Epigenetic Targeting of Ovarian Cancer Stem Cells

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Abstract

Emerging results indicate that cancer stem–like cells contribute to chemoresistance and poor clinical outcomes in many cancers, including ovarian cancer. As epigenetic regulators play a major role in the control of normal stem cell differentiation, epigenetics may offer a useful arena to develop strategies to target cancer stem–like cells. Epigenetic aberrations, especially DNA methylation, silence tumor-suppressor and differentiation-associated genes that regulate the survival of ovarian cancer stem–like cells (OCSC). In this study, we tested the hypothesis that DNA-hypomethylating agents may be able to reset OCSC toward a differentiated phenotype by evaluating the effects of the new DNA methyltransferase inhibitor SGI-110 on OCSC phenotype, as defined by expression of the cancer stem–like marker aldehyde dehydrogenase (ALDH). We demonstrated that ALDH+ ovarian cancer cells possess multiple stem cell characteristics, were highly chemoresistant, and were enriched in xenografts residual after platinum therapy. Low-dose SGI-110 reduced the stem-like properties of ALDH+ cells, including their tumor-initiating capacity, resensitized these OCSCs to platinum, and induced reexpression of differentiation-associated genes. Maintenance treatment with SGI-110 after carboplatin inhibited OCSC growth, causing global tumor hypomethylation and decreased tumor progression. Our work offers preclinical evidence that epigenome-targeting strategies have the potential to delay tumor progression by reprogramming residual cancer stem–like cells. Furthermore, the results suggest that SGI-110 might be administered in combination with platinum to prevent the development of recurrent and chemoresistant ovarian cancer. Cancer Res; 74(17) September 1, 2014; E-mail: dmatei@iupui.edu.

Introduction

Epithelial ovarian cancer causes more deaths than any other female reproductive tract cancer (1, 2). The majority of women diagnosed with advanced-stage epithelial ovarian cancer experience tumor recurrence associated with the development of chemoresistance, and platinum-resistant ovarian cancer is uniformly fatal (3). A new paradigm explaining tumor relapse involves the persistence of "cancer stem cells" that were characterized in several solid tumors, including ovarian cancer (4–6). Although chemotherapy may succeed initially at decreasing the size and number of tumors, it leaves behind residual malignant cells, which we hypothesize are enriched in tumor progenitors or "cancer stem cells."

Ovarian cancer stem cells (OCSC) have been isolated from established ovarian cancer cell lines, ascites, and primary and metastatic tumors (4, 7, 8). They share several characteristics with normal stem cells, including the ability to form anchorage-independent spherical aggregates, express stem cell markers, undergo membrane efflux, form clones in culture and in addition exhibit enhanced tumor-forming ability (9). Although a number of technical approaches have been successfully used to isolate OCSCs (sphere-forming, cell-surface markers, stem cell gene reporter assays), the use of an assay measuring aldehyde dehydrogenase isof orm 1 (ALDH) enzymatic activity has been recently proposed and is used to define CSCs in multiple other tumor types (10, 11).

Ovarian CSCs are hypothesized to be largely (or entirely) responsible for emergence of chemoresistant tumors, because they possess many of the phenotypes associated with drug resistance (e.g., enhanced DNA repair, diminished apoptotic responses, increased efflux mechanisms, and quiescent state; refs. 4, 12). Moreover, similar to normal embryonic or tissue stem cells, CSCs are believed to harbor a significantly altered epigenome (6, 13), and it has been hypothesized that DNA-hypomethylating agents could "reset" these cells toward
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differentiation (14). Indeed, several hypomethylating agents were originally characterized as inducers of cancer cell differ-
entiation (6, 15). However, it has become clear that hypo-
methylating agents or other epigenetic modulators alone cannot eradicate relapsed tumors. Preclinical studies from
our and other groups have established the rationale for com-
bining DNA methylation inhibitors with existing chemother-
apic agents to overcome acquired drug resistance in ovarian
cancer (16–20). On the basis of those studies, we recently
completed a phase II trial using a DNA methylation inhibitor as
a resensitizer to traditional chemotherapy in patients with
recurrent ovarian cancer and showed that this combination has
clinical and biologic activity (21), justifying other rationally
designed epigenetic treatment strategies in ovarian cancer.

