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# Effect of Cell Cycle Inhibition on Cisplatin-Induced Cytotoxicity*

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Running Title: Use of guanine derivatives to enhance cisplatin cytotoxicity

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Abbreviations used are: CDK, cyclin-dependent kinase; $O^6$-BG, $O^6$-benzylguanine; $S^6$-BG, $S^6$-benzyl-6-thioguanine; $S^6$-CMG, $S^6$-cyclohexylmethyl-6-thioguanine; $O^6$-CMG, $O^6$-[(cyclohexyl)methyl]guanine; 9-$CH_3$-BG, $O^6$-benzyl-9-methylguanine; 9-$CH_3$-CMG, $O^6$-[(cyclohexyl)methyl] -9-methylguanine; N7-BG, 7-benzylguanine; TSA, Trichostatin A; SAHA, suberoylanilide hydroxamic acid.

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## ABSTRACT

Pharmacological inhibitors of CDK2 are currently in preclinical and clinical development. The purpose of our work was to evaluate a series of guanine derivatives for their ability to inhibit CDK2, affect cell cycle progression, and enhance the cytotoxic and apoptotic effects of cisplatin. A panel of guanine derivatives including $O^6$-benzylguanine ($O^6$-BG), $S^6$-benzyl-6-thioguanine ($S^6$-BG), $S^6$-cyclohexylmethyl-6-thioguanine ($S^6$-CMG), $O^6$-[(cyclohexyl)methyl]guanine ($O^6$-CMG), $O^6$-benzyl-9-methylguanine (9-$CH_3$-BG), $O^6$-[(cyclohexyl)methyl] -9-methylguanine (9-$CH_3$-CMG), 7-benzylguanine (N7-BG) exhibited varying degrees of CDK2 inhibition with $O^6$-CMG being the most potent and 9-$CH_3$-BG, 9-$CH_3$-CMG, and N7-BG the least potent compounds. Treatment with $S^6$-CMG and $O^6$-CMG significantly decreased the percentage of cells in S phase. In SQ20b and SCC61 head and neck cancer cell lines, the most potent CDK2 inhibitor, $O^6$-CMG, was also the most effective at enhancing cisplatin-induced cytotoxicity and apoptosis. Cisplatin-induced DNA platination increased in SQ20b cells pretreated with $S^6$-BG, $S^6$-CMG, and $O^6$-CMG. Treatment with both $O^6$-BG and trichostatin A (TSA), an indirect cell cycle inhibitor, demonstrated additive effects on cisplatin-induced cytotoxicity. In summary, we have identified a group of guanine derivatives that were effective modulators of cisplatin-induced cytotoxicity and apoptosis.

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Combinations of conventional anti-cancer drugs with direct and indirect cell cycle inhibitors are currently being investigated clinically to determine if combining these inhibitors with cytotoxic chemotherapeutic agents will improve clinical response. The tightly controlled regulation of cell cycle progression involving cyclin dependent kinases (CDKs)[3] and their cyclin partners is disrupted in neoplastic cells compared to non-neoplastic tissues, thereby making this a target for anti-cancer therapies (Ruetz et al., 2003). Compounds that have a direct effect on cell cycle and that have entered clinical trials include flavopiridol, CTC-202, BMS-387032, and UCN-01. In addition to inhibiting cell cycle CDKs, these agents exhibit activities against other kinases that may reduce anti-tumor selectivity and result in toxicity that is not related to cell cycle inhibition (Bertrand et al., 1994; Carlson et al., 1996; Parker et al., 1998; Toogood, 2001). For example, UCN-01, although developed as a protein kinase C inhibitor (Mizuno et al., 1995), exerts effects on cell cycle via inhibition of CDKs (Akiyama et al., 1997) and abrogation of the DNA damage checkpoints (reviewed in (Senderowicz, 2003a).Unfortunately, flavopiridol and UCN-01 have demonstrated minimal activity as single agents in Phase I/II studies (reviewed in (Sausville et al., 2001; Grant and Roberts, 2003). Likewise, combinations of flavopiridol with conventional chemotherapy including paclitaxel, cisplatin, and irinotecan has not produced striking results (Grant and Roberts, 2003).

Cyclin D antagonists, p27 inducers, and differentiation-inducing agents such as all trans-retinoic acid, phorbol myristate acetate, and histone deacetylase inhibitors, i.e. trichostatin A and sodium butyrate, are all examples of indirect cell cycle inhibitors (Grant and Roberts, 2003). An indirect cell cycle inhibitor exerts its effects on targets upstream of the cell cycle rather than on the CDK enzymes themselves (Senderowicz, 2003b). Both direct

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and indirect cell cycle inhibitors have been able to potentiate the effects of cytotoxic agents including mitomycin C, cisplatin, radiation, gemcitabine and ara-C in vitro (reviewed in (Grant and Roberts, 2003; Kim et al., 2003; Senderowicz, 2003b), however improved therapies are needed in this area.

To overcome the limitations of first generation CDK inhibitors, tremendous effort, often supported by structural biology, has gone into discovering potent, more selective CDK inhibitors (Knockaert et al., 2002). Targets include CDK2/cyclin E and CDK2/cyclin A because they play a central role in driving the cells from G1 into S phase, and through S phase progression, respectively (Vermeulen et al., 2003). One such compound is $O^6$-CMG which is more specific for CDK1/2 than are other purine-derived compounds and has a differential pattern of tumor cell growth inhibition when compared with flavopiridol and olomoucine, the predecessor of CYC-202 (Schulze-Gahmen et al., 1995; De Azevedo et al., 1997; Arris et al., 2000). Important interactions between $O^6$-CMG and CDK2 include a triplet of hydrogen bonds (i.e., purine NH-9 to Glu 81, purine N-3 to Leu 83, and purine 2-$NH_2$ to Leu 83) and the cyclohexyl ring of $O^6$-CMG, that confers specificity for CDK 1 and 2 over CDK4 (Arris et al., 2000; Davies et al., 2002).

