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The novel, small molecule DNA methylation inhibitor SGI-110 as an ovarian cancer chemosensitizer

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Abstract

Purpose—To investigate SGI-110 as a “chemosensitizer” in ovarian cancer (OC) and to assess its effects on tumor suppressor genes (TSG) and chemo-responsiveness associated genes silenced by DNA methylation in OC.

Experimental Design—Several OC cell lines were used for *in vitro* and *in vivo* platinum resensitization studies. Changes in DNA methylation and expression levels of TSG and other cancer-related genes in response to SGI-110 were measured by pyrosequencing and RT-PCR.

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Disclaimers: none

Results—We demonstrate *in vitro* that SGI-110 resensitized a range of platinum-resistant OC cells to cisplatin (CDDP) and induced significant demethylation and reexpression of TSG, differentiation-associated genes and putative drivers of OC cisplatin resistance. *In vivo*, SGI-110 alone or in combination with CDDP was well tolerated and induced anti-tumor effects in OC xenografts. Pyrosequencing analyses confirmed that SGI-110 caused both global (*LINE1*) and gene specific hypomethylation *in vivo*, including TSGs (*RASSF1A*), proposed drivers of OC cisplatin resistance (*MLH1* and *ZIC1*), differentiation-associated genes (*HOXA10* and *HOXA11*), and transcription factors (*STAT5B*). Furthermore, DNA damage induced by CDDP in OC cells was increased by SGI-110, as measured by ICP-mass spectrometry analysis of DNA adduct formation and repair of cisplatin-induced DNA damage.

Conclusions—These results strongly support further investigation of hypomethylating strategies in platinum-resistant OC. Specifically, SGI-110 in combination with conventional and/or targeted therapeutics warrants further development in this setting.

Keywords

SGI-110; DNA methylation; chemotherapy; epigenetics; ovarian cancer

INTRODUCTION

Ovarian cancer (OC) is the deadliest gynecological cancer, causing 14,270 estimated deaths and 21,980 new cases in the United States (1). Current treatment for OC includes cytoreductive surgery and platinum-based chemotherapy (2). Although most patients initially respond to chemotherapy, more than 80% of women develop resistance, with an average time to progression ranging from 18 to 24 months (3). Therapeutic options are limited for platinum resistant OC and while new targeted agents are currently under clinical investigation, a personalized approach has not been easy to implement and has not resulted in improved outcomes. The recent genomic description of high grade serous OC revealed that even chemotherapy-naïve tumors harbor highly disorganized genomes (4), characterized by tens of genetic alterations per tumor. Such molecular chaos is expected to be further augmented in the platinum-resistant setting, rendering therapy targeted to single mutations highly unlikely to alter outcomes. Indeed, whereas several second-line therapeutic approaches have prolonged progression-free survival (PFS), the impact on overall survival remains modest (5–7).

Platinum resistance in OC is a complex phenomenon, resulting from alterations in a number of key pathways as well as epigenetic anomalies including changes in DNA methylation, histone modifications, and nucleosome positioning (8–11). Abnormal DNA methylation patterns, such as increased DNA methylation within CG-rich (“CpG islands”) promoter regions (often within tumor suppressor genes), is a well-studied transcriptionally repressive epigenetic modification (12) occurring frequently in OC. This epigenetic mark can be reversed using pharmacological approaches, such as by using DNA methyltransferase inhibitors (DNMTIs) (13). We and others previously demonstrated in preclinical studies that known DNMTIs decitabine (5-aza-dC) and zebularine resensitize chemoresistant OC cells to platinum (14, 15). Recent phase I/II trials showed that low-dose decitabine followed by carboplatin resulted in significant clinical and biological activity in women with platinum-

resistant OC (16–18). Current FDA approved therapeutic DNMTIs are subject to rapid degradation by hydrolytic cleavage and deamination by cytidine deaminase and are unstable after intravenous infusion limiting their potential as cancer therapeutics (19, 20).

SGI-110, a dinucleotide combining 5-aza-dC and deoxyguanosine (Astex Pharmaceuticals, Inc.), has been shown to be less prone to deamination by cytidine deaminase and more stable in aqueous solution (21), making it a promising alternative to 5-aza-dC. We conducted a preclinical combination study of SGI-110 with cisplatin (CDDP) in OC models and demonstrated that pre-treatment with SGI-110 resensitized a range of OC cells to CDDP, both *in vitro* and *in vivo*. In all, our preclinical studies reveal that SGI-110 is an effective DNA hypomethylator in OC and supports its future clinical development in OC and other solid tumors. Clinical trials using this combination are ongoing.

MATERIALS AND METHODS

Cell culture and drugs

A2780 OC cells were obtained and authenticated in 2012 from ATCC and cell culture reagents were purchased from Invitrogen. A2780-CDDP-resistant cells and CP70-CDDP resistant cells were prepared by exposure to incrementally increasing doses of cis-diamminedichloroplatinum (II) dichloride (CDDP, cisplatin) (Calbiochem) as previously described (14). SKOV3, 59M, and OAW28 cells were obtained from the European Collection of Cell Cultures (ECACC). 59M and OAW28 cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS). SKOV3 cells were maintained in McCoy's 5A medium supplemented with 1.5 Mm glutamine and 10% FBS. All other cells were maintained in RPMI 1640 media supplemented with 10% FBS and 1% antibiotics, as described previously (14). 5-aza-2'-deoxycytidine (5-aza-dC) was purchased from Sigma. SGI-110 was provided by Astex Pharmaceuticals, Inc. (Dublin, CA).

Platinum resensitization

Treatment with 5-aza-dC (5 μ M), SGI-110 (0.1, 0.3, 1 and 5 μ M), or vehicle (DMSO 1:2000) was performed for 48hr prior to CDDP treatment (15). MTT and alamar blue (Invitrogen) assays were used to determine both IC₅₀ values and growth curves, as described previously (15). Details can be found in the online Supplementary Methods.

qRT-PCR

RNA was isolated from cultured OC cells using AllPrep DNA/RNA/Protein Mini kit (Qiagen) following manufacturer's protocol and the quantity and quality determined by absorbance (260, 280nm). Total RNA (2 μ g) was reverse transcribed with the LightCycler 480 SYBR Green I Master kit (Roche, Switzerland) and analyzed by qRT-PCR according to the manufacturer's instructions. Primer sequences (Fisher Scientific) can be found in Supplementary Table S1. For *in vivo* qRT-PCR validation assay, the RNA was isolated from tumor tissues using TRizol® reagent (Invitrogen, CA) according to the manufacturer's instruction. qRT-PCR was performed using miScript reverse transcription and miScript SYBR® Green PCR kits (Qiagen, CA) in a Roche Lightcycler (Roche Applied Science, IN),

as described previously (14, 22). mRNA expression level was determined using LightCycler software version 3.5 (Roche Applied Science, IN), normalized to *EEF1α1b*, and using the 2^{-CT} method of relative quantification.

***In vivo* non-tumor bearing mice experiments and treatment schedule**

All animal studies adhered to protocols approved by the Institutional Animal Care and Use Committee of Indiana University. Female nude, athymic, BALB/c-nu/nu mice (4–5 weeks old) (Harlan) were treated with SGI-110, CDDP, or SGI-110 and CDDP in combination according to the treatment schedule provided in Supplementary Figure S1A. SGI-110 was administered at either 2mg/kg or 5mg/kg and CDDP was administered at 2mg/kg or 4mg/kg. Body weight (BW), eating habits and behavior were monitored biweekly.

***In vivo* xenograft experiments and treatment schedule**

Parental or CDDP-resistant A2780 cells (Sigma) were counted, resuspended in 100µl 1:1 RPMI 1640/Matrigel (BD Biosciences), and 7×10^5 cells were injected subcutaneously (s.c.) into the right flanks of 4–5 week old female nude athymic mice (BALB/c-nu/nu, Harlan). Tumors were allowed to grow to reach a predetermined size (~4–5 mm in diameter) before each treatment. Mice bearing similar tumor size (4–5 mm in diameter) were randomly assigned to different treatment arms: control, CDDP, SGI-110, or SGI-110 and CDDP combination, as summarized in Supplementary Figure S1B. Tumor sizes and BWs were measured biweekly. Tumor length (l) and width (w) were measured using digital calipers. Tumor volume (v) was calculated using the following equation: $v = \frac{1}{2} \times l \times w^2$. Mice were sacrificed if tumors reached a diameter of 2cm or at the end of study. Tumor growth curves were analyzed using general linear models. Xenografts were snap frozen for DNA/RNA extraction.

DNA extraction and pyrosequencing of blood, tumors and cell lines

DNA was extracted from 100 µl of blood or 25mg tumor tissue using DNeasy Blood & Tissue Kit (Qiagen). Sodium bisulfite conversion of genomic DNA, cleanup, and *LINE1* and specific gene pyrosequencing analysis was performed by EpigenDx Inc. Primers are listed in Supplementary Table S2. For cell lines, genomic DNA extraction was performed using the QIAamp DNA extraction kit and 100 ng–2 µg of genomic DNA was converted to bisulfite DNA using the EpiTect[®] Plus DNA Bisulfite Kit (Qiagen). Pyrosequencing analysis of *LINE1* elements, *MLH1* and *ZIC1* was performed using the PyroMark Q24 in conjunction with PyroMark Q24 CpG *LINE1*, CpG *MLH1* and CpG *ZIC1* assay kits (Qiagen).