On the basis of the above considerations, we hypothesized
that hypomethylating agents, in combination with chemother-
apic, may target drug-resistant OCSCs, possibly leading to
tumor eradication. In the current study, we isolated and
characterized ALDH− OCSCs from ovarian cancer cell lines
and human tumors. ALDH− cells were significantly more
chemoresistant and tumorigenic compared with ALDH+
cells in orthotopic tumor initiating assays. Treatment with
SGI-110, a second-generation DNA methyltransferase inhibitor
(DNMTI), resensitized OCSCs to platinum. A model recap-
pitating the emergence of recurrent tumors showed an
increased percentage of ALDH− OCSCs in residual tumors
after platinum. Maintenance therapy with SGI-110 during
platinum-induced remission inhibited the emergence of plat-
inum-resistant tumors. We suggest that epigenomic targeting
using SGI-110 may be useful as a “maintenance” clinical strategy after platinum-based therapy in ovarian cancer.

Materials and Methods

Cell lines, patient samples, culture conditions, and
reagents
Ovarian cancer cell lines (A2780, A2780_CR5, and SKOV3)
were maintained in RPMI-1640 medium (Invitrogen), with
supplements as described previously (see Supplementary
Materials and Methods; ref. 22). Cisplatin-resistant variant
A2780_CR5 was established from five-round IC50 survival
monoclonal selection by continuous exposure to increasing
concentration of cisplatin (22). A2780 and SKOV3 ovarian
cancer cells were authenticated in 2012 by the ATCC. Advanced
high-grade serous ovarian tumors were surgically collected
(IRB-approved protocol IUCRO-0280), enzymatically disasso-
ciated, and cultured, as previously described (4). SGI-110 was
provided by Astex Pharmaceuticals Inc. and cisplatin was
purchased from Calbiochem.

Aldefluor assay and flow cytometry
ALDH1 enzymatic activity was measured using the Aldefluor
assay (STEMCELL Technologies; details can be found in Sup-
plementary Materials and Methods; ref. 11).

Cell survival assay
MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium
bromide] assay was used to evaluate both the chemosensitivity
of ovarian cancer cells (A2780/C0, ALDH+/− derived from
A2780/C5) to cisplatin and the platinum resensitization by
SGI-110 by determining the 50% growth inhibitory (IC50) dose
values (see Supplementary Materials and Methods).

Cell-cycle analysis
Cell-cycle analysis was conducted as described in Sup-
plementary Materials and Methods.

Sphere and colony formation assays
Sphere formation assays were conducted as described pre-
viously (5) and in Supplementary Materials and Methods.
Colony formation assay was performed by sorting 500 untreat-
ed or drug-treated ALDH+/− cells into 6-well coated high-
adhension plates (Corning). Cells were seeded in 2 mL RPMI-
1640 (Invitrogen) medium with 10% FBS (Atlanta Biologicals),
1% t-glutamine (Corning), and 1% penicillin/streptomycin
(Corning), cultured for 8 days, plates were washed with 2 mL
PBS, fixed with 3 mL 10% formalin (Sigma) for 15 minutes,
and stained with crystal violet for 5 minutes (0.025% w/v; Sigma).
The number of colonies was counted in each well, excluding
small (<50 cells) colonies (23).

Differentiation assay
ALDH− cells were FACS sorted from control-treated (100
nmol/L DMSO) or SGI-110 (100 nmol/L per day for 3 days)-
treated Aldefluor-stained A2780_CR5 ovarian cancer cells, and
50,000 cells then were plated under adherent conditions with
either differentiation medium (DMEM/F12 with 10% FBS) or
standard RPMI-1640 medium containing 10% FBS, 1% t-glut-
amine and 1% penicillin/streptomycin, as described (5). The
number of ALDH+ cells on days 7, 14, 21, 28, and 42 was
determined using FACS analysis.

