agent, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and CDDP, whereas 2 other cell lines with high MGMT expression were resistant to both agents. Furthermore, the addition of the MGMT inhibitor, O^6-benzylguanine (O^6-BG), invariably enhanced CDDP sensitivity. *Conclusion:* CDDP depleted MGMT expression, and CDDP sensitivity was enhanced by O^6-BG. These results provide valuable information about the relationship between MGMT expression and CDDP sensitivity in oral cancer chemotherapy.

Keywords: MGMT; CDDP; O^6-BG; oral cancer

Introduction

Platinum agents are widely used in cancer chemotherapy, either alone or in combination with other antitumor agents, in the treatment of a variety of cancers such as lymphomas and testicular, ovarian, lung, and head and neck cancers [1]. Among the platinum agents, *cis*-Diaminedichloroplatinum(II) (CDDP) primarily forms cross-links on DNA that can block replication or inhibit transcription [2, 3], which produces cytotoxicity against cancers. The cross-links generated by CDDP on DNA are primarily intrastrand cross-links including 1,2-d(GpG), 1,2-d(ApG), and 1,3-d(GpNpG), and interstrand cross-links [4]. The intrastrand cross-links are thought to be removed by nucleotide excision repair proteins (NERs) [5, 6]. Several studies have suggested the existence of a correlation between NER expression and CDDP sensitivity in cancer cells. Furthermore, to improve the clinical response to CDDP, many studies have focused on the search for a preferential target or a predictor of CDDP sensitivity. Recently, it was demonstrated that CDDP is capable of abrogating O^6-methylguanine-DNA methyltransferase (MGMT) activity [7, 8] and that the promoter methylation of *MGMT* plays a role in achieving a favorable response to CDDP [9].

MGMT is a DNA repair enzyme that rapidly repairs adducts at the O^6-position of guanine [10-17]. Because MGMT is inactivated after O^6-alkylating DNA adducts are restored, MGMT activity is likely to be an important marker of the sensitivity to alkylating agents that generate a complex spectrum of adducts at the O^6-position of guanine similarly may be a predictor or the success of chemotherapeutic regimens using such alkylating agents. A small number of human tumor-derived cell lines have little or no methyltransferase activity and are hypersensitive to alkylating agents; these are the so-called Mer- or Mex- cell lines [18-21]. The depletion of MGMT in tumors has become a therapeutic target for sensitizing cells to O^6-alkylating agents [22]. To deplete MGMT in tumors, attempts have been made to inactivate it by pre-treatment with a methylating agent to induce O^6-methylguanine [23] or by using specific MGMT inhibitors [24]. O^6-benzylguanine (O^6-BG) is one such specific, rationally designed MGMT inhibitor that produces suicidal inactivation of MGMT with a restoration of sensitivity to chloroethylators or methylators [24-26]. O^6-BG has been approved in a phase I trial due to its demonstrated toxicity [27] and is

currently being used in combination with O^6-alkylguanine-generating drug, 1,3 bis(2-chloroethyl)-1-nitrosourea, for the treatment of glioblastoma.

In addition to such alkylating agents, the platinum agent, *cis*-Diaminedichloroplatinum(II) (CDDP) is also capable of inhibiting MGMT activity [7]. Therefore, given that CDDP is more widely used than alkylating agents in the clinical setting for the treatment of oral, colon, and other solid epithelial tumors, it would clearly be a major step forward in cancer chemotherapy if MGMT expression could also enhance the potential effectiveness of CDDP. In the present study, we examined whether or not CDDP could induce the MGMT depletion effect, and we also investigated whether MGMT expression status could correlate with the clinical cellular response to CDDP in four oral cancer cell lines.

Materials and Methods

Cell lines and culture

Four human oral cancer cell lines (HSC4, HSC3, SAS and Hep2) were obtained from the Cell Resource Center for the Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. All cell lines were maintained in Dulbecco's modified Eagle's medium (MDEM) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories Inc., UT, USA), 100 units/ml penicillin (Meiji Seika Ltd., Tokyo, Japan) and 100 μg/ml streptomycin (Meiji Seika Ltd., Tokyo, Japan) in a CO_2 incubator (Sanyo Electric Co., Ltd., Osaka, Japan) with in an atmosphere of 95% air plus 5% CO_2 at 37℃.

Table 1 shows the MGMT expression status in the 4 cell lines used in the present study. We previously examined MGMT expression status by Western blotting and RT-PCR [28]. Low levels of expression of MGMT protein and mRNA were observed in the SAS and Hep2 cells, and high levels were observed in the HSC4 and HSC3 cells.

Chemicals

N-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) (Nacalai Tosque, Inc. Kyoto, Japan),

CDDP (Sigma Chemical Co., St. Louis, MO, USA), bleomycin (BLM) (Nippon Kayaku Co., Ltd., Tokyo) and O^6-Benzylguanine (O^6-BG) (Sigma), diluted in water, were added to MDEM to the final concentration indicated in each treatment.

MNNG or BLM treatment

First, 5×10^5 cells were seeded in 5 ml MDEM in a flask (Nalge Nunc International, Roslilde, Denmark). Then, 24 h after seeding, the medium was changed for medium containing the appropriate drug (MNNG or BLM), and the flask was immersed in a 37℃ water bath (Taitec, Co., Ltd., Saitama, Japan). Following treatment with the drug for 1 h, the cells were rinsed three times with drug-free medium, and their survival rates were determined as described below.