Here, we evaluate a series of guanine-derived compounds related to $O^6$-CMG including $O^6$-BG, $S^6$-BG, $S^6$-CMG, 9-$CH_3$-BG, 9-$CH_3$-CMG and N7-BG (Figure 1), for their ability to directly inhibit CDK2 and inhibit cell cycle progression. Using these guanine derivatives, we compared the extent of CDK2 inhibition with the degree of enhancement of cisplatin-induced cytotoxicity and apoptosis to test the hypothesis that cell cycle perturbation by CDK inhibition results in enhancement of cisplatin cytotoxicity. Furthermore, we measured the degree of DNA platination following cisplatin treatment in the presence and

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absence of these guanine derivatives. We also evaluated whether TSA, an indirect cell cycle inhibitor, could further enhance cisplatin-induced cytotoxicity when combined with guanine derivatives.

## Materials and Methods

*Cell lines.* The head and neck cancer cell lines, SQ20b and SCC61 were kindly provided by Dr. Michael Beckett (Department of Radiation and Cellular Oncology, University of Chicago). These cell lines were maintained at 37°C and 5% $CO_2$ in Dulbecco's MEM/F12 (50/50 mixture, Mediatech, Inc., Herndon, VA) supplemented with 20% fetal bovine serum (Hyclone, Logan, UT), 0.4 μg/ml hydrocortisone (BD Biosciences, Bedford, MA), and 1% penicillin/ streptomycin (Mediatech, Inc., Herndon, VA).

*Materials*. Cisplatin and TSA were purchased from Sigma Aldrich (St. Louis, MO). $O^6$-BG, $S^6$-BG, N7-BG, and 9-$CH_3$-BG were synthesized as described previously (Dolan et al., 1990; Moschel et al., 1992; Chae et al., 1994).

$S^6$-CMG was synthesized from 6-thioguanine (Aldrich Chemical Co., Milwaukee, WI). UV spectra were determined on a Beckman Coulter DU 7400 spectrophotometer. $^1$H-NMR spectra were recorded in DMSO-$d_6$ with a Varian INOVA 400 MHz spectrometer. Chemical shifts are reported as δ values in parts per million relative to tetramethylsilane (TMS) as internal standard. Mass spectra were obtained on a Thermo Finnigan TSQ Quantum mass spectrometer. To a solution of 2 g of 6-thioguanine (0.012 mol) dissolved in 75 mL of MeOH/$H_2O$ (1:1) containing 12 mL of 2 N NaOH was added 1.6 mL of bromomethylcyclohexane (0.011 mol), and the suspension was stirred vigorously for 9 days at room temperature. The resulting homogeneous solution was treated with 1.4 mL glacial

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acetic acid, and the precipitated solid was filtered. The recovered solid was suspended with stirring in 170 mL of EtOH/$H_2O$ (1:1) which was brought to boiling. The solution was gravity filtered hot to remove a fine yellow precipitate. The filtrate was allowed to cool slowly to room temperature. After 48 hours, additional fine yellow precipitate was deposited, and this was also filtered. EtOH was allowed to slowly evaporate spontaneously which caused a white crystalline solid to precipitate. This was suction filtered (0.36 g). EtOH was evaporated under vacuum which caused precipitation of additional white precipitate. The pooled white solid was crystallized from 100 mL of acetone/$H_2O$ to afford 1.5 g of $S^6$-CMG. UV ($H_2O$) $\lambda_{max}$ 244, 256(sh), 312; $^1$H-NMR (DMSO-$d_6$/TMS) $\delta$ 12.47 (br s, 1 H, N**H**, exchanges with $D_2O$), 7.88 (s, 1 H, H-8), 6.27 (s, 2 H, N$^2$**H**$_2$, exchange with $D_2O$), 3.20 (d, J = 6.8 Hz, 2 H, SC**H**$_2$), 1.90-0.97 (m, 11 H, $C_6$**H**$_{11}$); MS Calcd. m/z for $C_{12}H_{17}N_5S$: 263.1205; Found: 263.1208.

$O^6$-CMG (also known as NU2058) and 9-$CH_3$-CMG (also known as NU6052) were synthesized as previously described (Arris et al., 1994; Lembicz et al., 1997).

*CDK2 Inhibition Assay.* Inhibition of CDK2/CyclinA3 was assayed as previously described (Arris et al., 2000). The final ATP concentration in the CDK assay was 12.5 μM, and the $IC_{50}$ concentration for each compound is the concentration required to inhibit enzyme activity by 50% under the assay conditions used. The assay buffer was comprised of 50 mM Tris-HCl, pH7.5 containing 5 mM $MgCl_2$.

*Cell cycle staining analysis*. To stain the cells for DNA content and analyze the percentage of cells in G0/G1, S, and G2/M, approximately 1.4x10$^6$ cells were plated in a T75 flask and

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allowed to attach overnight. After pretreatment with guanine derivative (10 μM) for 2 h, cisplatin was added for 2 h. Cells were washed twice with PBS after drug treatment, and normal growth medium returned to the flask. Cells were harvested 16 h after drug treatment and washed once with PBS. Cells were then fixed in 70% ice-cold EtOH and stored at 4°C until PI staining. To stain with PI, cells were sedimented by centrifugation, washed in PBS, and RNase was added (0.1 mg/mL). Cells were incubated at 37°C for 30 min in the RNase solution, sedimented by centrifugation, and washed in PBS to remove RNase. Cells were then resuspended in PI stain solution (0.1 mg/mL) at a final cell concentration $1x10^6$ cell/mL and were incubated on ice for 30 min prior to analysis by flow cytometry.

*Colony Formation Assay.* To evaluate cell survival after drug treatment, a colony formation assay was used as previously described (Fishel et al., 2003). Briefly, exponentially growing cells were exposed to guanine derivative at concentrations indicated for 2 h prior to the addition of up to 50 μM cisplatin. Following incubation with guanine derivative and cisplatin at 37°C, the cells were replated in triplicate at varying densities between 150 and 3000 cells per 100-mm dish. After approximately 12 days, colonies were stained with methylene blue (0.1% w/v) and scored. Percentage survival was calculated based on the plating efficiency of the appropriate set of control cells exposed to vehicle alone. In experiments with TSA (25 ng/mL), exponentially growing cells were treated for 24 h prior to addition of $O^6$-BG (2 h pretreatment and 2 h during cisplatin) or cisplatin (2 h).