In vivo xenograft experiments
All animal studies were conducted according to a protocol
approved by the Institutional Animal Care and Use Committee
of Indiana University. Female nude, athymic, BALB/c-nu/nu
mice (5–6 weeks old; Harlan) were injected subcutaneously
(s.c.) with 20,000 ALDH− or ALDH+ cells sorted from either
SGI-110 (100 nmol/L/day for 3 days) or vehicle (DMSO)-treated
aldefluor+ A2780_CR5, and ALDH+ or ALDH− were isolated
from three high-grade serous human tumors (1,500 cells per
mouse). Before subcutaneous injection, cells were resuspended
in 100 μL 1:1 RPMI-1640 mixed with Matrigel (BD Biosciences),
as described (5). Tumor length (l) and width (w) were measured
weekly using digital calipers and tumor volume (v) was cal-
culated as v = \( \frac{1}{2} \times l \times w^2 \). Mice were euthanized when tumors
were >2 cm in diameter or at end of study.

For the carboplatin-response studies, mice were injected
intraperitoneally with 2 × 106 A2780 cells and subsequently
treated with carboplatin (Hospira) at 50 mg/kg, intraperito-
neally or PBS (n = 6–9 animals per group) weekly for 3 weeks
beginning 3 days after injection of cells. For the maintenance
study, mice were injected with A2780 cells and treated with
carboplatin for 3 weeks, as described. At the completion of
the carboplatin treatment, mice were randomized to receive SGI-
110 (2 mg/kg) or vehicle, subcutaneous twice-a-week for 2

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weeks (n = 12 per group). Mice were euthanized and peritoneal tumors were counted, weighed, and volumes determined as described. Tumors were transferred to tubes containing the medium RPMI-1640 for immediate isolation of cancer cells, or snap frozen in liquid nitrogen or RLT buffer (Qiagen) and then stored at −80°C until DNA and RNA extraction.

Isolation of tumor cancer cells and growth of spheroids
Xenografts were minced and enzymatically dissociated in Dulbecco’s Modified Eagle Medium/F12 (Invitrogen), collagenase (100 U/mL; Sigma-Aldrich), and hyaluronidase (100 U/mL; Sigma-Aldrich), as previously described (5).

qRT-PCR
RNA was isolated from A2780, A2780_CR5_ALDH<sup>+/−</sup> cells, normal ovarian epithelial cells (nOSE), and primary tumors using AllPrep DNA/RNA/Protein Mini Kit (Qiagen,) following the manufacturer’s protocol (see Supplementary Materials and Methods).

DNA extraction, bisulfite conversion, and DNA methylation profiling
Genomic DNA was extracted from A2780 xenografts from mice treated with SGI-110 or control, by using the QIAamp DNA Mini Kit (Qiagen). Sodium bisulfite conversion was performed using the EZ DNA Methylation-Gold kit (Zymo Research), according to the manufacturer’s instructions. After bisulfite conversion, methylation of CpG sites was determined by Infinium HumanMethylation450 BeadChips (Illumina) following a procedure provided by Illumina, at the University of Chicago Genomics Core, Knapp Center for Biomedical Discovery (Chicago, IL). Data quality verification and levels of methylation of the 485,000 CpG sites included in the array were generated by the Illumina GenomeStudio Data Analysis Software. The Illumina Infinium 450k array was used to analyze DNA methylation in promoter site regions. The method measures the methylation levels over 482 k CpG probes. The average percentage of methylation levels were expressed as β-values and ranged from 0 (completely unmethylated) to 1 (completely methylated). Data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO series accession number GSE55613 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55613).

Western blot analysis
Protein extracts from control or treated cells were isolated and subjected to Western blot analysis as described (24). Antibodies for DNMT1, ALDH1A1, and GAPDH were from Cell Signaling Technology. After incubation with horseradish peroxidase–labeled secondary antibodies (Cell Signaling Technology), protein bands were visualized using the ECL reaction (Thermo Scientific).

DNA methylation assay by pyrosequencing
Methylation level for CpG islands of selected genes was determined by pyrosequencing assays as described (21), following a procedure provided by EpigenDX. Average methylation level for each CpG dinucleotide was calculated to indicate the methylation levels of each specific gene.