ICP-mass spectrometry analysis

Parental and CDDP-resistant A2780 cells were plated at 2×10^5 per well in 6-well plates. Triplicate wells were treated with either vehicle (DMSO) or 5µM SGI-110 for 48 hours. Media was replaced with fresh RPMI containing 10µM CDDP. Cells were incubated for 2hr at 37°C and 5% CO₂, media was removed and cells washed with phosphate-buffered saline. Cells received fresh RPMI without CDDP and were allowed to repair for 0, 2, 4 and 24 hours. DNA was extracted from cells by lysis in the well and spooling as described by Laird et al (23). DNA (30–50 µg) was hydrolyzed overnight in 1% nitric acid at 70°C in 500µL

total volume. Samples were then diluted to 1.5mL final volume in 1% nitric acid and analyzed by ICP-mass spectrometry as we have previously described (24). Briefly, a benchtop series Thermo ICPMS X-series II system with collision cell technology capability and PlasmaLab software were used to quantify CDDP concentrations. The argon plasma torch purity was at least 99.999% (Praxair Distribution, Inc.). Water was purified with a Milli-Q Advantage A10 System (Millipore). Optima nitric acid 67 to 70% (Fisher Scientific) was diluted to 2% and used as the solvent matrix while certified standard solutions were provided by Inorganic Ventures. ICP-MS calibration was conducted according to the manufacturer's specifications, followed by a multipoint curve fitted by linear regression with a minimum correlation coefficient (r^2) of 0.999. Samples were spiked with Yttrium (Y, 88.9 Da) and lead (Pb, 207.2 Da) to bracket the CDDP (Pt, 195.1 Da) signal and were used as machine controls. Triplicate injections were used to quantify the level of CDDP in each individual sample based on a standard curve with elemental CDDP. The standard curve was performed with each experimental run and achieved linearity over the range of concentrations tested with an r^2 of greater than 0.95.

Western blot analysis

Proteins extracted from treated cells were transferred to polyvinylidene difluoride membranes and blotted with rabbit anti- β -tubulin (1:4000) (Santa Cruz Biotechnology), anti-trimethyl-histone-H3 (Lys27) (1:1000) (Millipore), anti-acetyl-histone H4 (Lys16) (1:1000) (Millipore), mouse anti-histone H3 (1:1000) (Active Motif, CA), MLH1 (BD Biosciences) and actin (Abcam). Goat anti-Rabbit IgG (H+L), peroxidase labeled antibodies (1:4000) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) were used for detection of H4K16ac and H3K27me3. Infrared-dye-labeled anti-mouse antibodies (Licor Bioscience) and the Odyssey infrared imaging system (Licor Biotechnology) were used to detect *MLH1*.

Densitometry used Image J analysis software. Experiments were done in triplicate.

Statistical analysis

Statistical analysis employed the Student's *t*-test to compare BW and tumor volume and the paired *t*-test to compare *LINE1* and gene-specific methylation levels between mice treated with vehicle control, SGI-110, CDDP, or SGI-110 and CDDP. All *P*-values were corrected with the Bonferroni correction method for the number of comparisons. A *P*-value of 0.05 was considered statistically significant. All *in vitro* experiments were reported as means \pm S.E.M of 3 independent experiments.

RESULTS

SGI-110 modulates sensitivity to CDDP, causes demethylation and gene reexpression *in vitro*

We investigated whether the DNMTIs 5-aza-dC and SGI-110, a dinucleotide antimetabolite of 5-aza-dC, modulates the response of OC cells to CDDP. Parental A2780 cells, A2780-CDDP resistant cells, and CP70-CDDP resistant cells were primed with vehicle, SGI-110, or 5-aza-dC for 48 hours and then were treated with CDDP. Cell viability was measured by

MTT assay. “Priming” with moderate doses (5 μM) of either SGI-110 (Fig. 1A) or 5-aza-dC (Supplementary Figure S2) increased the sensitivity of OC cells to CDDP causing a >2-fold reduction in the IC_{50} for CDDP: 28 μM CDDP IC_{50} for A2780-CDDP resistant cells after SGI-110 priming compared to 42 μM CDDP IC_{50} for parental A2780 cells by CDDP, Supplementary Table S3). Interestingly, we observed that SGI-110 increased sensitivity to CDDP for both the parental and the resistant A2780 cells. Although among other OC cell lines, the parental A2780 is considered to be CDDP “sensitive”, it has a relatively high IC_{50} for the drug (Supplementary Table S3).

Previous studies have demonstrated an association between CDDP-resistance of OC cells and hypermethylation mediated silencing of several genes including the mismatch repair protein *MLH1*, TSGs, *RASSF1A*, and the differentiation associated gene *HOXA11* (10, 14, 15), prompting us to measure the effects of SGI-110 on the DNA methylation and expression levels of those genes. SGI-110-modulated chemoresensitization of the A2780 cells was accompanied by demethylation and reexpression of *MLH1* gene (Fig. 1B), *RASSF1A* (Fig. 1C) and *HOXA11* (Fig. 1D). We also measured the effects of SGI-110 on other genes whose hypomethylation correlated with clinical response to decitabine in a previous clinical trial (16), noting that SGI-110 induced promoter demethylation of these additional genes. Additional results are provided in Supplementary Figure S3.

Next, we evaluated whether a lower dose of SGI-110 (0.3 μM) also modulates CDDP-response in OC cell lines. Cells were pre-treated for 3 days with a low dose of SGI-110 (25), prior to exposure to CDDP and cell viability was measured by the alamar blue assay. We confirmed that low dose SGI-110 increased sensitivity of A2780 cells to CDDP (9-fold sensitization, data not shown) consistent with the observations using higher doses of SGI-110 (Fig. 1A). Pre-treatment with SGI-110 increased up to 9-fold and 4-fold CDDP sensitivity of OVCAR8 and OAW28 OC cell lines, respectively (Supplementary Figure S4). However, the 59M, OVCAR3 and SKOV3 cells were not sensitized to CDDP by exposure to low dose SGI-110, suggesting differential cell response to the hypomethylating strategy.

To determine whether the 3 day exposure to low dose SGI-110 induced effective hypomethylation in the cell lines analyzed, *LINE1* methylation was measured by pyrosequencing. Significant demethylation of *LINE1* ranged from 17% to 45% (Fig. 1E), however no correlation between *LINE1* hypomethylating activity of SGI-110 and resensitization to CDDP was recorded. As *MLH1* reexpression correlated with resensitization of A2780 cells to CDDP (Fig. 1B), we next measured the effects of low dose SGI-110 on *MLH1* expression levels in other OC cells. We found that *MLH1* was not silenced in the OVCAR8 or OAW28 cells and treatment with SGI-110 did not modulate *MLH1* expression (Supplementary Fig. S5B, Supplementary Table S4), thus suggesting that the SGI-110-regulated mechanisms of CDDP-resistance in these cell lines are distinct from those of A2780 cells and not related to *MLH1* expression levels.

Epigenetic gene silencing is a potential mechanism of OC CDDP-resistance

Unlike the A2780 models, the contribution of epigenetic silencing of key genes to CDDP-resistance of OVCAR8 and OAW28 has not been previously characterized. To investigate whether epigenetic mechanisms play a role in CDDP resistance in OVCAR8 and OAW28

cells, we analyzed gene expression levels of a panel of candidate drivers of OC CDDP resistance (Supplementary Table S5). The candidate genes were previously proposed by us (26) and Lum et al. (27), who identified genes that were epigenetically silenced (by hypermethylation) in primary samples derived from platinum-resistant OC patients. Expression levels of the candidate genes were determined by using real-time PCR, following exposure of the cells to SGI-110. Of the 19 candidate genes analyzed, a marked induction of *DOK2* 65-fold at 1 μ M SGI-110 and *ZIC1* 11-fold at 1 μ M SGI-110 expression levels were observed in response to SGI-110 in the OVCAR8 cell line (Fig. 2A, B). Furthermore, an induction of *DOK2* 41-fold at 1 μ M SGI-110 and *ZIC1* 13-fold at 1 μ M SGI-110 was also observed in the OAW28 cells, in addition to a modest induction of *TWIST1* (3-fold at 1 μ M SGI-110), *NR2E1* (7-fold at 1 μ M SGI-110) and *SOX9* (3-fold at 1 μ M SGI-110) (Supplementary Figure S6). To determine whether the reexpression of *ZIC1* and *DOK2* was associated with resensitization of OC cells to CDDP, the effect of SGI-110 on *ZIC1* and *DOK2* expression levels was analyzed in the cells not sensitized to CDDP by SGI-110 (59M, OVCAR3 and SKOV3, Supplementary Figure S4). A dose-dependent increase in *DOK2* expression was observed in all of the cell lines tested, irrespective of response to CDDP (Fig. 2A), suggesting that the induction of *DOK2* did not contribute to the CDDP-resensitization observed in these cell lines. However, SGI-110-dependent induction of *ZIC1* was only observed in the cell lines in which SGI-110 conferred resensitization to CDDP (OVCAR8 and OAW28; Fig. 2B, Supplementary Table S6), suggesting that the epigenetic-silencing of *ZIC1* is a potential mechanism of CDDP resistance in these cells.