Cell survival assay for MNNG and BLM

Cell survival rates were assayed by measuring the colony-forming ability of the cells in triplicate samples. Only colonies containing more than 50 cells were counted. After drug treatment, the cells were dispersed with trypsin, seeded at adequate concentrations, and incubated at 37°C in a CO_2 incubator. Surviving cells were fixed in 10% formaldehyde and stained with 10% Giemsa staining solution. Cell survival rates were corrected for the seeding efficacy of untreated controls.

RNA isolation and RT-PCR

Extraction of total cellular RNA was carried out using Trizol reagent (Invitrogen Co., Carlsbad, CA) according to the manufacturer's instructions. For CDDP-treated groups, 1×10^5 cells incubated for 38 h in medium containing 20 μM of CDDP were rinsed three times with PBS, and then the RNA (or protein for Western blotting) was extracted. The RNA was reverse-transcribed with Superscript II Reverse Transcriptase and oligo dT primers (Invitrogen Co., Carlsbad, CA). Amplification of the cDNAs was performed under the following PCR conditions: 7 min at 94℃ for 1 cycle; then 26 cycles at 94℃ for 30 s, 59℃ for 30 s, 72℃ for 30 s; and a final elongation step at 72℃ for 10 min. The following primers used for the amplification:

MGMT; sense: 5'-GCCGGCTCTTCACCATCCCG-3', antisense: 5'-GCTGCAGACCACTCTGTGGCACG-3', *GAPDH;* sense: 5'-GAAGGTGAAGGTCGGAGTC-3', antisense: 5'-CAAAGTTGTCATGGATGACC-3' [29]. The *MGMT* primers amplified a 211-bp product spanning sequence (339-527) from GenBank, accession number M29971. The amplified *GAPDH* fragment was used as a positive control. The RT-PCR products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and viewed by UV. The intensities of the bands were quantified using Image J 1.33u (National Institutes of Health, USA).

Western blotting

The proteins in the cell-free extracts were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), and the individual proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) using a semi-dry electrophoretic transfer apparatus (LKB-Produkter AB, Bromma, Sweden) at room temperature. The blotted membranes were blocked for 1 h in TBS-T (containing 0.1% Tween 20) plus 5% powdered skin milk. The memgranes were then probed for 2 h with mouse anti-MGMT monoclonal antibody MT 3.1 Ab-1 (Neomarkers, Fremont, CA) diluted 1:800 in TBS-T. The membranes were then washed three times in TBS buffer, and incubated for 1 h with the appropriate secondary antibody horseradish peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (ImmunoResearch Laboratories Inc., West Grove, PA) in TBS-T. Bound antibody was detected using ECL + plus kit (Amersham Pharmacia Biotech Inc., Little Chalfont, UK) according to the manufacturer's instructions. The mouse monoclonal antibody for beta actin, beta actin AC-15-ab6276, was purchased from Abcam Limited (Cambridge, UK): this antibody was diluted 1:5000 in TBS-T and was utilized as that described above. The intensities of the bands were quantified using Image J 1.33u (National Institutes of Health, USA).

CDDP sensitivity

The alteration of CDDP sensitivity for each condition was evaluated using MTT

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) assay. The MTT assay was carried out using MTT Cell Growth Kit (Chemicon International, Inc. Temecula, CA) according to the manufacturer's instructions. Eight replicate wells per assay condition were seeded at a density of 1.5×10^4 cells in 0.1 ml of medium. The wells were incubated for 24 h at 37° C. Stock solutions of CDDP were prepared by dissolving the drug at a concentration of 10 mM in distilled water for no more than 2 h prior to use in an experiment, and the final concentrations were obtained by diluting the stock solution directly into the tissue culture medium. The cells were incubated with several concentrations of CDDP ranging from 10-100 μM for an additional 38 h. At the end of the exposure to CDDP exposure, 10 μl of MTT (5 mg/ml) were added to each well for 4 h at 37℃ to allow MTT to form formazan crystals by reaction with metabolically active cells. Next, 100 μl of color development solution (isopropanol with 0.04 N HCl) were added to each well. Within one hour, the absorbance of each well was measured in a microplate reader (Corona microplate reader MTP-120, Corona Electric Co., Ltd, Japan) with a test wavelength of 570 nm. The percentage of cell growth inhibition was calculated by comparison of the absorbance reading from treated versus untreated control cells under each experimental condition.

O^6-BG treatment and alteration of CDDP sensitivity

For the O^6-BG-treated groups, 1×10^5 cells incubated in medium containing 75 μM of O^6-BG for 4 days were rinsed three times with fresh medium, and then the cells were seeded into 96-well plates. Eight replicate wells per assay condition were seeded at a density of 1.5×10^4 cells in 0.1 ml of medium containing the appropriate amount of O^6-BG (37.5 or 75 μM). To serve as O^6-BG-untreated control groups, cells were also seeded into 96-well plates at the same density in medium lacking O^6-BG. The cells were then incubated for 24 h at 37° C. The cells were incubated with several concentrations of CDDP ranging from 10-100 μM for an additional 38 h. At the end of the period of exposure to CDDP, the MTT assay was carried out as described above.

