Cancer Therapy: Preclinical

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 and to assess its effects on tumor suppressor genes (TSG) and chemoresponsiveness-associated genes silenced by DNA methylation in ovarian cancer.

Experimental Design: Several ovarian cancer 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.

Results: We demonstrate in vitro that SGI-110 resensitized a range of platinum-resistant ovarian cancer cells to cisplatin (CDDP) and induced significant demethylation and reexpression of TSG, differentiation-associated genes, and putative drivers of ovarian cancer cisplatin resistance. In vivo, SGI-110 alone or in combination with CDDP was well tolerated and induced antitumor effects in ovarian cancer xenografts. Pyrosequencing analyses confirmed that SGI-110 caused both global (LINE1) and gene-specific hypomethylation in vivo, including TSGs (RASSF1A), proposed drivers of ovarian cancer cisplatin resistance (MLH1 and ZIC1), differentiation-associated genes (HOXA10 and HOXA11), and transcription factors (STAT5B). Furthermore, DNA damage induced by CDDP in ovarian cancer cells was increased by SGI-110, as measured by inductively coupled plasma-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 ovarian cancer. Specifically, SGI-110 in combination with conventional and/or targeted therapeutics warrants further development in this setting. Clin Cancer Res; 20(24); 6504–16. ©2014 AACR.

Introduction

Ovarian cancer is the deadliest gynecologic cancer, causing 14,270 estimated deaths and 21,980 new cases in the United States (1). Current treatment for ovarian cancer 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 ovarian cancer 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 ovarian cancer 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, although several...
Platinum-resistant ovarian cancer 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 ovarian cancer 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 ovarian cancer. 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 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 ovarian cancer models and demonstrated that pretreatment with SGI-110 resensitized a range of ovarian cancer cells to CDDP, both in vitro and in vivo. In all, our preclinical studies reveal that SGI-110 is an effective DNA hypomethylator in ovarian cancer and supports its future clinical development in ovarian cancer and other solid tumors. Clinical trials using this combination are ongoing.

Materials and Methods

Cell culture and drugs
A2780 ovarian cancer 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-diammine-dichloroplatinum (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 DMEM supplemented with 10% FBS. SKOV3 cells were maintained in McCoy's 5A medium supplemented with 1.5 mmol/L 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.

Platinum resensitization
Treatment with 5-aza-dC (5 μmol/L), SGI-110 (0.1, 0.3, 1, and 5 μmol/L), or vehicle (DMSO 1:2000) was performed for 48 hours before CDDP treatment (15). MIT 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 ovarian cancer cells using AllPrep DNA/RNA/Protein Mini kit (Qiagen) following the manufacturer's protocol and the quantity and quality determined by absorbance (260 and 280 nm). Total RNA (2 μg) was reverse transcribed with the LightCycler 480 SYBR Green I Master kit (Roche) 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) according to the manufacturer's instruction. qRT-PCR was performed using miScript reverse transcription and miScript SYBR Green PCR kits (Qiagen) in a Roche Lightcycler (Roche Applied Science), as described previously (14, 22). mRNA expression level was determined using LightCycler software version 3.5 (Roche Applied Science), normalized to EEF1αb, and using the 2^(-ΔΔCt) method of relative quantification.
In vivo nontumor-bearing mice experiments and treatment schedule

All animal studies adhered to protocols approved by the Institutional Animal Care and Use Committee of Indiana University (Indianapolis, IN). 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 Fig. S1A. SGI-110 was administered at either 2 or 5 mg/kg and CDDP was administered at 2 or 4 mg/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 100 μL 1:1 RPMI-1640/Matrigel (BD Biosciences), and 7 × 10⁵ cells were injected subcutaneously (s.c.) into the right flanks of 4- to 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) before each treatment. Mice bearing similar tumor size (4–5 mm in diameter) before each treatment. Mice bearing similar tumor size (4–5 mm in diameter) before each treatment. Mice bearing similar tumor size (4–5 mm in diameter) before each treatment. Mice bearing similar tumor size (4–5 mm in diameter) before each treatment. Mice bearing similar tumor size (4–5 mm in diameter) before each treatment. Mice bearing similar tumor size (4–5 mm in diameter) before each treatment. Mice bearing similar tumor size (4–5 mm in diameter) before each treatment. Mice bearing similar tumor size (4–5 mm in diameter) before each treatment. Mice bearing similar tumor size (4–5 mm in diameter) before each treatment. Tumor length (l) and width (w) were measured using digital calipers. Tumor volume (v) was calculated using the following equation: v = 1/2 l × w². Mice were sacrificed if tumors reached a diameter of 2 cm 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 25 mg 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 to 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 × 10⁶ per well in 6-well plates. Triplicate wells were treated with either vehicle (DMSO) or 5 μmol/L SGI-110 for 48 hours. Media were replaced with fresh RPMI containing 10 μmol/L CDDP. Cells were incubated for 2 hours at 37°C and 5% CO₂, media were removed, and cells washed with PBS. 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 and colleagues (23). DNA (30–50 μg) was hydrolyzed overnight in 1% nitric acid at 70°C in 500 μL total volume. Samples were then diluted to 1.5 mL. final volume in 1% nitric acid and analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) as we have previously described (24). Briefly, a benchtop series Thermo ICP-MS 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 (r²) 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 r² 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 (1:4,000; Santa Cruz Biotechnology), anti-trimethyl-histone-H3 (Lys27; 1:1,000; Millipore), anti-acetyl-histone H4 (Lys16; 1:1,000; Millipore), mouse anti-MLH1 (1:1,000; Active Motif, CA), MLH1 (BD Biosciences), and actin (Abcam). Goat anti-Rabbit IgG (H+L), peroxidase-labeled antibodies (1:4,000), and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) were used for detection of H4K16ac and H3K27me3. Infra-red-dye-labeled anti-mouse antibodies (Licor Bioscience) and the Odyssey infrared imaging system (Licor Biotechnology) were used to detect MLH1.