The methylation levels of *ZIC1* in the parental A2780, OVCAR8, OAW28, 59M, OVCAR3 and SKOV3 cells were next determined by pyrosequencing (Fig. 2C). The highest level of *ZIC1* methylation was observed in the OVCAR8 (21%), OAW28 (44%), and SKOV3 cells (31%). Levels of *ZIC1* methylation were much lower in the parental A2780 (4%), 59M (4%) and OVCAR3 (4%) cell lines. Interestingly in SKOV3 cells, although *ZIC1* was highly methylated, SGI-110 treatment did not induce *ZIC1* reexpression, indicating that factors other than promoter methylation contribute to the repression of *ZIC1* in these cells. In contrast, SGI-110 reversed the methylation of *ZIC1* in a dose-dependent manner in OAW28 cells (Fig. 2D). Taken together these data suggest that the CDDP resistance observed in OVCAR8 and OAW28 cells is at least in part due to hypermethylation of the *ZIC1* promoter and that the reversal of epigenetic silencing of *ZIC1* by SGI-110 can resensitize the cells to CDDP. These results point to new potential biomarkers that can explain development of platinum resistance in OC and predict response to epigenetic therapies.

SGI-110 tolerability studies in non-tumor bearing mice

To investigate whether SGI-110 alone or in combination with CDDP was tolerable *in vivo*, female nude athymic non-tumor bearing mice were treated with two different schedules (QD5 and biweekly) (Supplementary Figure S1A). The QD5 daily schedule was SGI-110 (2 mg/kg or 5 mg/kg) treatment for five consecutive days alone or followed by CDDP (2 mg/kg or 4 mg/kg) on day 8 and was designed to model the regimen used in our previous clinical trial testing decitabine (5-aza-dc) as a chemosensitizer (16). The biweekly schedule used different doses of SGI-110 (2 mg/kg or 5 mg/kg), CDDP (2 mg/kg or 4 mg/kg), or both twice a week for 4 weeks. Animals were observed for three additional weeks post-treatment.

In the QD5 schedule, SGI-110 2 mg/kg had no effect on BW compared to SGI-110 5 mg/kg (Supplementary Figure S7A). Furthermore, the lower dose SGI-110-CDDP combination was well tolerated (Supplementary Figure S7A, orange line) but initial BW loss was observed using the higher dose combinations (Supplementary Figure S7A, pink and green lines). In the biweekly schedule, all SGI-110 and CDDP combinations were well tolerated, based on steady increases in BW at each time point examined (Supplementary Figure S7B). Overall, SGI-110 in combination with CDDP at physiologically achievable doses was well tolerated in non-tumor bearing mice.

SGI-110 inhibits tumor growth *in vivo*

Based on the above results in non-tumor bearing mice, low dose SGI-110 (2 mg/kg) was used for subsequent OC xenograft experiments. CDDP-resistant A2780 cells were injected subcutaneously into the right flanks of 4–5 week old female nude athymic mice, and tumors were allowed to form as described. SGI-110 2 mg/kg and SGI-110 2 mg/kg + CDDP (2 mg/kg or 4 mg/kg), both the QD5 and biweekly schedules, delayed ($P < 0.05$) tumor growth (Fig. 3A QD5 treatment schedule and 3B biweekly treatment schedule; tumor growth curves for parental A2780 xenografts in Supplementary Figure S8; AUC graphs in Supplementary Figure S9). SGI-110 alone or in combination with CDDP was well tolerated overall in tumor bearing mice using either treatment schedule (Supplementary Figure S10).

The effect of SGI-110 on *LINE1* methylation in PBMCs and TSG methylation and gene expression in xenograft tumors was examined in mice bearing parental A2780 or CDDP-resistant A2780 xenografts. PBMC *LINE1* demethylation was observed in SGI-110-treated mice but not in control or single agent CDDP-treated mice (Fig. 4, QD5 and biweekly treatment schedules). Interestingly, SGI-110 hypomethylation activity in the biweekly regimen was similar to the daily treatment (Fig. 4A–D, QD5 left panels; biweekly right panels). In mice harboring parental A2780-derived xenografts and treated with the QD5 schedule, demethylation and reexpression of *AKT1S1*, *RASSF1A*, *HOXA10*, *HOXA11*, *STAT5B*, and *MLH1* were observed (Fig. 5A). Similarly, using the biweekly schedule, SGI-110 treated groups showed significant demethylation and reexpression of all genes including *BRCA1* (Fig. 5B). Essentially similar demethylation and reexpression results were observed for the A2780 CDDP-resistant-derived xenografts in both QD5 and biweekly regimens (Fig. 5C, D). CDDP treatment alone had no effect on target gene methylation in either biweekly or QD5 treatment of parental or CDDP-resistant A2780 cells (data not shown).

SGI-110 increases platinum DNA adducts *in vitro*

To gain additional insight into a possible mechanism by which SGI-110 resensitized OC models to CDDP, we analyzed CDDP adduct formation in parental A2780 cells and A2780-CDDP resistant cells. Parental A2780 cells and A2780-CDDP resistant cells were pre-treated with SGI-110 for 48hr and then exposed to CDDP for 2hr followed by a 0, 2, 4, and 24hr recovery period (described in Methods section). DNA was extracted and CDDP-DNA adduct formation was measured by inductively coupled plasma mass spectrometry (ICP-MS). SGI-110 pretreatment of the parental A2780 cell resulted in an increase in the level of CDDP adducts by approximately 40% (0 hour), 20% (2 hours) and 40% (4 hours) after

CDDP treatment compared to control, mock treated cells (Fig. 6A; number of CDDP adducts provided in Supplementary Figure S11). Despite the higher level of CDDP-DNA adducts in the SGI-110 treated cells, the repair rates were largely independent of SGI-110 treatment. Similar results were obtained for A2780-CDDP resistant cells pretreated with SGI-110 compared to control (Fig. 6A). Interestingly, over the 24hr time, pretreatment with SGI-110 of both parental A2780 cells and A2780-CDDP resistant cells resulted in an overall increase in the level of CDDP-DNA adducts (Fig. 6A). The increased CDDP adduct formation may be attributed to the ability of SGI-110 to “relax” chromatin conformation, allowing better access of CDDP to DNA (28) with the resulting overall increase in the level of CDDP-DNA adducts contributing to the increase efficacy observed in the SGI-110 treated cells.

SGI-110 treatment alters global levels of active and repressive histone marks

In addition to DNA methylation changes, it was of interest to examine whether the effects of SGI-110 on chromatin included altered levels of repressive and active histone transcription marks. We examined global levels of H3K27 trimethylation (H3K27me₃), a repressive histone mark and the H4K16 acetylation (H4K16ac) activating mark, as acetylated histones are known to be associated with unmethylated DNA and correlated with a euchromatic state (13, 29). Western blot analysis using highly specific antibodies demonstrated that SGI-110 treatment of parental and resistant A2780 cells decreased levels of H3K27me₃ (−10.4 and −0.6321-fold compared to vehicle treatment) and increased H4K16ac levels (2.71 and 1.17-fold respectively vs. control, Fig. 6B and C). Histone H3 protein levels (control) were unchanged after SGI-110 treatment. These results further support the observation that increased CDDP adduct formation is associated with a more accessible chromatin environment induced by SGI-110.

DISCUSSION

Combining DNMTIs with existing chemotherapeutic agents to overcome acquired drug resistance in OC has been proposed by pre-clinical studies from our and other groups (10, 15, 30–32). Recently a completed phase II trial using DNMTIs as resensitizers to traditional chemotherapy in patients with recurrent OC showed clinical and biological activity, justifying further examination of other rationally designed epigenetic treatment strategies in OC (16, 18). In this study, we demonstrate for the first time that the novel DNMTI SGI-110 is an effective chemosensitizer in platinum-resistant OC cells *in vitro* and *in vivo* and demonstrate that SGI-110 induces demethylation of distinct drivers of OC cisplatin resistance. We further show that SGI-110 alone, and in combination with CDDP, is well tolerated and reduces tumor volumes in OC xenograft models. SGI-110 causes both global (*LINE1*) and gene-specific demethylation, and derepresses key TSGs and differentiation-associated genes *in vivo*. In addition, this is the first report that increased CDDP-DNA adduct interactions contribute to chemosensitization by a DNMTI.

While epigenetic therapies hold promise for resensitization of chemoresistant tumors (33–36), DNMTIs are subject to rapid intracellular deamination and aqueous instability (20). Compared to other nucleoside analogs currently used for cancer therapy, e.g., 5-azacytidine

and decitabine, SGI-110 is resistant to cytidine deaminase and has been shown to have antigrowth effects against bladder and colon cancer cells (21, 37). More recently preliminary results from a phase I/II in MDS and AML patients (38) showed that delivering SGI-110 as a small volume and pharmaceutically stable subcutaneous injection allows longer effective half-life and more extended 5-aza-dC exposure window than intravenous infusion. The differentiated pharmacokinetic profile offers the potential for improved biological and clinical activity and safety over currently available hypomethylating agents. Preliminary results from an ongoing phase 2 study of SGI-110 and carboplatin in platinum-resistant, recurrent ovarian cancer patients confirmed this improved pharmacokinetic and pharmacodynamic profile (39). In a recently completed phase I trial in AML and MDS patients, SGI-110 has also been shown to be better tolerated and demonstrate activity in those patients who had progressed on decitabine or 5-azacytidine (40). Our preclinical study further demonstrates that SGI-110 in combination with a cytotoxic is well tolerated in two different treatment regimens and support the concept that SGI-110 provides equivalent or perhaps improved drug exposure compared to 5-aza-dC when given 5X daily, as used in the aforementioned phase II study using decitabine and carboplatin (16).