*Determination of Apoptotic Cells.* To analyze cells for apoptosis, Annexin-V-FITC antibody and PI were added to the cells according to the manufacturer's indicated protocol (BD

Biosciences Clontech, Palo Alto, CA). Samples were analyzed by flow cytometry using FACS DiVa (Becton Dickinson, San Jose, CA). As described previously, cells that were Annexin-V-FITC-positive and PI-negative were considered positive for apoptosis (Fishel et al., 2003). For Figure 3, apoptosis was analyzed using the Alexa Fluor® 488 Annexin-V from Vybrant® Apoptosis Assay Kit in combination with MitoTracker® Red CMXRos, a dye that is retained in active mitochondria (Molecular Probes, Eugene, OR). To distinguish the cells with permeable membranes, DAPI (4',6-diamidino-2-phenylindole dihydrochloride) was used, and cells that were DAPI-positive were not included in the analysis for apoptosis. Samples were analyzed by flow cytometry using FACS DiVa (Becton Dickinson, San Jose, CA). The following controls were used: unstained cells, cells stained with Alexa Fluor® 488 Annexin-V only, cells stained with MitoTracker® Red, and cells stained with DAPI only. FloJo FACS analysis software (Tree Star, Inc. Ashland, OR) was used to determine cells that were Annexin-V-FITC-positive, MitoTracker Red-negative, and DAPI-negative (positive for apoptosis).

*Measurement of total platinum in DNA.* Atomic absorption spectroscopy was used to quantitate total platinum on DNA as described previously (Fishel et al., 2003). Exponentially growing cells were treated with modulator or vehicle for 2h and then with 50 μM cisplatin for an additional 2h. Cells were collected at 0, 24, or 48 h after completion of cisplatin treatment. Total genomic DNA was isolated by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Platinum concentration was assessed with a Perkin-Elmer model 1100 flameless atomic absorption spectrometer (Perkin-Elmer, Norwalk,

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Conn.) monitoring 265.9 nm. Platinum concentrations were determined by comparison with a standard curve performed on the same day as the assay (Erkmen et al., 1995).

*Statistical Analysis.* Statistics for the apoptosis experiments were analyzed using analysis of variance (ANOVA) models with cisplatin dose (0, 10, and 20 μM) and treatment (cisplatin alone, and cisplatin plus $O^6$-BG, $S^6$-CMG, $S^6$-BG, and $O^6$-CMG). In experiments with $O^6$-BG and TSA treatment, ANOVA models were used to test for an overall effect of cisplatin dose, treatment (none, $O^6$-BG, TSA, and $O^6$-BG plus TSA), and the interaction between cisplatin dose and treatment. The outcome employed in the statistical model was the natural logarithm of the proportion of cells surviving. If a statistically significant interaction or main effect of treatment was found using the ANOVA model (i.e. the p-value<0.05), then pairwise comparisons were made between treatment groups to determine which treatments differed. In addition, two-way ANOVA models were used to examine the interaction between $O^6$-BG and TSA. A separate model was fit for each of the four cisplatin doses (0, 6, 12.5, and 25 μM). A significant interaction would provide evidence for a synergy between the two treatment modalities.

In the DNA platination experiments, the ANOVA model was fitted to the platinum concentrations to test for an overall effect of time (0, 24, and 48 hours post treatment), and treatment, and the interaction between time and treatment. The outcome employed in the statistical model was the square root of the platination levels due to non-normality. Of primary interest were the comparisons between samples treated with cisplatin alone to the samples treated with guanine derivatives plus cisplatin.

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## RESULTS

*In vitro kinase assays of the guanine derivatives.* Figure 1 illustrates the structures of the guanine derivatives used in these studies. We determined the $IC_{50}$ for CDK2/CyclinA3 of several guanine derivatives. CDK2 was inhibited to varying degrees with the greatest inhibition by $O^6$-CMG and the least potent compounds those with a substituent at the 9-position (9-$CH_3$-BG, 9-$CH_3$-CMG) or at the 7-position (N7-BG), (Table 1) as expected from the crystal structure (Davies et al., 2002).

*Cisplatin sensitivity of SQ20b after treatment with guanine derivatives.* To determine the relationship between potency of CDK2 inhibition and enhancement of cisplatin cytotoxicity, we evaluated the effect of the four most potent derivatives on cisplatin-induced cytotoxicity (Figure 2). In SQ20b cells, cisplatin cytotoxicity ($ED_{50}$) was enhanced to varying degrees by the guanine derivatives at 10 μM: $S^6$-BG (1.6-fold*)*, $O^6$-BG (1.7-fold), $S^6$-CMG (1.5-fold), and $O^6$-CMG (2.6-fold) and at 100 μM: $S^6$-BG (2.3-fold*)*, $O^6$-BG (1.5-fold), $S^6$-CMG (3-fold), and $O^6$-CMG (3.3-fold) (Figure 2 A, B). There was a significant increase in the percentage of SQ20b cells undergoing apoptosis when cisplatin was used in combination with 10 μM $S^6$-CMG (1.4-fold), and $O^6$-CMG (1.5-fold), but not with $O^6$-BG (1.2-fold) or $S^6$-BG (Figure 2C). In the apoptosis experiment, ANOVA detected a significant main effect of treatment between cisplatin dose and treatment group (p=0.003). The most potent CDK2 inhibitor resulted in the greatest degree of enhancement in cisplatin-induced cytotoxicity and apoptosis (Table 1 and Figure 2).

*Effect of poor CDK2 inhibitors on cisplatin-induced cytotoxicity.* To determine whether CDK inhibition was critical for enhancement of cisplatin-induced cytotoxicity, we utilized three

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guanine derivatives that required >100 μM concentrations to inhibit CDK2 in vitro. 9-$CH_3$-BG and 9-$CH_3$-CMG were designed with a methyl group at the N-9 position to disrupt the H-bonds which are thought to be critical for CDK2 inhibition (Arris et al., 2000). As shown in Figure 3, the $ED_{50}$ of cisplatin was enhanced 1.6-fold with 9-$CH_3$-CMG and was not enhanced by 9-$CH_3$-BG (1.1-fold) or N7-BG (1.0-fold) at 50 μM nor did they result in increased apoptosis (Figure 3, inset), consistent with the hypothesis that CDK2 inhibition is coincident with potentiation of cisplatin-induced cytotoxicity. The dose of 50 μM guanine derivative was chosen in these experiments due to limited solubility of some of these compounds.