Statistical analysis
All data are presented as mean values ± SD of triplicate measurements. IC<sub>50</sub> dose values were determined by Prism 6 (GraphPad Software), using logarithm-normalized sigmoidal dose curve fitting. The Student t test was used to statistically analyze the significant difference among different groups by using Prism 4.0 (GraphPad Software). P value of 0.05 being considered significant. The genome-wide analysis experiments were conducted using the Partek Genomics Suite (version 6.5). The differences in methylation levels between samples (i.e., the differential methylation levels) were calculated using a mixed-model ANOVA. The resultant P values < 0.05 signified highly significant differential methylation levels at a specific nucleotide site. The analysis built a gene integration network, incorporating physical and predicted interactions, colocalization, shared pathways, and shared protein domains. The visualization of the interactions between the genes in the top functional category was realized using Cytoscape (additionalinformatics analysis can be found in Supplementary Materials and Methods; ref. 25).

Results
ALDH<sup>+</sup> cells are enriched in platinum-resistant ovarian cancer
ALDH activity has been demonstrated to be a global and well-established marker for OCSCs (10, 11). To determine the baseline level of ALDH<sup>+</sup> subpopulation in ovarian cancer, the percentages of ALDH<sup>+</sup> cells in ovarian cancer cell lines A2780, A2780_CR5, and SKOV3 and biopsies from chemotherapy naïve high-grade-serous ovarian cancer (HGSOC) patients were examined using FACS analysis. The percentage of ALDH<sup>+</sup> in A2780 was 0.3% and enrichment (P < 0.05) of ALDH<sup>+</sup> cells was observed in the A2780_CR5 (1.07%, >3-fold increase) and SKOV3 (0.65%, 2-fold increase; Fig. 1A). In primary tumors, the ALDH<sup>+</sup> percentage varied (0.4% to 15%); however, the average percentage of ALDH<sup>+</sup> cells was similar to ovarian cancer cell lines (3.53% vs. 1.07%; Fig. 1A and Supplementary Table S2). The presence of ALDH<sup>+</sup> cells in ovarian cancer cell lines and
primary tumors and the relative increase in percentage of ALDH\(^+\) cells in platinum-resistant cell lines supports the potential contribution of OCSCs to platinum resistance and poor clinical outcome.

**Low-dose SGI-110 treatment depletes ALDH\(^+\) cells in ovarian cancer**

As transient exposure to low doses of the DNMTI decitabine has been shown to target CSCs in leukemia and breast cancer (15), we examined the effect of the second-generation DNMTI SGI-110 on OCSCs. Ovarian cancer cell lines and dissociated cells from HGSO patients' tumors were treated with SGI-110 (100 mmol/L for 3 days) and the number of ALDH\(^+\) cells was determined by FACS analysis. SGI-110 treatment decreased \((P < 0.05)\) the percentage of ALDH\(^+\) cells in A2780 (0.3% to 0.14%), A2780_CR5 (1.07% to 0.33%), and primary ovarian tumors (2.63% to 0.4%), but not in SKOV3 (Fig. 1A), indicating that low-dose SGI-110 has the potential to target OCSCs in most ovarian cancer cells, but not all.

To investigate whether low-dose SGI-110 resensitizes ovarian cancer cells to platinum therapy, MTT assays were conducted after treatment with cisplatin alone or in combination with SGI-110. As expected, A2780_CR5 cells were more \((P < 0.001)\) resistant to cisplatin than A2780 cells (13.6 vs. 3.4 \(\mu\)mol/L; Fig. 1B), and low-dose SGI-110 treatment decreased \((P < 0.05)\) the IC\(_{50}\) for cisplatin in both cell lines (A2780, 3.4 to 1.6 \(\mu\)mol/L; A2780_CR5, 13.6 to 6.7 \(\mu\)mol/L; Fig. 1B). Similarly, ALDH\(^+\) cells derived from A2780_CR5 displayed increased \((P < 0.05)\) resistance to cisplatin compared with ALDH\(^-\) cells (38.7 vs. 12.2 \(\mu\)mol/L), and SGI treatment increased \((P < 0.05)\) cisplatin sensitivity of A2780_CR5-derived ALDH\(^+\) cells (38.7 to 6.2 \(\mu\)mol/L) as well as ALDH\(^-\) cells (12.2 to 6.0 \(\mu\)mol/L) but to a lesser extent (Fig. 1B). These results suggest that ALDH\(^+\) cells contribute to chemoresistance and can be resensitized to cisplatin by epigenetic therapy.