Platinum resistance in OC is believed to be multifactorial, resulting from transmembrane drug efflux, impairment of DNA mismatch repair, apoptosis, and senescence-promoting pathways, and/or gain of base-excision repair, growth-promoting, and metabolic pathways. Methylation-induced silencing of various genes and pathways in OC have been reported (8), including *LINE1* repetitive elements, *BRCA1*, and *MLH1* as well as *RASSF1A* (41), *HSulf-1* (growth factor signaling) (42), and *TUBB3* (class III β -tubulin). Recently discovered, candidate TSGs hypermethylated in OC include *SPARC* (secreted protein acidic and rich in cysteine) (43), and *ANGPTL2* (angiopoietin-like protein 2) (44). Also, methylation of the embryonic developmentally regulated genes *HOXA10* and *HOXA11* was also found to be highly discriminative between normal and malignant ovarian tissues (45). Adding to this list and reaffirming other hypomethylated genes, we show that SGI-110 reactivates *AKT1S1* (subunit of mTORC1), *IFNAR1* and *IL2RG* (receptor subunit in Jak/STAT pathway) (46), *AKT1* (serine/threonine kinase in apoptosis) (47), *STAT5B* (transcription factor) (48), *LRP6* (cell surface protein in Wnt/beta-catenin signaling cascade), *AXINI* (cytoplasmic G-protein signalling regulator) (49), *CTNNB1* (β -catenin), and *CSNK1D* (casein kinase I), and *ZIC1* (zinc finger protein of the cerebellum 1) (26, 27) in OC cells and mouse xenografts. These data support the concept that platinum resistance in OC is driven in part by hypermethylated TSGs.

The multifactorial nature of OC CDDP resistance presents a clinical problem. In this study, we use a range of OC cell lines to demonstrate that SGI-110 is able to reverse distinct mechanisms of CDDP resistance. We have previously demonstrated that DNA methylation and gene silencing of a sonic hedgehog (Hh) pathway member and putative TSG *ZIC1*, in OC tumors results in loss of negative regulation of the Hh pathway and contribute to OC progression (26). Here we demonstrate that *ZIC1* hypermethylation is associated with CDDP resistance in the OAW28 and OVAR8 cell lines. We postulate that SGI-110-induced demethylation and reexpression of *ZIC1* (Fig. 2B, D) confers negative regulation of the Hh signaling pathway and inhibition of OC cell proliferation. We further show that SGI-110 is

an effective pharmacological approach for reversing this “deep silencing” epigenetic mark and resensitizing chemoresistant OC cells to platinum.

Consistent with previous studies in bladder cancer cells (37), both 5-aza-dC and SGI-110 comparably decreased IC₅₀ values in OC cells treated with CDDP (Fig. 1A, Supplementary Tables S3, 4). Cisplatin functions by forming platinum-DNA lesions, forcing cells to undergo DNA repair or apoptosis. In addition to reactivating TSGs and other cancer-related genes and pathways previously silenced by promoter DNA methylation, we hypothesize that DNMTIs create a more active (open) chromatin environment, allowing better access of CDDP to DNA, and greater adduct formation. Mass spectrometry analysis supports our hypothesis that SGI-110 enhances platinum access to chromatin, and the observed changes in H3K27me3 and H4K16ac (Fig. 6B, C), repressive and active transcription marks (13, 29), respectively further support the concept that SGI-110 induces a “transcriptionally favorable” chromatin environment. Furthermore, as acetylated histones are associated with unmethylated DNA, nearly absent from methylated DNA regions and correlate with a euchromatic state (13), DNMTIs may reestablish chemotherapy drug response cascades by creating a more active (open) chromatin environment (5).

In summary, we show that the novel DNMTI SGI-110 sensitizes a range of OC models to CDDP. SGI-110 is well tolerated and has global DNA hypomethylating activity, thus reactivating numerous genes linked to chemotherapy response and previously associated with clinical outcome in OC (16–18). We provide pre-clinical and biological evidence supporting further investigation of hypomethylating strategies in platinum-resistant OC in general and particularly SGI-110, which compared to current nucleoside analogs is more stable, resulting in better drug exposure (37). As therapeutic options for women with recurrent and platinum resistant OC are extremely limited (5, 7), SGI-110 in combination with conventional and/or targeted therapeutics warrants further development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA: a cancer journal for clinicians*. 2013; 63:11–30. [PubMed: 23335087]
2. Coleman RL, Monk BJ, Sood AK, Herzog TJ. Latest research and treatment of advanced-stage epithelial ovarian cancer. *Nature reviews Clinical oncology*. 2013; 10:211–224.
3. Ozols RF. Recurrent ovarian cancer: evidence-based treatment. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2002; 20:1161–1163. [PubMed: 11870155]
4. Cancer Genome Atlas Research N. Integrated genomic analyses of ovarian carcinoma. *Nature*. 2011; 474:609–615. [PubMed: 21720365]

5. Matei DE, Nephew KP. Epigenetic therapies for chemoresensitization of epithelial ovarian cancer. *Gynecologic oncology*. 2010; 116:195–201. [PubMed: 19854495]
6. Lenzi R, Edwards R, June C, Seiden MV, Garcia ME, Rosenblum M, et al. Phase II study of intraperitoneal recombinant interleukin-12 (rhIL-12) in patients with peritoneal carcinomatosis (residual disease < 1 cm) associated with ovarian cancer or primary peritoneal carcinoma. *Journal of translational medicine*. 2007; 5:66. [PubMed: 18076766]
7. Vaughan S, Coward JI, Bast RC Jr, Berchuck A, Berek JS, Brenton JD, et al. Rethinking ovarian cancer: recommendations for improving outcomes. *Nature reviews Cancer*. 2011; 11:719–725.
8. Barton CA, Clark SJ, Hacker NF, O'Brien PM. Epigenetic markers of ovarian cancer. *Advances in experimental medicine and biology*. 2008; 622:35–51. [PubMed: 18546617]
9. Wei SH, Chen CM, Strathdee G, Harnsomburana J, Shyu CR, Rahmatpanah F, et al. Methylation microarray analysis of late-stage ovarian carcinomas distinguishes progression-free survival in patients and identifies candidate epigenetic markers. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2002; 8:2246–2252. [PubMed: 12114427]
10. Plumb JA, Strathdee G, Sludden J, Kaye SB, Brown R. Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. *Cancer research*. 2000; 60:6039–6044. [PubMed: 11085525]
11. Balch C, Fang F, Matei DE, Huang TH, Nephew KP. Minireview: epigenetic changes in ovarian cancer. *Endocrinology*. 2009; 150:4003–4011. [PubMed: 19574400]
12. Yoo CB, Jones PA. Epigenetic therapy of cancer: past, present and future. *Nature reviews Drug discovery*. 2006; 5:37–50.
13. Jones PA, Baylin SB. The epigenomics of cancer. *Cell*. 2007; 128:683–692. [PubMed: 17320506]
14. Li M, Balch C, Montgomery JS, Jeong M, Chung JH, Yan P, et al. Integrated analysis of DNA methylation and gene expression reveals specific signaling pathways associated with platinum resistance in ovarian cancer. *BMC medical genomics*. 2009; 2:34. [PubMed: 19505326]
15. Balch C, Yan P, Craft T, Young S, Skalnik DG, Huang TH, et al. Antimitogenic and chemosensitizing effects of the methylation inhibitor zebularine in ovarian cancer. *Molecular cancer therapeutics*. 2005; 4:1505–1514. [PubMed: 16227399]
16. Matei D, Fang F, Shen C, Schilder J, Arnold A, Zeng Y, et al. Epigenetic resensitization to platinum in ovarian cancer. *Cancer research*. 2012; 72:2197–2205. [PubMed: 22549947]
17. Fang F, Balch C, Schilder J, Breen T, Zhang S, Shen C, et al. A phase 1 and pharmacodynamic study of decitabine in combination with carboplatin in patients with recurrent, platinum-resistant, epithelial ovarian cancer. *Cancer*. 2010; 116:4043–4053. [PubMed: 20564122]
18. Fu S, Hu W, Iyer R, Kavanagh JJ, Coleman RL, Levenback CF, et al. Phase 1b–2a study to reverse platinum resistance through use of a hypomethylating agent, azacitidine, in patients with platinum-resistant or platinum-refractory epithelial ovarian cancer. *Cancer*. 2011; 117:1661–1669. [PubMed: 21472713]
19. Jabbour E, Garcia-Manero G, Batty N, Shan J, O'Brien S, Cortes J, et al. Outcome of patients with myelodysplastic syndrome after failure of decitabine therapy. *Cancer*. 2010; 116:3830–3834. [PubMed: 20564137]
20. Jabbour E, Issa JP, Garcia-Manero G, Kantarjian H. Evolution of decitabine development: accomplishments, ongoing investigations, and future strategies. *Cancer*. 2008; 112:2341–2351. [PubMed: 18398832]
21. Yoo CB, Jeong S, Egger G, Liang G, Phiasivongsa P, Tang C, et al. Delivery of 5-aza-2'-deoxycytidine to cells using oligodeoxynucleotides. *Cancer research*. 2007; 67:6400–6408. [PubMed: 17616700]
22. Fan M, Yan PS, Hartman-Frey C, Chen L, Paik H, Oyer SL, et al. Diverse gene expression and DNA methylation profiles correlate with differential adaptation of breast cancer cells to the antiestrogens tamoxifen and fulvestrant. *Cancer research*. 2006; 66:11954–1166. [PubMed: 17178894]
23. Laird PW, Zijderfeld A, Linders K, Rudnicki MA, Jaenisch R, Berns A. Simplified mammalian DNA isolation procedure. *Nucleic acids research*. 1991; 19:4293. [PubMed: 1870982]