*Cisplatin sensitivity of SCC61 after treatment with guanine derivatives.* To extend our results observed in SQ20b head and neck cancer cell lines, experiments were performed in SCC61, another head and neck cancer cell line. Similar to SQ20b cells, $O^6$-CMG and $S^6$-BG were the most and least effective modulators of cisplatin-induced cytotoxicity, respectively (Figure 4A, B). ANOVA demonstrated a significant interaction between treatment and cisplatin dose for these cell survival experiments ($p<0.001$). The observed increase in cytotoxicity with the modulators correlated well with an increase in the percentage of SCC61 cells undergoing apoptosis when exposed to 20 μM cisplatin [$S^6$-BG (1.5-fold), $O^6$-BG (1.7-fold), $S^6$-CMG (1.8-fold), and $O^6$-CMG (2.0-fold)], as shown in Figure 4B. Again, ANOVA detected a significant interaction between cisplatin dose and treatment group ($p<0.0001$) in the apoptosis experiment as well.

*CDK2 inhibition and cell cycle perturbation.* In an effort to elucidate whether cell cycle perturbation was related to the degree of enhancement of cisplatin-induced cytotoxicity, the

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percentage of cells in G1, S, and G2/M phase was analyzed following treatment with the four most potent CDK2 inhibitors (Figure 5). In the absence of cisplatin, there is no difference between the cells that were treated with guanine derivative (10 μM) and the vehicle-treated cells in G1, S, or G2/M phase distributions. However, in the presence of cisplatin, cells treated with 10 μM $S^6$-CMG and $O^6$-CMG demonstrated a statistically significant decrease in the number of cells in S phase at t= 16 h following treatment, as determined using the two-tailed Student's t test ($p<0.001$). Using the same statistical test, there was also a statistically significant increase in the percentage of cells in G2/M with all modulators in the presence of cisplatin compared to control cells ($p<0.05$) (Figure 5).

*Effect of modulators on platination of DNA following treatment with cisplatin.* To ascertain whether the increased cytotoxicity and apoptosis induced by the guanine derivatives was related to higher levels of DNA platination damage, atomic absorption spectroscopy was employed. DNA isolated from cisplatin-treated SQ20b cells had higher levels of platination at 0, 24, and 48 h when pretreated with 100 μM of guanine derivative compared to cells treated with cisplatin alone (Figure 6). This increase was observed up to 48 h. ANOVA detected a significant effect of treatment ($p=0.0001$). There was not a significant increase in the amount of platinum on DNA with guanine derivatives at 10 μM (data not shown). DNA platination experiments were also conducted using 9-$CH_3$-BG, 9-$CH_3$-CMG and N7-BG at 50 μM (data not shown). 9-$CH_3$-CMG and N7-BG demonstrated no increase in DNA platination, and 9-$CH_3$-BG treatment demonstrated slightly significantly higher levels of platination at 0, 24, and 48 h compared to cisplatin alone ($p<0.05$).

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*Effect of combination of TSA with $O^6$-BG and cisplatin.* Previously, $O^6$-BG was shown to inhibit CDK2/cyclinA3 (Arris et al., 2000) and to enhance the sensitivity of head and neck cancer cells to cisplatin (Fishel et al., 2003). We therefore sought to determine whether further enhancement would occur in the presence of TSA, an indirect cell cycle inhibitor. In SQ20b cells, TSA alone or $O^6$-BG alone decreased the $ED_{10}$ of cisplatin 1.4-fold and 1.9-fold, respectively (Figure 7). However, the combination of TSA (25 ng/mL) and $O^6$-BG (50 μM) resulted in a more dramatic enhancement of cisplatin-induced cytotoxicity, decreasing the $ED_{10}$ of cisplatin 2.5-fold compared to cisplatin alone (Figure 7). ANOVA demonstrated that cell survival was different across the four treatment groups ($p<0.0001$). The combination of both modulators, $O^6$-BG plus TSA, on cisplatin cytotoxicity was additive compared to either modulator plus cisplatin based on the interaction terms in the ANOVA models for increasing cisplatin doses in the presence of TSA and $O^6$-BG not being statistically significant.

## DISCUSSION

Cisplatin and other platinum derivatives are used widely against several types of human cancers including testicular cancer, metastatic lung cancer, relapsed lymphomas, head and neck cancer, and gynecologic cancers, and the goal of this research is to improve platinating agent efficacy. Previously, we had shown that $O^6$-BG, a potent $O^6$-alkylguanine-DNA alkyltransferase (AGT) inactivator and a weak CDK2 inhibitor, enhanced cisplatin-induced cytotoxicity, apoptosis, and DNA platination. In this report, we extend these findings to evaluate a group of similarly structured guanine derivatives for their ability to enhance cisplatin-induced cytotoxicity. To ascertain the role of CDK2 inhibition, we compared more

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and less potent CDK2 inhibitors with the extent of cisplatin-enhanced cytotoxicity and apoptosis. Our data suggests that cell cycle perturbation resulting from CDK2 inhibition is likely the mechanism of the enhancement of cisplatin-induced cytotoxicity. We also tested the combination of both a direct ($O^6$-BG), and an indirect cell cycle inhibitor (TSA), which resulted in a dramatic, albeit additive, effect on cisplatin-induced cytotoxicity. This implies that $O^6$-BG and TSA are enhancing cisplatin-induced cytotoxicity through different mechanisms.

$O^6$-BG-mediated enhancement of cisplatin- and carboplatin-induced cytotoxicity is independent of AGT status (Fishel et al., 2003), nucleotide excision repair (NER) capacity or glutathione (GSH) concentrations (Fishel et al., 2004). Similar to many other drugs developed with a known mechanism, on closer examination additional mechanisms of action and applications of $O^6$-BG are becoming evident. Evidence in favor of CDK2 as a mechanism of cisplatin enhanced cytotoxicity is as follows: 1) the more potent CDK inhibitors proved to be the more potent modulators of cisplatin-induced cytotoxicity; 2) all 3 poor inhibitors of CDK2 inhibitors, 9-$CH_3$-CMG, 9-$CH_3$-BG and N7-BG, were less or ineffective at enhancing cisplatin-induced cytotoxicity; 3) the observed enhancement of cisplatin-induced cytotoxicity by $O^6$-BG is dependent of the sequence of administration, with a dramatic increase in cisplatin-induced cytotoxicity, apoptosis and DNA platination observed only in cells incubated with $O^6$-BG prior to and during cisplatin exposure (Fishel et al., 2004). Sequence dependency is consistent with many cell cycle inhibitors (Shah and Schwartz, 2001); and 4) a significant decrease in the percentage of cells in S phase and an increase in cells in G2/M when cisplatin was used in combination with $O^6$-CMG and $S^6$-

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CMG was observed. Our findings therefore implicate cell cycle modulation by CDK inhibition as a key event.