Densitometry used ImageJ analysis software. Experiments were done in triplicate. Statistical analysis

Statistical analysis used the Student 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 mean ± SEM of three independent experiments.

Results

SGI-110 modulates sensitivity to CDDP and 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 ovarian cancer 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 μmol/L) of either SGI-110 (Fig. 1A) or 5-aza-dC (Supplementary Fig. S2) increased the sensitivity of ovarian cancer cells to CDDP causing a 2-fold reduction in the IC50 for CDDP: 28 μmol/L CDDP IC50 for A2780-CDDP-resistant cells after SGI-110 priming compared with 42 μmol/L CDDP IC50 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 ovarian cancer cell lines, the parental A2780 is considered to be CDDP “sensitive,” it has a relatively high IC50 for the drug (Supplementary Table S3).

Previous studies have demonstrated an association between CDDP resistance of ovarian cancer 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.

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 μmol/L SGI-110, or vehicle (DMSO 1:2000) for 48 hours followed by CDDP ranging from 0 to 50 μmol/L CDDP. Mean values ± SEM of eight 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 changes in RNA levels were calculated as 2(ΔΔCt) 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 μmol/L SGI-100, 0.3 μmol/L 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 ± SEM from triplicate experiments.
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 Fig. S3.

Next, we evaluated whether a lower dose of SGI-110 (0.3 μmol/L) also modulates CDDP response in ovarian cancer cell lines. Cells were pretreated for 3 days with a low dose of SGI-110 (25), before 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). Pretreatment with SGI-110 increased up to 9-fold and 4-fold CDDP sensitivity of OVCAR8 and OAW28 ovarian cancer cell lines, respectively (Supplementary Fig. 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 MLH expression levels in other ovarian cancer 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 and 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 ovarian cancer 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 ovarian cancer CDDP resistance (Supplementary Table S5). The candidate genes were previously proposed by us (26) and Lum and colleagues (27), who identified genes that were epigenetically silenced (by hypermethylation) in primary samples derived from platinum-resistant ovarian cancer 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 μmol/L SGI-110) and ZIC1 (11-fold at 1 μmol/L SGI-110) expression levels were observed in response to SGI-110 in the OVCAR8 cell line (Fig. 2A and B). Furthermore, an induction of DOK2 (41-fold at 1 μmol/L SGI-110) and ZIC1 (13-fold at 1 μmol/L SGI-110) was also observed in the OAW28 cells, in addition to a modest induction of TWIST1 (3-fold at 1 μmol/L SGI-110), NR2E1 (7-fold at 1 μmol/L SGI-110), and SOX9 (3-fold at 1 μmol/L SGI-110; Supplementary Fig. S6). To determine whether the reexpression of ZIC1 and DOK2 was associated with resensitization of ovarian cancer 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 Fig. 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 and 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 ovarian cancer and predict response to epigenetic therapies.

SGI-110 tolerability studies in nontumor-bearing mice

To investigate whether SGI-110 alone or in combination with CDDP was tolerable in vivo, female nude athymic nontumor-bearing mice were treated with two different schedules (QD5 and biweekly; Supplementary Fig. S1A). The QD5 daily schedule was SGI-110 (2 or 5 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 or 5 mg/kg), CDDP (2 or 4 mg/kg), or both twice a week for 4 weeks. Animals were observed for three additional weeks posttreatment. In the QD5 schedule, SGI-110 2 mg/kg had no effect on BW compared with SGI-110 5 mg/kg (Supplementary Fig. S7A). Furthermore, the lower dose SGI-110-CDDP combination was well tolerated (Supplementary Fig. S7A, orange line) but initial BW loss was observed using the higher dose combinations (Supplementary Fig. 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 Fig. S7B). Overall, SGI-110 in combination with CDDP at physiologically achievable doses was well tolerated in nontumor-bearing mice.