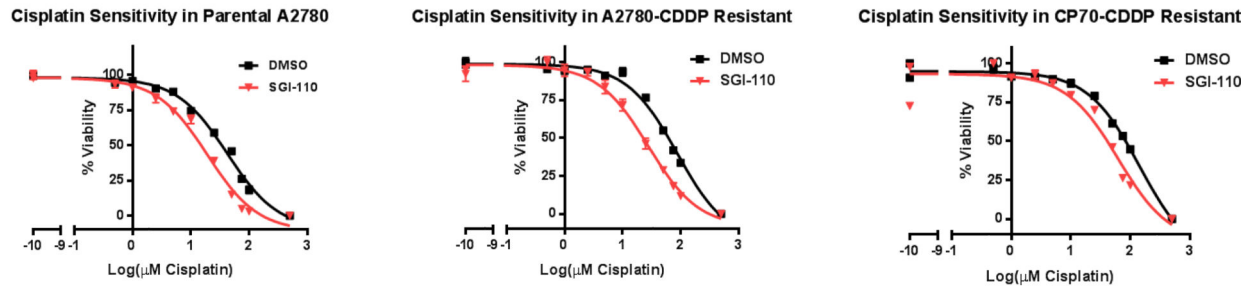
24. Neal JA, Sugiman-Marangos S, VanderVere-Carozza P, Wagner M, Turchi J, Lees-Miller SP, et al. Unraveling the complexities of DNA-dependent protein kinase autophosphorylation. *Molecular and cellular biology*. 2014; 34:2162–2175. [PubMed: 24687855]
25. Tsai HC, Li H, Van Neste L, Cai Y, Robert C, Rassool FV, et al. Transient low doses of DNA-demethylating agents exert durable antitumor effects on hematological and epithelial tumor cells. *Cancer cell*. 2012; 21:430–446. [PubMed: 22439938]
26. Huang RL, Gu F, Kirma NB, Ruan J, Chen CL, Wang HC, et al. Comprehensive methylome analysis of ovarian tumors reveals hedgehog signaling pathway regulators as prognostic DNA methylation biomarkers. *Epigenetics: official journal of the DNA Methylation Society*. 2013; 8:624–634. [PubMed: 23774800]
27. Lum E, Vigliotti M, Banerjee N, Cutter N, Wrzeszczynski KO, Khan S, et al. Loss of DOK2 induces carboplatin resistance in ovarian cancer via suppression of apoptosis. *Gynecologic oncology*. 2013; 130:369–376. [PubMed: 23684582]
28. David GL, Yegnasubramanian S, Kumar A, Marchi VL, De Marzo AM, Lin X, et al. MDR1 promoter hypermethylation in MCF-7 human breast cancer cells: changes in chromatin structure induced by treatment with 5-Aza-cytidine. *Cancer biology & therapy*. 2004; 3:540–548. [PubMed: 15034303]
29. Li R, Mav D, Grimm SA, Jothi R, Shah R, Wade PA. Fine-tuning of epigenetic regulation with respect to promoter CpG content in a cell type-specific manner. *Epigenetics: official journal of the DNA Methylation Society*. 2014; 9:747–759. [PubMed: 24521667]
30. Gomyo Y, Sasaki J, Branch C, Roth JA, Mukhopadhyay T. 5-aza-2'-deoxycytidine upregulates caspase-9 expression cooperating with p53-induced apoptosis in human lung cancer cells. *Oncogene*. 2004; 23:6779–6787. [PubMed: 15273730]
31. Li Y, Hu W, Shen DY, Kavanagh JJ, Fu S. Azacitidine enhances sensitivity of platinum-resistant ovarian cancer cells to carboplatin through induction of apoptosis. *American journal of obstetrics and gynecology*. 2009; 200:177 e1–177 e9. [PubMed: 19110234]
32. Frost P, Abbruzzese JL, Hunt B, Lee D, Ellis M. Synergistic cytotoxicity using 2'-deoxy-5-azacytidine and cisplatin or 4-hydroperoxycyclophosphamide with human tumor cells. *Cancer research*. 1990; 50:4572–4577. [PubMed: 1695122]
33. Aparicio A, Eads CA, Leong LA, Laird PW, Newman EM, Synold TW, et al. Phase I trial of continuous infusion 5-aza-2'-deoxycytidine. *Cancer chemotherapy and pharmacology*. 2003; 51:231–239. [PubMed: 12655442]
34. Schrupp DS, Fischette MR, Nguyen DM, Zhao M, Li X, Kunst TF, et al. Phase I study of decitabine-mediated gene expression in patients with cancers involving the lungs, esophagus, or pleura. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2006; 12:5777–5785. [PubMed: 17020984]
35. Manetta A, Blessing JA, Look KY. A phase II study of fazarabine in patients with advanced ovarian cancer. A Gynecologic Oncology Group study. *American journal of clinical oncology*. 1995; 18:156–157. [PubMed: 7534978]
36. Appleton K, Mackay HJ, Judson I, Plumb JA, McCormick C, Strathdee G, et al. Phase I and pharmacodynamic trial of the DNA methyltransferase inhibitor decitabine and carboplatin in solid tumors. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. 2007; 25:4603–4609. [PubMed: 17925555]
37. Chuang JC, Warner SL, Vollmer D, Vankayalapati H, Redkar S, Bearss DJ, et al. S110, a 5-Aza-2'-deoxycytidine-containing dinucleotide, is an effective DNA methylation inhibitor in vivo and can reduce tumor growth. *Molecular cancer therapeutics*. 2010; 9:1443–1450. [PubMed: 20442312]
38. Kantarjian HM, Roboz GJ, Rizzieri DA, Stock W, O'Connell CL, Griffiths EA, et al. Results from the dose escalation phase of a randomized phase 1–2 first-in-human (FIH) study of SGI-110, a novel low volume stable subcutaneous (SQ) second generation hypomethylating agent (HMA) in patients with relapsed/refractory MDS and AML. *Blood (ASH Annual Meeting Abstracts)*. 2012; 120 Abstract 414.
39. Fleming G, Ghamande S, Lin Y, Second AA, Nemunaitis J, Markham MJ, et al. Clinical epigenetic resensitization of platinum-resistant, recurrent ovarian cancer patients with SGI-110, a novel,

- second-generation, subcutaneously administered hypomethylating agent (HMA). Proceedings of the 105th Annual Meeting of the American Association for Cancer Research. 2014 Abstract 2320.
40. Issa JP, Roboz GJ, Rizzieri DA, Faderl S, O'Connell CL, Stock W, et al. Interim results from a randomized Phase 1–2 first-in-human study of PK/PD guided escalating doses of SGI-110, a novel subcutaneous second generation hypomethylating agent in relapsed/refractory MDS and AML. Proceedings of the 103th Annual Meeting of the American Association for Cancer Research. 2012; 2012 p. Abstract 2320.
 41. Sellar GC, Watt KP, Rabisz GJ, Stronach EA, Li L, Miller EP, et al. OPCML at 11q25 is epigenetically inactivated and has tumor-suppressor function in epithelial ovarian cancer. *Nature genetics*. 2003; 34:337–343. [PubMed: 12819783]
 42. Backen AC, Cole CL, Lau SC, Clamp AR, McVey R, Gallagher JT, et al. Heparan sulphate synthetic and editing enzymes in ovarian cancer. *British journal of cancer*. 2007; 96:1544–1548. [PubMed: 17437011]
 43. Socha MJ, Said N, Dai Y, Kwong J, Ramalingam P, Trieu V, et al. Aberrant promoter methylation of SPARC in ovarian cancer. *Neoplasia*. 2009; 11:126–135. [PubMed: 19177197]
 44. Kikuchi R, Tsuda H, Kozaki K, Kanai Y, Kasamatsu T, Sengoku K, et al. Frequent inactivation of a putative tumor suppressor, angiopoietin-like protein 2, in ovarian cancer. *Cancer research*. 2008; 68:5067–5075. [PubMed: 18593905]
 45. Fiegl H, Windbichler G, Mueller-Holzner E, Goebel G, Lechner M, Jacobs IJ, et al. HOXA11 DNA methylation--a novel prognostic biomarker in ovarian cancer. *International journal of cancer*. 2008; 123:725–729.
 46. Tsuno T, Mejido J, Zhao T, Phillips T, Myers TG, Bekisz J, et al. BID is a critical factor controlling cell viability regulated by IFN-alpha. *Journal of immunotherapy*. 2012; 35:23–31. [PubMed: 22130162]
 47. Hanrahan AJ, Schultz N, Westfal ML, Sakr RA, Giri DD, Scarperi S, et al. Genomic complexity and AKT dependence in serous ovarian cancer. *Cancer discovery*. 2012; 2:56–67. [PubMed: 22328975]
 48. Jinawath N, Vasoontara C, Jinawath A, Fang X, Zhao K, Yap KL, et al. Oncoproteomic analysis reveals co-upregulation of RELA and STAT5 in carboplatin resistant ovarian carcinoma. *PLoS one*. 2010; 5:e11198. [PubMed: 20585448]
 49. Dai W, Teodoridis JM, Zeller C, Graham J, Hersey J, Flanagan JM, et al. Systematic CpG islands methylation profiling of genes in the wnt pathway in epithelial ovarian cancer identifies biomarkers of progression-free survival. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2011; 17:4052–4062. [PubMed: 21459799]