To further investigate the impact of SGI-110 on OCSCs, we treated ovarian cancer cells with cisplatin (1.67 \(\mu\)mol/L), SGI-110 (100 mmol/L) alone or in combination and examined ALDH\(^+\) cell viability. Cisplatin treatment alone reduced \((P < 0.05)\) the number of viable A2780 (1.80 \(\times 10^6\) vs. 4.57 \(\times 10^5\)) and SKOV3 (1.50 \(\times 10^6\) vs. 8.95 \(\times 10^5\)) cells (Fig. 1C), but the number of A2780_CR5-derived ALDH\(^+\) cells increased (1.06 \(\times 10^6\) vs. 1.64 \(\times 10^5\)) after cisplatin treatment (Fig. 1C). SGI-110 alone inhibited \((P < 0.001)\) the growth of cisplatin-resistant A2780_CR5 (4.58 \(\times 10^6\) vs. 2.32 \(\times 10^5\)), SKOV3 cells (1.50 \(\times 10^6\) vs. 2.92 \(\times 10^5\)) as well as A2780_CR5-derived ALDH\(^+\) cells (1.06 \(\times 10^5\) vs. 6.09 \(\times 10^4\)) and reduced \((P < 0.05)\) the percentage of ALDH\(^+\) cells in A2780 (0.30% vs. 0.14%) and A2780_CR5 (1.5% vs. 0.38%; Fig. 1C and D). As expected, combined treatment with cisplatin–SGI-110 effectively inhibited \((P < 0.001)\) ovarian cancer cell viability (A2780, 1.80 \(\times 10^6\) vs. 3.14 \(\times 10^5\); A2780_CR5, 5.48 \(\times 10^5\) vs. 1.18 \(\times 10^5\); SKOV3, 1.50 \(\times 10^5\) vs. 2.04 \(\times 10^5\)) as well as the growth of ALDH\(^+\) cells derived from A2780_CR5 (1.06 \(\times 10^5\) vs. 5.43 \(\times 10^4\)), and decreased \((P < 0.05)\) the ALDH\(^+\) subpopulation in A2780 (0.30% vs. 0.20%) and A2780_CR5 (1.5% vs. 0.20%; Fig. 1C and D).

To determine whether SGI-110 restored cisplatin sensitivity, effects of treatment with SGI-110 + cisplatin were compared with those induced by cisplatin only. The number of platinum-resistant A2780_CR5, and A2780_CR5-derived ALDH\(^+\) ovarian cancer cells was reduced \((P < 0.01)\) by the combination therapy compared with cisplatin alone (Fig. 1C). The total number of viable A2780_CR5 cells was not reduced by treatment with cisplatin only, and the number of A2780_CR5-derived ALDH\(^+\) cell increased after cisplatin treatment alone (Fig. 1C). The observation that the cisplatin response of A2780_CR5 and A2780_CR5-derived ALDH\(^+\) cells to cisplatin alone was not significant indicates that the effect of SGI-110 + cisplatin on these platinum-resistant cells was not simply additive. In addition, modest activity of single-agent SGI-110 was evident in platinum-resistant cells, inducing more prominent G0–G1 arrest in the platinum-resistant compared with platinum-sensitive ovarian cancer cells (Supplementary Fig. S1), indicating that low-dose SGI-110 exerted a chemosensitization effect. As different cellular backgrounds likely contribute to epigenetic therapy response, SKOV3-derived ALDH\(^+\) population seemed to be more resistant to SGI-110 compared with A2780 and primary tumor–derived cells (Fig. 1C). However, the overall SKOV3 cell population was responsive to the drug, based on increased G0–G1 arrest in SKOV3 cells treated with SGI-110 (Supplementary Fig. S1). Taken together, these data suggest that low-dose SGI-110 exerted antitumor and chemoresensitization effects on OCSC.