CDK2 is overexpressed and oftentimes correlated with prognosis in head and neck cancers (Dong et al., 2001; Mihara et al., 2001; Shintani et al., 2002), ovarian cancer (Barboule et al., 1998; Marone et al., 1998; Sui et al., 2001), and melanoma (Tang et al., 1999) providing a rationale for exploring cell cycle modulation of cisplatin-induced cytotoxicity. CDK2/cyclin E is important in the G1 to S phase transition, and CDK2/cyclin A is important during S phase making it a reasonable target for cancer therapy especially in cancers where expression is elevated (reviewed in (Wadler, 2001)). Typically, when cells are arrested at the G1/S or G2/M checkpoints following DNA damage, and not allowed to progress to S phase or mitosis, respectively, it is advantageous to the cell as it may avoid replication on a damaged template or the transmission of damaged genetic material to the daughter cells. Higher doses of CDK inhibitors tend to accumulate cells in G2/M while lower doses block cells at G1 (Meijer et al., 1999), and these effects could explain the decrease of cells in S phase observed in the current study with two of the more potent CDK inhibitors, $O^6$-CMG and $S^6$-CMG. Perturbation of the cell cycle during treatment with cisplatin could cause changes in the cellular response to the drug relating to the formation, detection and processing of platinum lesions, or more generally in the expression of cell cycle-regulated genes such as histones. Whatever the mechanism operating, the effect of CDK inhibition on cisplatin-induced cytotoxicity apparently overrides any reduction in cellular sensitivity that could, in theory, arise as a result of the inhibition of cell cycle progression by the CDK inhibitor.

The mechanism of action of TSA is believed to be through inhibition of histone deacetylases (HDAC) leading to an alteration of chromatin acetylation and structure

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(Yoshida et al., 1990). Modest enhancement of cisplatin cytotoxicity by TSA has been observed in MCF-7 breast cancer cells, and this enhancement was more dramatic when TSA treatment preceded cisplatin exposure (Kim et al., 2003). In the current study, the potentiation of cisplatin by TSA was confirmed and greater potentiation of cisplatin-induced cytotoxicity achieved with the combination of $O^6$-BG and TSA. There might therefore be value in considering this three drug combination clinically.

In contrast to other cell cycle inhibitors, $O^6$-BG has shown only limited side effects in Phase II/III clinical trials (Friedman et al., 1998; Spiro et al., 1999; Friedman et al., 2000; Schilsky et al., 2000). However, at the doses used to modulate AGT activity, $O^6$-BG may lack potency as a cell cycle modulator. The most potent modulator of cisplatin activity and the most potent CDK inhibitor of the guanine derivatives identified in the current study, $O^6$-CMG, enhanced cisplatin at a 10-fold lower concentration than $O^6$-BG, and hence $O^6$-CMG may have clinical potential as a cisplatin modulator. In addition, we are currently delving deeper into the mechanism of modulation of cisplatin-induced cytotoxicity with the guanine derivatives along with studies of the structure-activity relationship in efforts to design a clinically feasible modulator of platinating agents.

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**Footnotes.**

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Dr. Dolan is co-inventor of $O^6$-benzylguanine, a compound that has been licensed to Access Oncology, Inc.

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## Figure Legends

Figure 1. Chemical structures of guanine derivatives used in combination with cisplatin.

Figure 2. Effect of guanine derivatives on cisplatin cytotoxicity in SQ20b cells. **A.** Colony formation assay of SQ20b cells treated with guanine derivative at 10 μM for 2 h prior and during the 2h cisplatin exposure. **B.** Colony formation assay of SQ20b cells treated with guanine derivative at 100 μM for 2 h prior and during cisplatin exposure. Percentage survival is calculated as described in Materials and Methods. Each data point represents the mean +/– SE from at least 2 separate experiments with each experiment representing 6 replicate dishes per treatment group. **C.** SQ20b cells treated with guanine derivative at 10 μM for 2 h prior and during cisplatin exposure (2 h) and assayed for apoptosis 96 h following cisplatin treatment. Shown here is a representative experiment of the assay performed in triplicate. $S^6$-CMG and $O^6$-CMG treatment in combination with cisplatin induced significantly greater levels of apoptosis than cisplatin alone ($p<0.01$).

Figure 3. Effect of guanine derivatives that are not CDK inhibitors on cisplatin cytotoxicity. Colony formation assay of SQ20b cells treated with guanine derivative at 50 μM for 2 h prior and during cisplatin exposure (2h). Percentage survival is calculated as described in Materials and Methods. Each data point represents the mean +/– SE from at least 5 separate experiments with each experiment representing 6 replicate dishes per treatment group. Based on subsequent pairwise comparisons of the various treatments versus cisplatin alone, the effect of 9-$CH_3$-BG and 9-$CH_3$-CMG plus cisplatin on cell survival compared to cisplatin alone depended on the cisplatin dose (***** $p<0.05$). The cytotoxic effects of N7-BG plus cisplatin were not significantly

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different from cisplatin alone (p=0.21). Inset: SQ20b cells treated with 50 μM guanine derivative for 2 h prior to and during 2 h cisplatin exposure and assayed for apoptosis 96 h later. Each column represents the mean +/– SE from at least 4 separate experiments.

Figure 4. Effect of guanine derivatives on cisplatin cytotoxicity in SCC61 cells. **A.** Colony formation assay of SCC61 cells treated with guanine derivative at 100 μM for 2 h prior and during the 2h cisplatin exposure. Percentage survival is calculated as described in Materials and Methods. Each data point represents the mean +/– SE from at least 3 separate experiments with each experiment representing 6 replicate dishes per treatment group. Based on pairwise comparisons, all modulators in combination with cisplatin treatment were statistically significant compared to cisplatin alone (****** $S^6$-CMG, $O^6$-CMG, $p \leq 0.01$), with $O^6$-BG and $S^6$-BG being moderately significant.(***** $p \leq 0.05$). **B.** SCC61 cells treated with guanine derivative at 100 μM for 2 h prior and during cisplatin exposure (2 h) and assayed for apoptosis 96 h after cisplatin treatment. Shown here is the average of 3 separate experiments (± SE). $S^6$-BG, $O^6$-BG, $S^6$-CMG, and $O^6$-CMG treatment in combination with cisplatin induced significantly greater levels of apoptosis than cisplatin treatment alone ($p<0.0001$, $p<0.05$, $p<0.0001$, and $p<0.00001$, respectively).