Statistical analysis

Statistical analysis was conducted with JMP 5.0.1 J software (SAS Institute Inc. Cary, NC).

Results

MNNG and BLM sensitivity

In order to confirm the contribution of MGMT expression status to cellular sensitivity to the alkylating agents MNNG, we treated each of the oral cancer cell lines considered here with varying concentrations of MNNG and with a non-alkylating chemotherapeutic agent, BLM. The cell lines with high MGMT expression (HSC4 and HSC3) were resistant to the effects of MNNG, whereas the cell lines with low MGMT expression (SAS and Hep2) exhibited sensitivity. In contrast, we found no evidence of a relationship between MGMT expression status and BLM sensitivity in the 4 cell lines studied here (Figure 1a and b).

Effects of CDDP on levels of MGMT expression level

In order to test whether or not MGMT expression was altered by CDDP, we examined the levels of MGMT expression by Western blotting and RT-PCR after treating the cell lines with 20 μM CDDP for 38 h. Interestingly, in all 4 cell lines, CDDP reduced the levels of MGMT protein expression compared to those of the CDDP un-treated control (Figure 2a). Furthermore, the RT-PCR results revealed that *MGMT* mRNA expression was also attenuated by treatment with CDDP (Figure 2b).

CDDP sensitivity

Because MGMT is inactivated after O^6-alkylating DNA adducts are restored, MGMT activity may be an important marker of sensitivity to alkylating agents that are known to generate a complex spectrum of adducts at the O^6-position of guanine. Considering our finding that CDDP also depleted MGMT expression (Figure 2), we hypothesized that MGMT may play a role in cellular sensitivity to CDDP. Figure 3 shows the results of treatment with varying concentrations of CDDP using 4 oral cancer cell lines. Among these 4 cell lines, SAS and Hep2 cells, both of

which exhibited low levels of MGMT expression, were more sensitive to CDDP than were HSC4 and HSC3 cells, which exhibited high levels of MGMT expression (Figure 3). Interestingly, the difference between MGMT expression levels seemed to relate to the respective cellular sensitivities to CDDP.

Effect of O^6-BG on cellular sensitivity to CDDP

To examine whether MGMT depletion enhances the potential utility of CDDP in the treatment of oral cancer, we evaluated the effects of an MGMT inhibitor, O^6-BG, on CDDP sensitivity in 4 oral cancer cell lines. For the O^6-BG-treated groups, cells were exposed to 75 μM O^6-BG for 4 days. Then, the cells were washed and seeded into 96-well plates with medium containing the appropriate concentrations of O^6-BG (37.5 or 75 μM). After 24-h incubation, the cells were then exposed to CDDP at various concentrations for an additional 38 h. In addition, in order to exclude the effects of the cytotoxicity of O^6-BG when used alone, we evaluated the cell survival rates of group treated with O^6-BG alone at the end of the course of treatment (Figure 4a). Given that the cell survival in the O^6-BG-treated groups was consistently higher than 80% of that of the drug-untreated control groups, the cytotoxicity of O^6-BG was confirmed to have remained at a minimum. Figure 4b shows that all cell lines inhibited growth inhibition in a dose-dependent manner following treatment with CDDP for 38 h over a concentration range from 10 to 100 μM. The combined treatment with O^6-BG and CDDP produced supra-additive effects compared to the result obtained with CDDP alone. Interestingly, 2 cell lines with low MGMT expression levels (SAS and Hep2) also showed restored sensitivities to the cytocidal effects of CDDP by pre-treatment with O^6-BG.

Discussion

CDDP is a commonly used chemotherapeutic agent that is effective when used alone or in combination with other drugs, radiotherapy, and/or surgery in the treatment of various malignancies, including head and neck cancers [1, 30, 31]. A major limitation to successful treatment with platinum agents is the development of acquired drug resistance by the cancer cells [4]. Cellular

resistance to these drugs is multifactorial, and the mechanisms by which such resistance is achieved are not yet fully understood. Modulation of CDDP resistance is thus a potential new therapeutic target.

MGMT is a DNA repair enzyme that rapidly repairs adducts at the O^6-position of guanine, and its expression is known to modulate the effectiveness of alkylating agents [18-21]. Alkylating agents may generate DNA adducts (such as O^6-methyl guanine) and may produce suicidal inactivation of MGMT. Moreover, not only such alkylating agents inhibit the activity of MGMT; Wang and Settlow reported that CDDP is also capable of inhibiting MGMT activity [7]. This CDDP-induced attenuation of MGMT renders novel chemotherapy approaches such as temozolamide plus CDDP particularly attractive in the treatment of a number of cancers [7]. Koul and co-workers reported that the transcriptional inactivation of *MGMT* by epigenetic alterations confers exquisite sensitivity to CDDP [9].