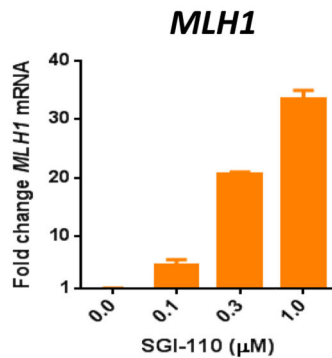
Translational relevance

Platinum-resistant ovarian cancer (OC) is uniformly fatal. Platinum resistance is associated with epigenetic anomalies including aberrant DNA methylation, a reversible epigenetic mark. We hypothesized that DNA methyltransferase inhibitors (DNMTIs) restore OC sensitivity to platinum and our recent phase I/II trial showed that low-dose 5-aza-dC followed by carboplatin resulted in promising clinical activity in women with platinum-resistant OC. However, current DNMTIs are rapidly degraded by hydrolytic cleavage, deaminated by cytidine deaminase and unstable during intravenous infusion, limiting their potential as cancer therapeutics. SGI-110, a dinucleotide combining 5-aza-dC and deoxyguanosine (Astex Pharmaceuticals, Inc.), is less prone to deamination and more stable. The current preclinical study demonstrates that pre-treatment with SGI-110 resensitizes OC cells to cisplatin *in vitro* and *in vivo*. Mechanistically, the reversal of platinum resistance by SGI-110 was due to demethylation and reactivation of numerous chemotherapy response-related genes. Our data support clinical evaluation of this combination in platinum resistant OC.

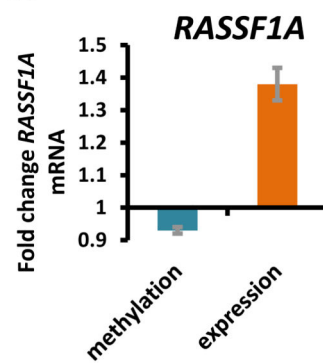
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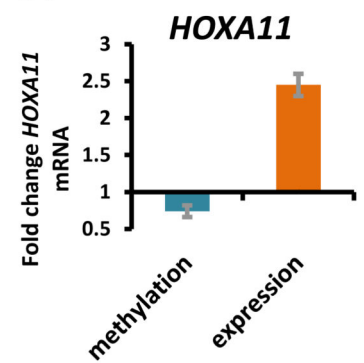
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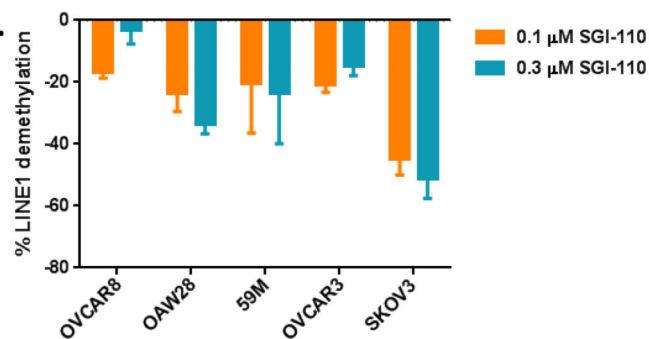
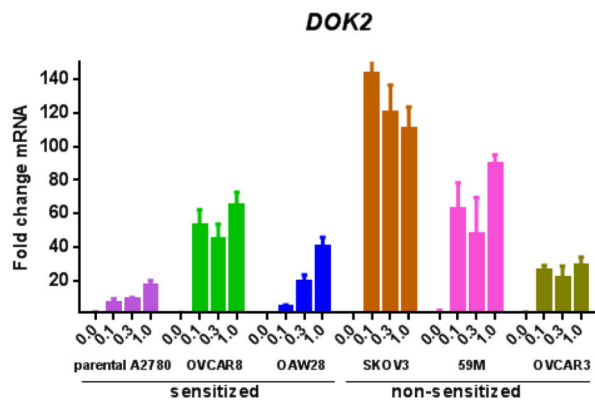


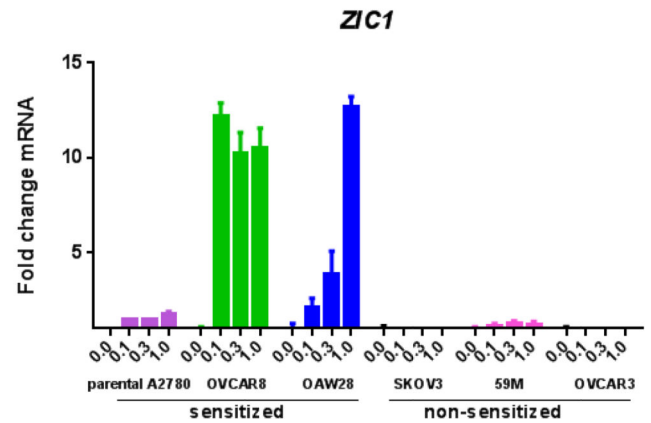
Figure 1. 5-aza-dC and SGI-110 treatment modulates CDDP sensitivity and alters DNA methylation and gene expression *in vitro*

(A). Comparison of cell growth rates of parental A2780 cells, A2780-CDDP resistant cells, and CP70 CDDP resistant cells treated with 5 μ M SGI-110, or vehicle (DMSO 1:2000) for 48 hours followed by CDDP ranging from 0–50 μ M CDDP. Mean values \pm S.E.M. of 8 independent experiments in duplicate are reported. All treatments were significantly different, at $P < 0.05$, than vehicle control cells. IC₅₀ values are listed in Supplementary Table S3. (B). RT-PCR analysis of *MLH1* RNA levels in A2780 cells. Fold change in RNA levels were calculated as $2^{(-CT)}$ relative to DMSO-treated cells. (C and D). *RASSF1A* and *HOXA11* were significantly demethylated by SGI-110 and the mRNA expression was upregulated in A2780-CDDP resistant cells. All changes are significant ($P < 0.05$). (E). Cells were treated for 3-consecutive days with 0.1 μ M SGI-110, 0.3 μ M SGI-110 or DMSO (control). *LINE1* methylation status was determined by pyrosequencing analysis and expressed as % of DMSO-treated cells. Data shown represent mean values \pm S.E.M. from triplicate experiments.

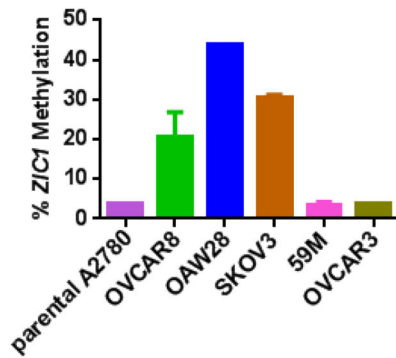
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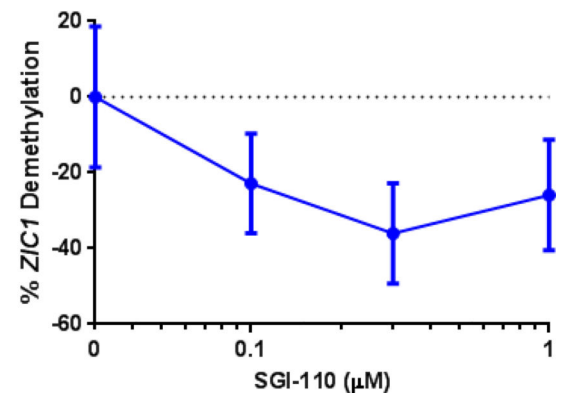


Figure 2. SGI-110 induces the expression of potential CDDP-resistance biomarkers in panel of OC cell lines

(A, B) Fold-change in mRNA expression of *DOK2* and *ZIC1* in OC cell lines following 3-consecutive day treatment with 0.1 μM SGI-110, 0.3 μM SGI-110, 1 μM SGI-110 or vehicle (DMSO) quantified by qRT-PCR. Data shown represent mean values ± S.E.M. from triplicate samples. (C) Pyrosequencing analysis of basal *ZIC1* methylation levels in untreated OC cell lines. Data shown represent mean values ± S.E.M. from triplicate samples. (D) Pyrosequencing analysis of *ZIC1* methylation levels in OAW28 cells, following 3-consecutive day treatment with SGI-110. Methylation levels expressed as % of DMSO-treated cells. Data shown represent mean values ± S.E.M. from triplicate experiments.