Figure 5. Effect of guanine derivatives on cell cycle phase distribution. Cell cycle distribution was determined using PI staining of SQ20b cells treated with guanine derivatives (10 μM) 2 h prior to and during cisplatin exposure (50 μM, 2h). Cells were collected and fixed 16 h after cisplatin treatment. Each data point represents the mean +/– SE from 3 separate experiments. (∗

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p<0.05, compared to cisplatin treatment alone; *** p<0.001, compared to cisplatin treatment alone; # p<0.05, compared to cells not treated with cisplatin.)

Figure 6. Effect of guanine derivatives on DNA adduct levels following cisplatin treatment. Platination on DNA of SQ20b cells treated 100 μM guanine derivative plus cisplatin (50 μM) or cisplatin alone. Cells were collected 0, 24, and 48 h after cisplatin treatment. Total DNA platinum adducts were measured using atomic absorption spectrometry as described in Materials and Methods. Pt levels are expressed relative to cisplatin treatment alone using the average from at least 3 separate experiments. The effect of treatment averaged across the 3 time points for $S^6$-BG, $S^6$-CMG, and $O^6$-CMG treatment in combination with cisplatin was significantly greater than cisplatin treatment alone ($p \leq 0.001$).

Figure 7. Effect of $O^6$-BG and TSA treatment on cisplatin cytotoxicity. The following four treatment groups were used: cisplatin alone (closed squares), TSA (25ng/mL) plus cisplatin (open circles), $O^6$-BG (50 μM) plus cisplatin (closed triangles), and TSA plus $O^6$-BG plus cisplatin (crossed squares). TSA treatment was 24h prior to $O^6$-BG and/or cisplatin treatment. $O^6$-BG treatment schedule was 2h prior to and during the 2h cisplatin exposure. Cytotoxicity was measured as colony forming ability 10-12 days after treatment. Each data point represents the mean +/– SE from at least 3 separate experiments with each experiment representing 6 replicate dishes per treatment group. Based on pairwise comparisons of the combined treatments versus cisplatin alone, $O^6$-BG + cisplatin and $O^6$-BG plus TSA plus cisplatin resulted in significantly greater enhancement of cytotoxicity compared to cisplatin alone (******* $p \leq 0.0001$). TSA treatment

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alone had significantly greater cytotoxicity than vehicle-treated cells, and this difference remained fairly constant across the cisplatin doses (***** $p<0.05$).

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Table 1. Inhibition of CDK2 by guanine derivatives.

| *Compound* | *IC50 (μM) CDK2/cyclinA3* |
|---|---|
| $O^6$-CMG | 17 ± 2 (Arris et al., 2000) |
| $S^6$-CMG | 26 ± 7 |
| $O^6$-BG | 35 ± 6 (Gibson et al., 2002) |
| $S^6$-BG | 92 ± 12 |
| 9-$CH_3$-BG | > 100 |
| 9-$CH_3$-CMG | > 100 (Arris et al., 2000) |
| N7-BG | > 100 |