In our study, the administration of CDDP was associated with decreased levels of MGMT protein and mRNA contents, in comparison with those of the un-treated control (Figure 2a and 2b). The question that arises in this context is the identity of the pathway involved in MGMT depletion by CDDP. First, a simple explanation for MGMT depletion by CDDP would be that CDDP may also generate DNA adducts (such as the O^6-alkylating DNA adducts generated by alkylating agents). MGMT may repair those adducts, which results in the suicidal inactivation of MGMT. Second, considering that CDDP primarily forms cross-links on DNA that can block replication or inhibit transcription [2, 3], those cross-links on the DNA that are induced by CDDP may inhibit the transcription of *MGMT*. A third possible explanation would be that CDDP might affect the CpG methylation in the promoter region of the *MGMT* gene, perhaps resulting in the decreased transcription of *MGMT*. Evidence that *MGMT* expression levels are greatly reduced following the methylation of its promoter has already been reported in a study by Esteller and co-wokers [32], who also suggested that the differential methylation of the MGMT promoter might be responsible for the marked differences in prognosis observed among glioma patients following treatment with carmustine treatment. A previous study has indicated that human tumor cells exposed to high concentrations of CDDP induce alterations in 5-methyl cytosine *in vitro* [33]. Koul and

colleagues [9] reported that promoter hypermethylation of the *MGMT* and *RARB* genes is associated with CDDP sensitivity, and that the complete promoter methylation of *MGMT* plays a role in achieving a favorable response of male germ cell tumors to CDDP treatment. It remains unclear whether or not CDDP induces CpG methylation in the *MGMT* promoter; however, in our previous study, we did discover a link between the methylation status of the upstream promoter of the *MGMT* gene and transcriptional inhibition in oral cancer cell lines, including the same cell lines tested in the present study [28]. Another possible explanation could be suggested at this point, namely, that the CDDP-induced depletion of a natural amino acid, methionine (Met), may be responsible for the attenuation of MGMT expression. Scanlon *et al* [34, 35] and Mineura *et al* [36] demonstrated that CDDP affected the metabolism of Met in tumor cells and that CDDP interfered with Met transport by acting as an inhibitor of amino acid entry [34, 37]. Recently, Kokkinakis and co-wokers [38] observed in brain cancer cells and non-small cell lung cancer cells that MGMT activity was markedly down-regulated in response to Met deprivation *in vitro*.

An additional question remains to be addressed in this context: What is the biological goal of CDDP-induced MGMT depletion? Because MGMT is inactivated after O^6-alkylating DNA adducts are restored, MGMT activity may be an important marker of tumor and normal tissue sensitivity to alkylating agents which generate a complex spectrum of adducts at the O^6-position of guanine. When considering our results (Figure 2), we hypothesized that MGMT may play a role in cellular sensitivity to CDDP. Interestingly, SAS and Hep2 cells, which exhibited low levels of MGMT protein expression, were growth-inhibited by treatment with CDDP in a dose-dependent manner. Difference between original MGMT expression levels appeared to be relate to cellular sensitivity to CDDP (Figure 3).

Next, to investigate the possible relationship between MGMT expression and CDDP sensitivity, we examined whether MGMT depletion by O^6-BG would lead to the sensitization of cells to CDDP (Figure 4). Recently, MGMT activity has been regarded as an important marker of sensitivity to alkylating agents, and many attempts have been made to deplete MGMT by using the specific inhibitor O^6-Benzylguanine (O^6-BG) to enhance the sensitivity of tumors to alkylating agent [24]. O^6-BG is a MGMT substrate that was rationally designed to produce suicidal inactivation via a

restoration of sensitivity to chloroethylators or methylators [24-26]. In our O^6-BG regimen, O^6-BG treatment depleted levels of MGMT protein expression and restored sensitivity to an alkylating agent, MNNG (data not shown). To exclude the cytocidal effects induced by O^6-BG pre-treatment, we re-seeded cells into 96-well plates after O^6-BG treatment at the same density as that used for the O^6-BG-untreated control groups. In our combined regimen with O^6-BG and CDDP, O^6-BG treatment alone was found to exert only minimal cytocidal effects on cancer cells (Figure 4a). We also found that the combined regimen with O^6-BG and CDDP produced supra-additive cytocidal effects in all cells lines, compared with the results obtained by CDDP treatment alone (Figure 4b). Single administration of O^6-BG to the cells has been known as non-toxic [39, 40]. Although pretreatment of cancer cells by O^6-BG showed minimal cytocidal effect in our study, our co-incubation time of CDDP and O^6-BG in our protocol was a little long compared to other study. Anyhow, we found that the O^6-BG/CDDP combined regimen produced supra-additive cytocidal effects in all cells examined. Clearly, it would be a major step forward in cancer chemotherapy if MGMT protein expression could be related to the likely effectiveness of CDDP, and O^6-BG could be a promising modulating agent for CDDP as well. However, we could not exclude the involvement of different pathways, other than the apparent one in which MGMT is involved in the control of the combined regimen with O^6-BG and CDDP. Fishel *et al.* also reported that O^6-BG treatment resulted in additive effect on CDDP- and carboplatin-induced cytotoxicity, however, its enhancement seems to be independent of MGMT status [41]. They focused on the aspect of O^6-BG as a cell cycle inhibitor and reported that O^6-BG enhances CDDP -induced cytotoxicity resulting from its effect on the cell cycle. Mack *et al.* reported that the cyclin-dependent kinase inhibitor (*i.e.* 7-hydroxystaurosporine, UCN-01) could potentiate CDDP activity through targeting the cell cycle [42]. O^6-BG is also known to inhibit CDK1/cyclin B and CDK2/cyclin A by competing for the ATP binding domain in the CDK enzyme [43, 44]. However, the mechanism of O^6-BG to modulate platinating agent has not been definitively demonstrated, improved studies are needed in this area.