**SGI-110 inhibits tumor growth in vivo**

On the basis of the above results in nontumor-bearing mice, low-dose SGI-110 (2 mg/kg) was used for subsequent ovarian cancer xenograft experiments. CDDP-resistant A2780 cells were injected subcutaneously into the right flanks of 4- to 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; Fig. 3B, biweekly treatment schedule; tumor growth curves for parental A2780 xenografts in Supplementary Fig. S8; AUC graphs in Supplementary Fig. S9). SGI-110 alone or in combination with CDDP was well tolerated overall in tumor-bearing mice using either treatment schedule (Supplementary Fig. 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; biweekly right). 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 and 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 ovarian cancer models to CDDP, we analyzed CDDP adduct formation in parental A2780 and A2780–CDDP–resistant cells. Parental A2780 cells and A2780–CDDP–resistant cells were pretreated with SGI-110 for 48 hours and then exposed to CDDP for 2 hours followed by a 0, 2, 4, and 24-hour recovery period (described in Materials and Methods section). DNA was extracted and CDDP-DNA adduct formation was measured by ICP-MS. SGI-110 pretreatment of the parental A2780 cell resulted in an increase in the level of CDDP-DNA adducts by approximately 40% (0 hour), 20% (2 hours), and 40% (4 hours) after CDDP treatment compared with control, mock-treated cells (Fig. 6A; number of CDDP adducts provided in Supplementary Fig. 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 with control (Fig. 6A). Interestingly, over the 24-hour 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 (−0.4 and −0.6321-fold compared with 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 ovarian cancer has been proposed by preclinical 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 ovarian cancer showed clinical and biologic activity, justifying further examination of other rationally designed epigenetic treatment strategies in ovarian cancer (16, 18). In this study, we demonstrate for the first time that the novel DNMTI SGI-110 is an effective chemosensitizer in platinum-resistant ovarian cancer cells in vitro and in vivo and demonstrate that SGI-110 induces demethylation of distinct drivers of ovarian cancer cisplatin resistance. We further show that SGI-110 alone, and in combination with CDDP, is well tolerated and reduces tumor volumes in ovarian cancer 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 to show that increased CDDP-DNA
Adduct interactions contribute to chemosensitization by a DNMTI.

Although epigenetic therapies hold promise for resensitization of chemoresistant tumors (33–36), DNMTIs are subject to rapid intracellular deamination and aqueous instability (20). Compared with other nucleoside analogs currently used for cancer therapy, for example, 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 patients with myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML; ref. 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 biologic and clinical activity and safety over currently available hypomethylating agents. Preliminary results from an ongoing phase II 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 patients with AML and MDS, SGI-110 has also been shown to be better tolerated and demonstrate the 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 with 5-aza-dC when given 5 times daily, as used in the aforementioned phase II study using decitabine and carboplatin (16).

Platinum resistance in ovarian cancer 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 ovarian cancer have been reported (8), including LINE1 repetitive elements, BRCA1, and MLH1 as well as RASSF1A (41), SULF-1 (growth factor signaling; ref. 42), and TUBB3 (class III β-tubulin). Recently discovered, candidate TSGs hypermethylated in ovarian cancer include SPARC (secreted protein acidic and rich in cysteine; ref. 43) and ANGPTL2 (angiopoietin-like protein 2; ref. 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; ref. 46), AKT1 (serine/threonine kinase in apoptosis; ref. 47), STAT5B (transcription factor; ref. 48), LRP6 (cell surface protein in Wnt/β-catenin signaling cascade), AXIN1 (cytoplasmic G-protein signaling regulator;...
ref. 49), CTNNB1 (β-catenin), and CSN1K1D (casein kinase 1), and ZIC1 (zinc finger protein of the cerebellum 1; ref. 26, 27) in ovarian cancer cells and mouse xenografts. These data support the concept that platinum resistance in ovarian cancer is driven in part by hypermethylated TSGs.

The multifactorial nature of ovarian cancer CDDP resistance presents a clinical problem. In this study, we use a range of ovarian cancer 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 ovarian cancer tumors result in loss of negative regulation of the Hh pathway and contribute to ovarian cancer 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 and D) confers negative regulation of the Hh signaling pathway and inhibition of ovarian cancer cell proliferation. We further show that SGI-110 is an effective pharmacologic approach for reversing this "deep silencing" epigenetic mark and reestablishing chemoresistant ovarian cancer cells to platinum.

Consistent with previous studies in bladder cancer cells (37), both 5-aza-dC and SGI-110 comparably decreased IC_{50} values in ovarian cancer cells treated with CDDP (Fig. 1A and Supplementary Tables S3 and S4). 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 and 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 ovarian cancer 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 ovarian cancer (16–18). We provide preclinical and biologic evidence supporting further investigation of hypomethylating strategies in platinum-resistant ovarian cancer in general and particularly SGI-110, which compared with current nucleoside analogs is more stable, resulting in better drug exposure (37). As therapeutic options for women with recurrent and platinum-resistant ovarian cancer are extremely limited (5, 7), SGI-110 in combination with conventional and/or targeted therapeutics warrants further development.

Disclosure of Potential Conflicts of Interest

M. Wagner is a consultant/advisory board member and has provided expert testimony for Wagner and Associates on clinical and forensic toxicology as it relates to medical legal investigations. J. Lyons is an employee of Astex Pharmaceuticals. D. Matei is a consultant/advisory board member for Astex Pharmaceuticals. K.P. Nephew reports receiving a commercial research grant from and is a consultant/advisory board member for Astex Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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