$O^6$-BG, $R_1$ =
$R_2$ = H




$O^6$-CMG, $R_1$ =
$R_2$ = H




$S^6$-BG, $R_1$ =
$R_2$ = H




$S^6$-CMG, $R_1$ =
$R_2$ = H

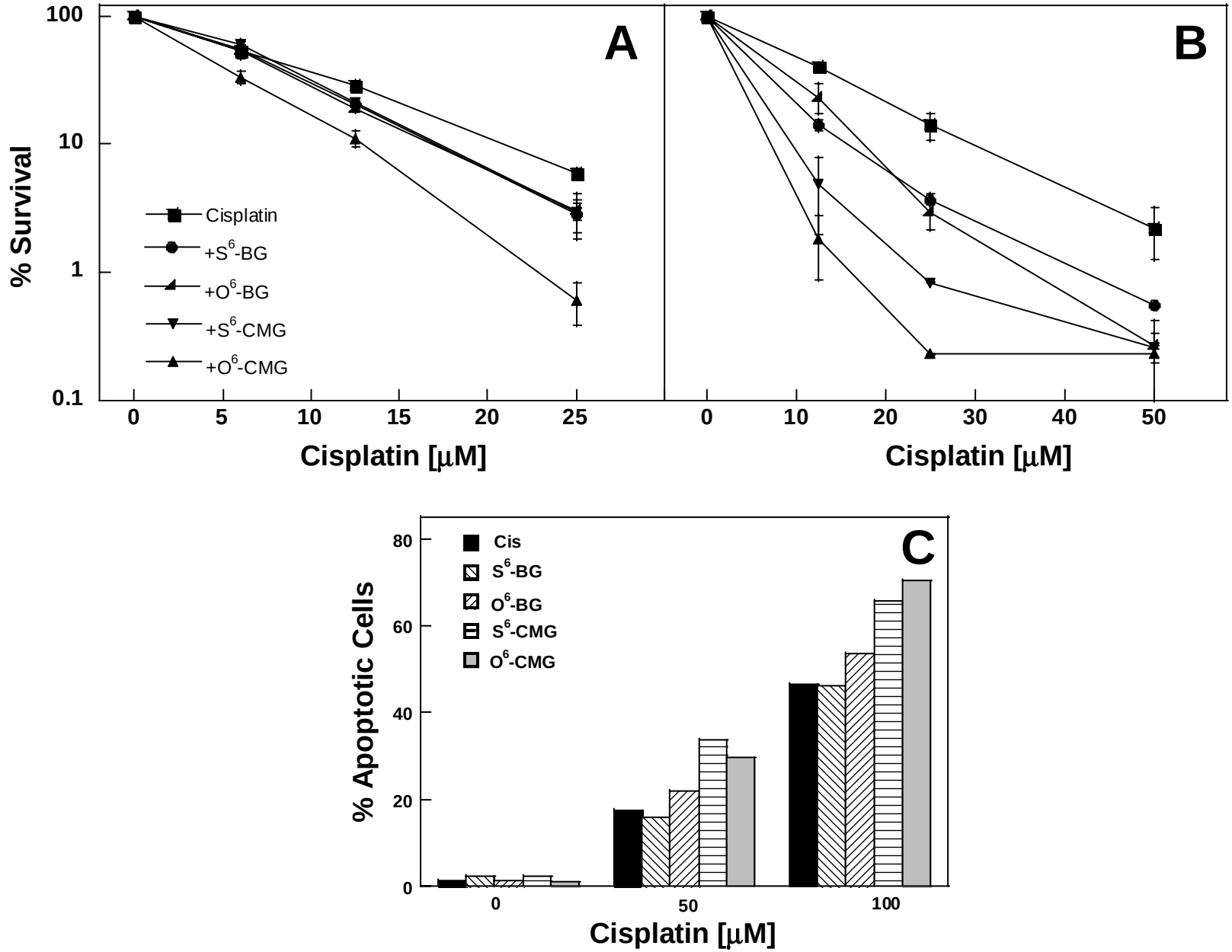



9-$CH_3$-CMG, $R_1$ =
$R_2$ = $CH_3$




9-$CH_3$-BG, $R_1$ =
$R_2$ = $CH_3$

N7-BG



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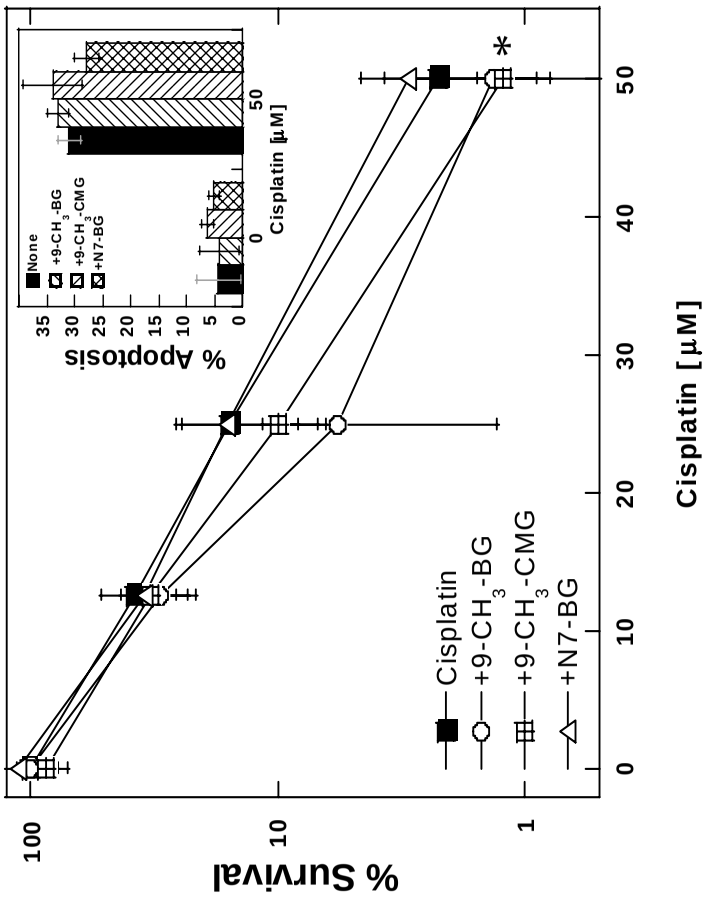

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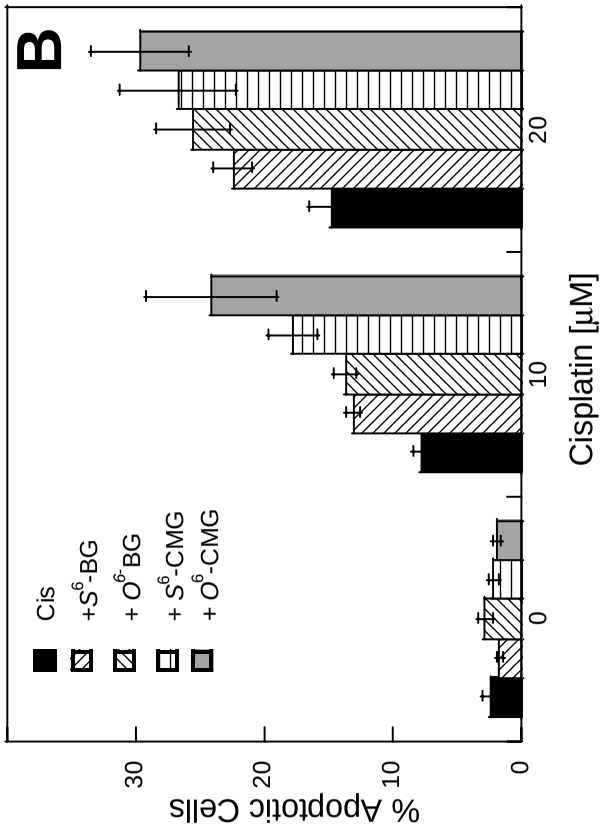