In summary, MGMT depletion occurs in response to CDDP treatment in oral cancer cell lines. Moreover, MGMT expression may play a role in cellular sensitivity to CDDP; an

enhancement of the anti-tumor effects of CDDP by MGMT depletion was observed in the present study. Although our findings are the results of *in vitro* studies, we believe that the resent results may have important clinical implications in the potential utility of CDDP in the treatment of cancer.

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Figure Legends

Figure 1: MNNG and BLM sensitivity in oral cancer cell lines.

a: Cellular sensitivity to the alkylating agent MNNG. HSC4 and HSC3 cells with high MGMT expression were resistant to the lethal effects of MNNG, whereas SAS and Hep2 cells with low MGMT expression showed sensitivity. The cell-survival rates in groups with high MGMT expression were significantly different ($p < 0.01$) from those with low MGMT expression by a Student's *t*-test.

b: Cellular sensitivitiy to BLM. HSC3 cells with high levels of MGMT expression were the most sensitive to the lethal effects of BLM, whereas the 3 other lines proved to be much less sensitive. We found no evidence of a relationship between the MGMT expression status of any of the 4 cell lines studied here and their sensitivity to BLM. Symbols: HSC4, ◆; HSC3, ▲; SAS, □; Hep2, △. Standard errors are shown for each concentration.

Figure 2: The effect of CDDP treatment on MGMT expressions.

For CDDP-treated groups, 1×10^5 cells were incubated for 38 h in medium containing 20 μM of CDDP. After 38-h incubation in the presence of CDDP, *MGMT* mRNA and protein expression were measured by Western blotting or RT-PCR, and then the results were compared were compared to those obtained with non-treated cells.

a: CDDP treatment attenuated MGMT protein in all 4 cancer cell lines. Extracted protein from the indicated cell lines was loaded onto a 10% SDS-PAGE gel and electrophoresed. In the Western blot analysis, an equal amount of proteins were electroblotted. Proteins were electroblotted onto a PVDF membrane, which was probed with monoclonal antibody MT 3.1 specific for human MGMT.

b: CDDP treatment attenuated *MGMT* mRNA in all 4 cancer cell lines. CDDP treatment reduced the content of MGMT protein and mRNA compared to the CDDP un-treated control in all four cell lines examined here.

c: Intensities of the bands were quantified by the proportion of MGMT versus beta actin or GAPDH with Image J 1.33u (National Institutes of Health, USA). The relative band intensity

represents the intensity of CDDP treated sample / CDDP un-treated control sample. Closed columns represent cells with no CDDP treatment Open columns represent cells treated with CDDP. The significance of the differences was tested by the Student's *t*-test: $p < 0.01$. Each column is the average of three measurements; bars, SD. The band intensities in groups treated with CDDP were significantly different from those without CDDP.

Figure 3: CDDP sensitivity.

Cellular sensitivity to CDDP among 4 cell lines was examined using an MTT assay. HSC4 and HSC3 cells with high MGMT expression were resistant to the lethal effects of CDDP. In contrast, SAS and Hep2 cells with low MGMT expression were sensitive to CDDP. The cell-survival rates in groups with high MGMT expression were significantly different ($p < 0.01$) from those with low MGMT expression by a Student's *t*-test, except for the asterisk-added groups.

Figure 4: Effect of O^6-BG on cellular sensitivity to CDDP treatment.

To examine whether or not MGMT depletion y enhances the potential utility of CDDP in oral cancer therapy, we evaluated the combined effect of CDDP and the MGMT inhibitor O^6-BG in 4 cell lines. For the O^6-BG-treated groups, cells were exposed to 75 μ M O^6-BG for 4 days.

a: The cellular sensitivity to O^6-BG in 4 cell lines. Given that the cell survival in the O^6-BG alone-treated groups was consistently higher than 80% compared to that of drug-untreated control groups, the cytotoxicity of O^6-BG was confirmed to be minimal.

b: The combined effect with O^6-BG on cellular sensitivity to CDDP treatment. All cells exhibited dose-dependent growth inhibition due to treatment with CDDP, and the combined regimen with O^6-BG and CDDP produced supra-additive effects, compared with the results obtained by CDDP treatment alone. The closed symbols represent the survival rates of CDDP alone-treated groups, whereas the open symbols represent the survival rates of cells treated with O^6-BG and CDDP. The cell-survival rates in groups treated with CDDP together with O^6-BG were significantly different ($p < 0.05$) from those without O^6-BG by a Student's *t*-test, except for the asterisk-added groups.