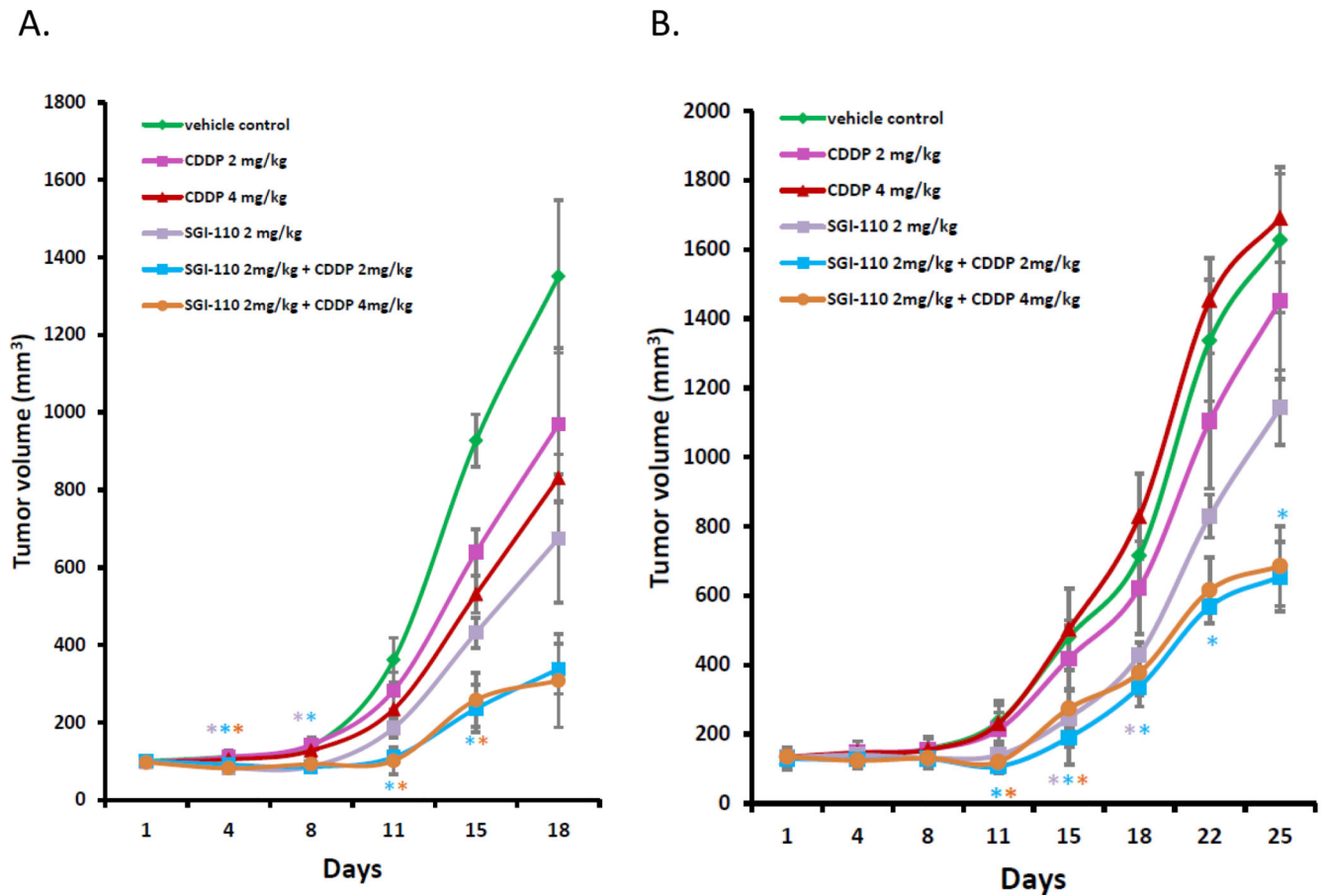


Figure 3. SGI-110 and CDDP in the QD5 and biweekly treatment regimens decreases A2780-CDDP resistant-derived xenograft tumor growth treated with
 CDDP-resistant A2780 xenograft tumor volume was compared among single agent and combination treatment to vehicle control in two treatment schedules (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). (A). QD5 treatment. (D). Biweekly treatment. Data shown represent mean values \pm S.E.M. from 5 tumor samples.

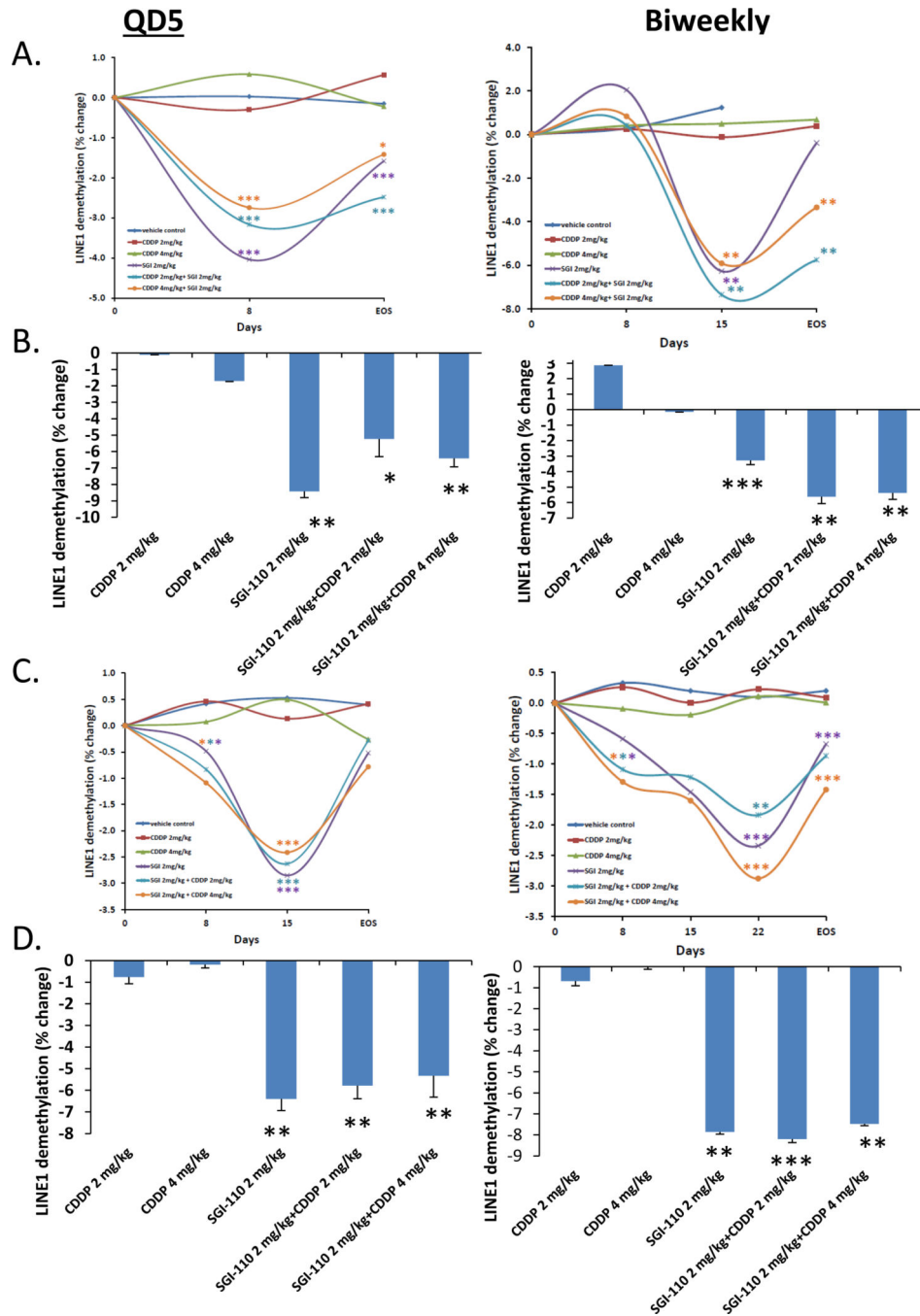


Figure 4. QD5 and biweekly SGI-110 treatments induce changes in *LINE1* methylation in PBMCs and tumor samples from mice with parental A2780 or CDDP-resistant xenografts Tumor bearing mice were treated with SGI-110 and CDDP according to biweekly or QD5 schedule. Blood samples were collected (biweekly: on days 1, 8, 15, 22, and end of study (EOS); QD5: on days 1, 8, and EOS). Tumors were collected at (EOS) after the mice were sacrificed. DNAs were extracted from PBMCs and tumor and subjected to bisulfite conversion and pyrosequencing for *LINE1* methylation (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$). (A). PBMC *LINE1*. Left panel- parental A2780 xenograft mice with QD5

regimen, right panel- parental A2780 xenograft mice with biweekly regimen. (B). Tumor *LINE1*. Left panel- parental A2780 xenograft mice with QD5 regimen, right panel- parental A2780 xenograft mice with biweekly regimen. (C). PBMC *LINE1*. Left panel- A2780-CDDP resistant xenograft mice with QD5 regimen, right panel- A2780-CDDP resistant xenograft mice with biweekly regimen. (D). Tumor *LINE1*. Left panel- A2780-CDDP resistant xenograft mice with QD5 regimen, right panel- A2780-CDDP resistant xenograft mice with biweekly regimen. Data shown represent mean values \pm S.E.M. from 5 xenograft samples.