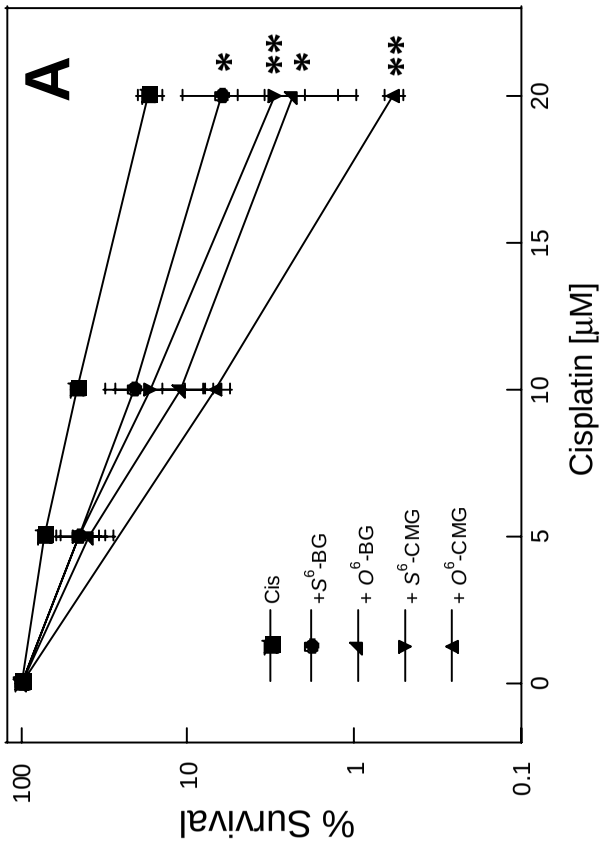

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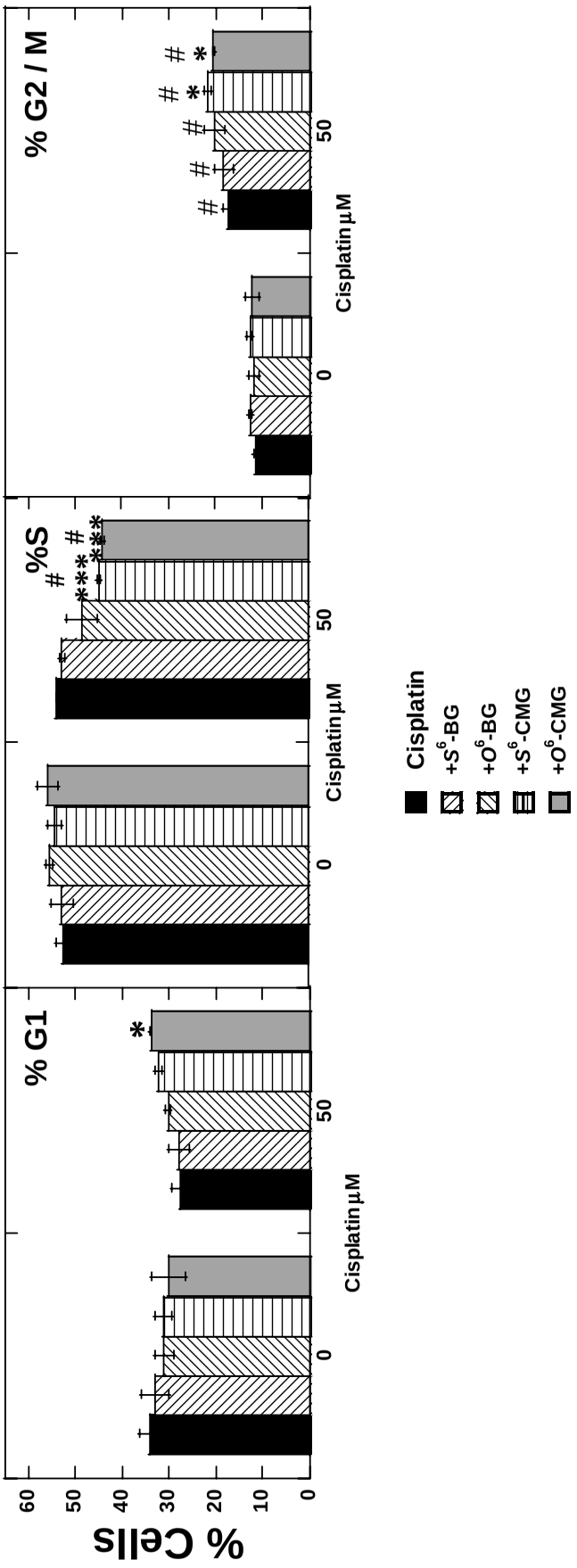

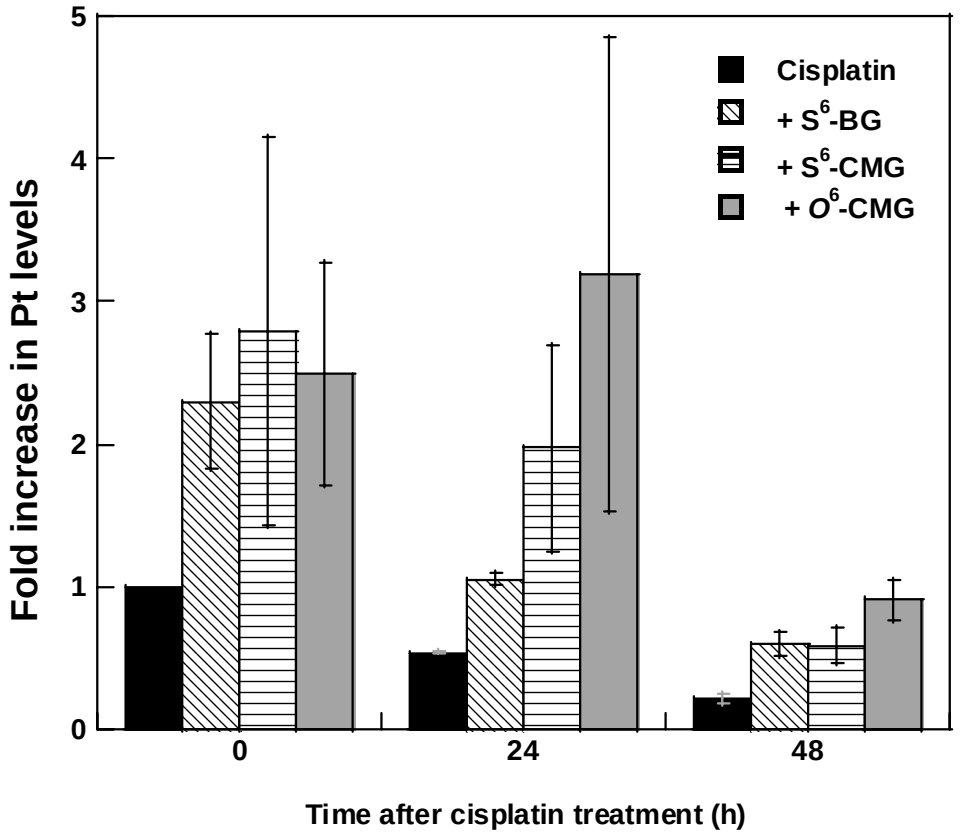


* p<0.05
***p<0.001

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