Table 1. MGMT expression status in the oral cancer cell lines

cell line	origin	histological type	MGMT expression
HSC-4	tongue	squamous cell carcinoma	+++
HSC-3	mouth	squamous cell carcinoma	+++
SAS	tongue	squamous cell sarcoma	+/-
Hep2	larynx	epidermoid carcinoma	+/-

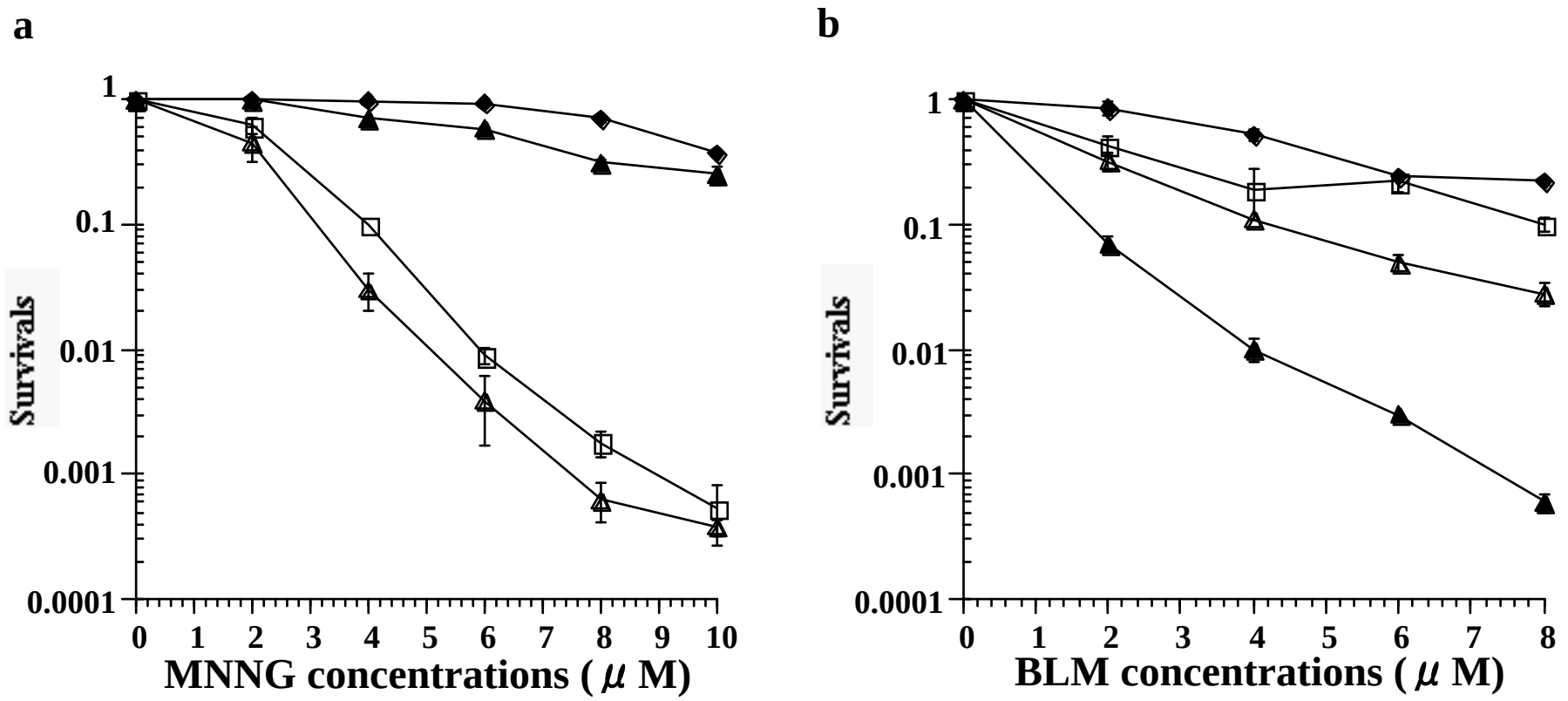
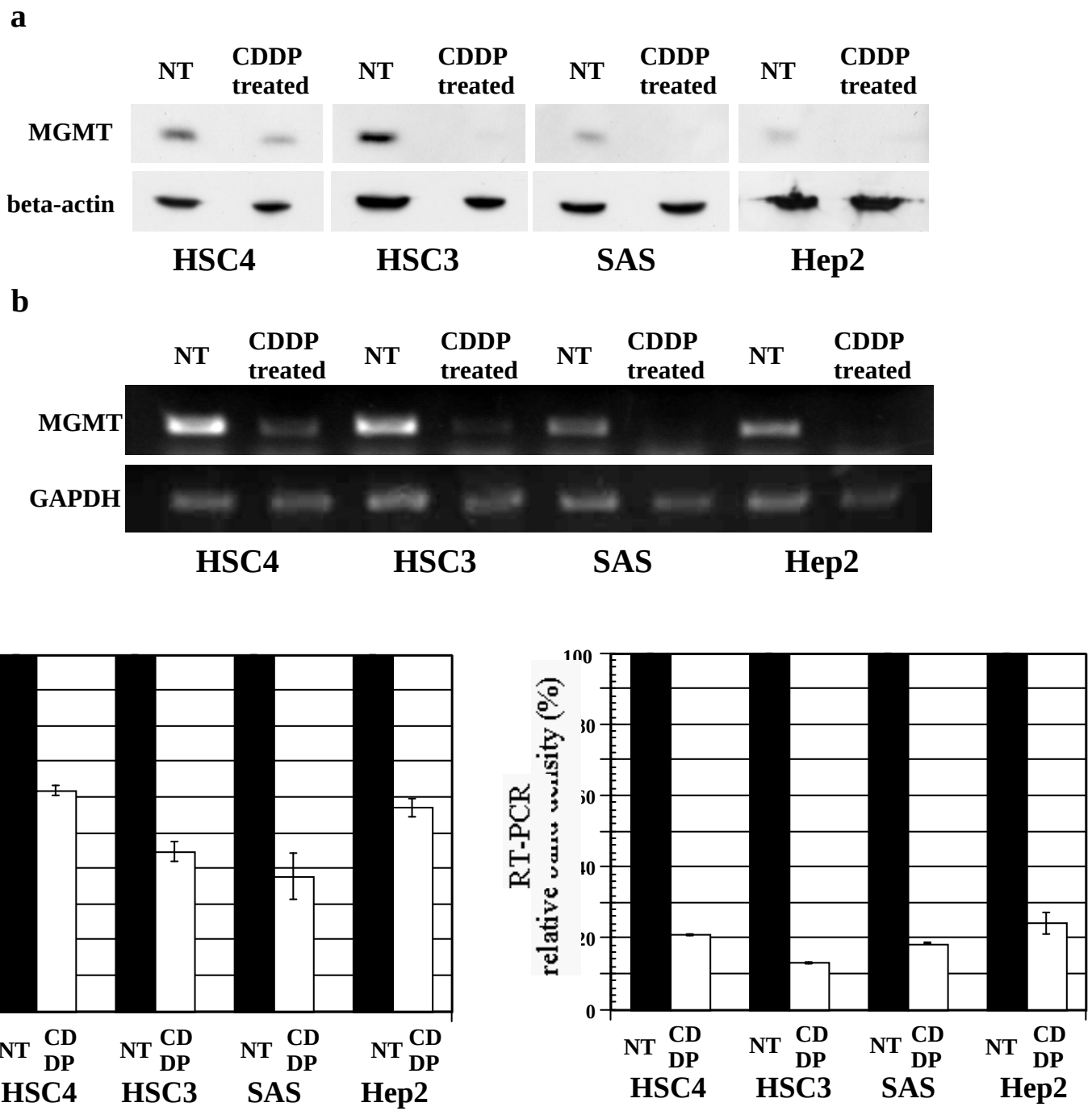