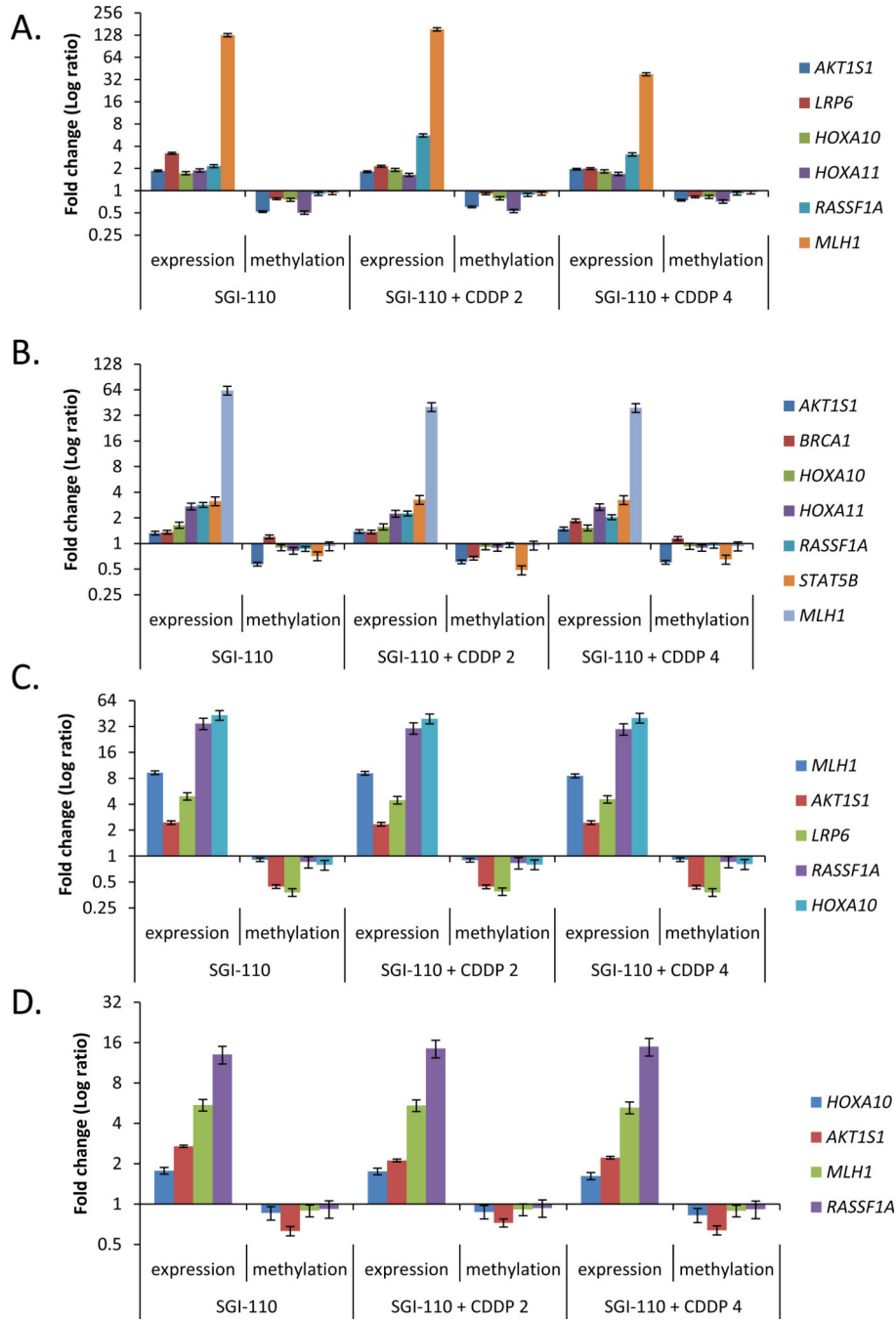


Figure 5. qRT-PCR and pyrosequencing analysis of specific genes in QD5 and biweekly schedule mice with parental and CDDP-resistant A2780 xenografts

Selected specific genes showed significantly demethylated and upregulated from A2780 xenografts in the 2 treatment schedules. (A). Parental A2780 xenografts from mice treated with QD5 schedule. (B). Parental A2780 xenografts from mice treated with biweekly schedule. (C). A2780-CDDP resistant xenografts from mice treated with QD5 schedule. (D). A2780-CDDP resistant xenografts from mice treated with biweekly schedule. Data shown represent mean values \pm S.E.M. from 5 xenograft samples.

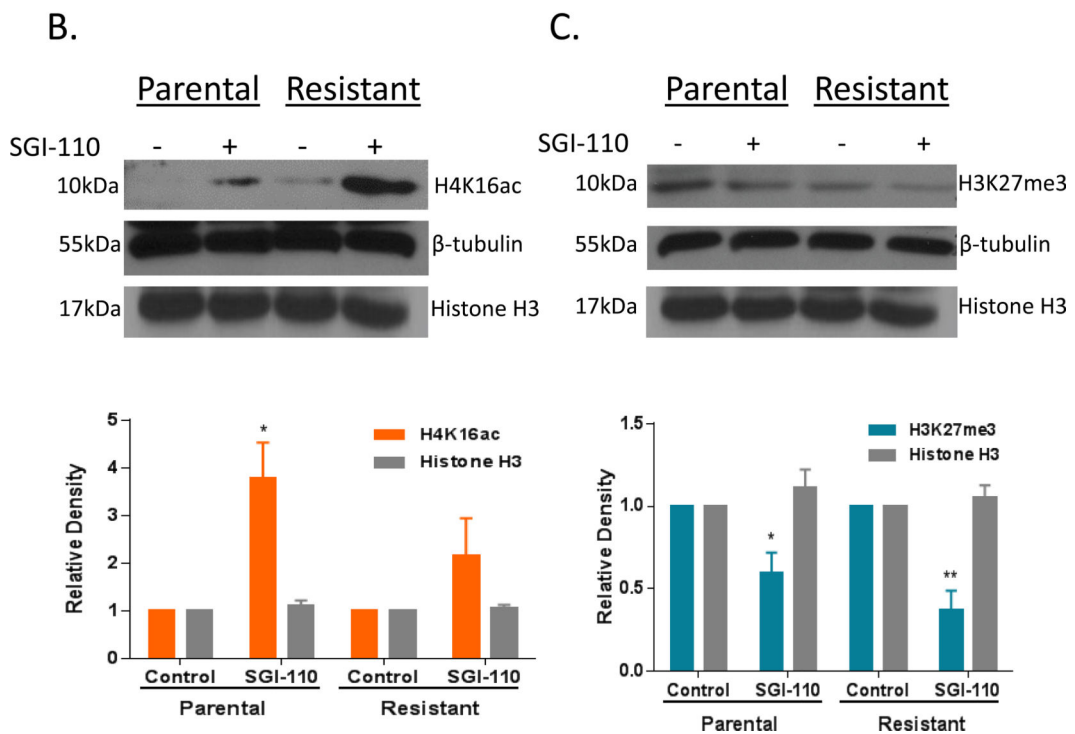
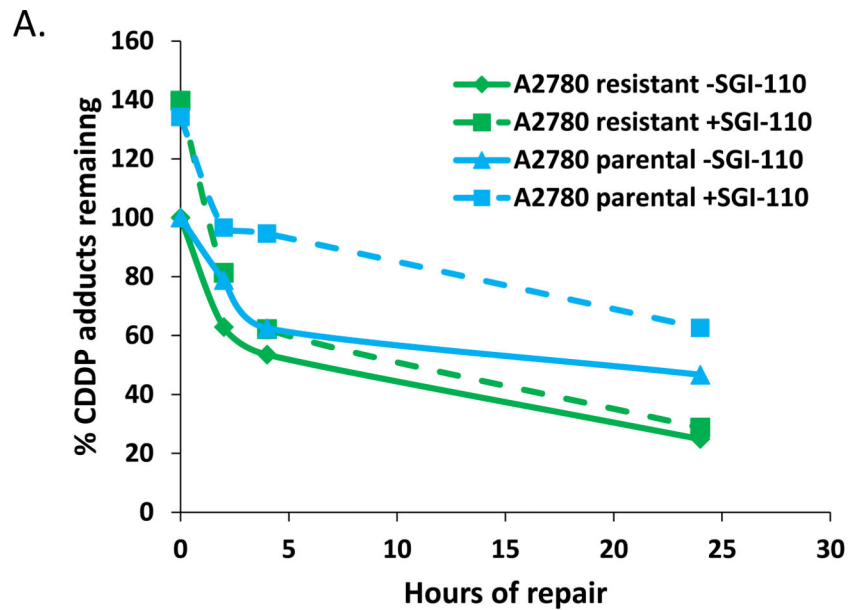


Figure 6. SGI-110 increased CDDP DNA adducts and alters epigenetic marks *in vitro*

Parental A2780 and A2780 CDDP-resistant cells were treated in triplicate with either no SGI-110 or 5 μ M SGI-110 in fresh RPMI and grown for 48 hours. Media was removed and replaced with fresh RPMI containing 10 μ M CDDP for 2hr and were allowed to repair for 0, 2, 4 and 24 hours DNA was extracted from cells analyzed by ICP-mass spectrometry. (A). SGI-110 increased CDDP DNA adducts *in vitro*. (B and C). Western blot and quantification of protein from parental A2780 and A2780 CDDP-resistant cells treated with 5 μ M SGI-110 for 48hr and blotted with rabbit anti- β -tubulin, mouse anti-histone H3, and anti-acetyl-

histone H4 (B), or anti-trimethyl-histone-H3 (C). Data shown represent mean values \pm S.E.M. from triplicate experiments.