a
b
Survivals
1
0.1
0.01
0.001
0.0001
0 1 2 3 4 5 6 7 8 9 10
MNNG concentrations (μ M)
0 1 2 3 4 5 6 7 8
BLM concentrations (μ M)

Figure 1

Figure 2


a
NT
CDDP treated
NT
CDDP treated
NT
CDDP treated
NT
CDDP treated
MGMT
beta-actin
HSC4
HSC3
SAS
Hep2
b
NT
CDDP treated
NT
CDDP treated
NT
CDDP treated
NT
CDDP treated
MGMT
GAPDH
HSC4
HSC3
SAS
Hep2
c
Western blotting
relative band density (%)
100
80
60
40
20
0
NT
CD DP
NT
CD DP
NT
CD DP
NT
CD DP
HSC4
HSC3
SAS
Hep2
RT-PCR
100
80
60
40
20
0
NT
CD DP
NT
CD DP
NT
CD DP
NT
CD DP
HSC4
HSC3
SAS
Hep2

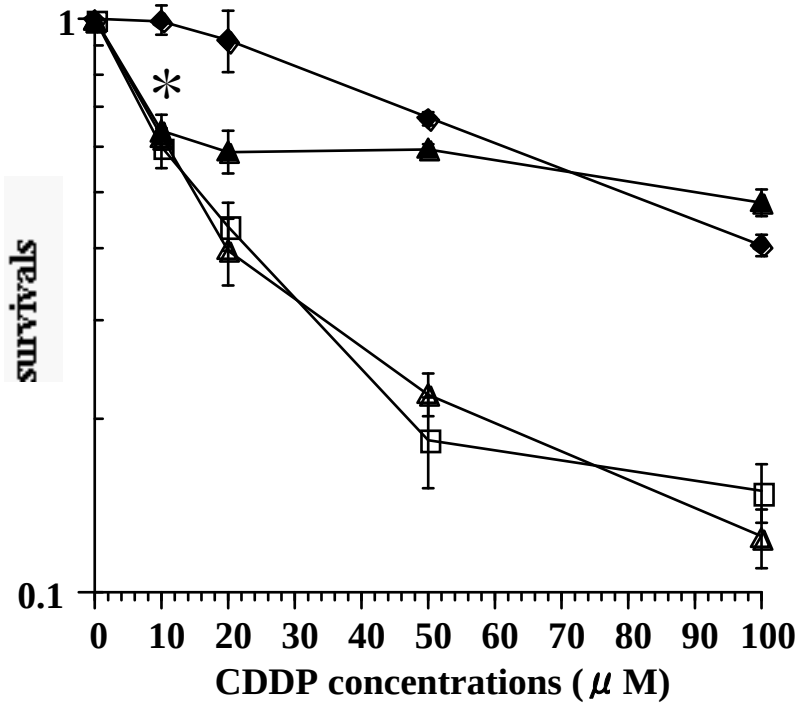
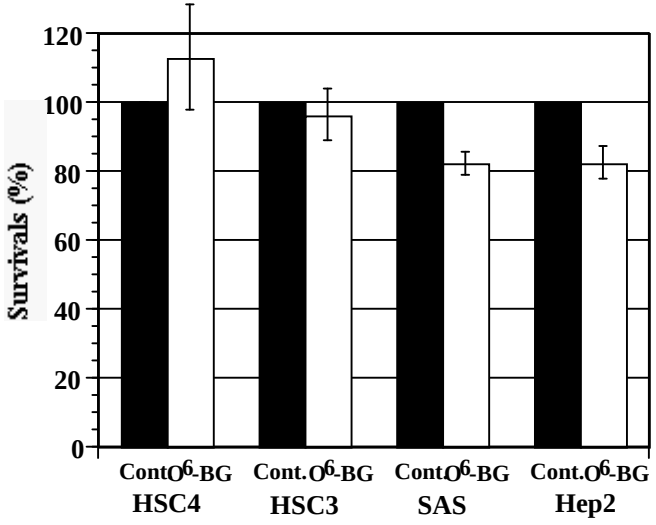

1
0.1
survivals
*
0
10
20
30
40
50
60
70
80
90
100
CDDP concentrations (μ M)

Figure 3

a


Survivals (%)
0
20
40
60
80
100
120
ContO6-BG
Cont.O6-BG
ContO6-BG
Cont.O6-BG
HSC4
HSC3
SAS
Hep2

b

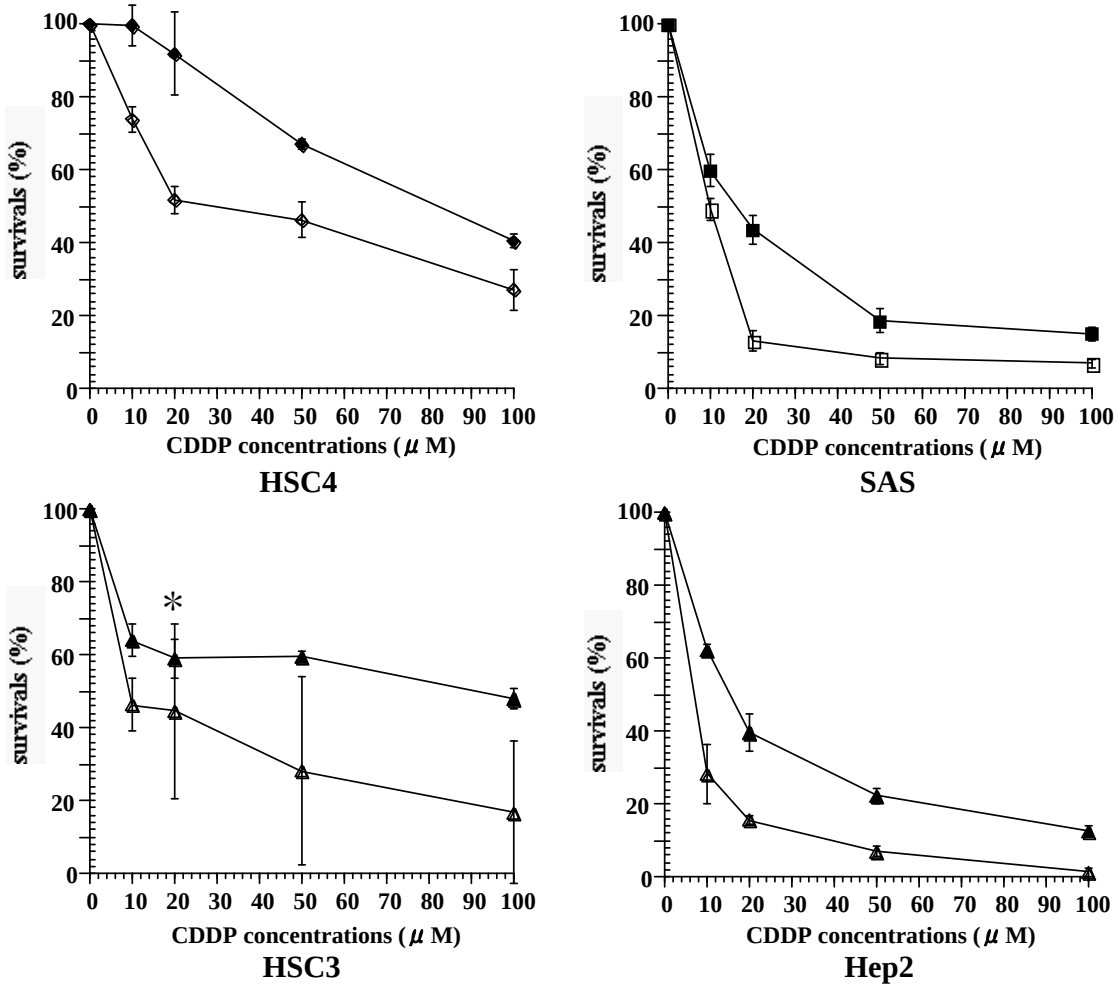

survivals (%)
CDDP concentrations (μ M)
HSC4
SAS
HSC3
Hep2
*

Figure 4