**Low-dose SGI-110 reduces ovarian cancer self-renewal and clonogenicity**

We and others previously demonstrated enhanced sphere forming and self-renewal ability of OCSCs when grown in stem cell–selective culture conditions (26–29). To investigate the effect of SGI-110 on sphere formation, A2780 and A2780_CR5 cells were treated with SGI-110/cisplatin alone.

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**Figure 2.** SGI-110 decreases self-renewal and clonogenicity of ovarian cancer. A. 30,000 dissociated sphere-forming cells derived from A2780 cells (platinum-sensitive and -resistant) were treated with cisplatin (1.67 \(\mu\)mol/L) or SGI-110 (100 mmol/L) alone or in combination. Representative images are shown. Scale bar, 200 \(\mu\)m. B, quantitative analysis of sphere formation assay. C, 500 ALDH\(^+\) cells derived from A2780_CR5 were treated with cisplatin (1.67 \(\mu\)mol/L), SGI-110 (100 mmol/L), alone or in combination, and allowed to recover for 4 days. The number of spheres (left) and colonies (right) was counted in 14 days and 7 days, respectively. D, sphere formation assay of 500 untreated and SGI-110 (100 mmol/L)-treated ALDH\(^-\)/(ALDH\(^+\) isolated from cultured A2780_CR5 (platinum-resistant). Representative images are shown at the top; scale bar, 200 \(\mu\)m. Quantification of sphere formation assay is shown below the images. Cells were plated in triplicate and spheres were mechanically dissociated every 7 days and counted on day 14. E, colony formation assay of 500 untreated and SGI-110 (100 mmol/L)-treated ALDH\(^-\)/(ALDH\(^+\) isolated from cultured A2780_CR5. Cells were plated in triplicate. Colonies were stained with crystal violet and counted on day 8. F, ALDH\(^+\) cell differentiation assay. Average number of ALDH\(^+\) population present in untreated or SGI-110 (100 mmol/L)-treated A2780_CR5_ALDH\(^+\) cells. ALDH\(^+\) cells were cultured in RPMI-1640 or DMEM for 7, 14, 21, 28, and 42 days. Mean values \(\pm\) SD of three independent experiments in triplicate are reported (*, \(P < 0.05\); **, \(P < 0.51\); ***, \(P < 0.001\)).
or in combination, and tumor-sphere formation assays were performed. A2780_CR5 cells demonstrated greater \( (P < 0.001) \) sphere-forming ability than A2780 (224 ± 20 vs. 54 ± 13 spheres; Fig. 2A and B). Moreover, SGI-110 treatment alone markedly inhibited \( (P < 0.001) \) the sphere-forming ability of A2780_CR5 (224 ± 20 vs. 115 ± 11). The combined SGI-110–cisplatin treatment inhibited \( (P < 0.05) \) sphere-forming capability of both the parental line and the resistant subline (A2780, 54 ± 13 vs. 27 ± 7; A2780_CR5, 224 ± 20 vs. 85 ± 12).

To examine the long-term impact of SGI-110 on OCSCs, sphere formation and colony formation assays were performed using ALDH\(^+\) cells derived from A2780_CR5 treated with cisplatin and (or) SGI-110 and allowed to recover for 4 days. As shown in Fig. 2C and Supplementary Fig. S3, low-dose SGI-110, either alone or in combination with cisplatin, inhibited \( (P < 0.05) \) sphere (30 ± 16 vs. 13 ± 2 SGI-110 or 30 ± 16 vs. 10 ± 5 SGI-110 + cisplatin) and colony (72 ± 17 vs. 58 ± 5 SGI-110 or 72 ± 17 vs. 42 ± 10 SGI-110 + cisplatin) formation capability of ALDH\(^+\) cells. To further examine the short-term effect of low-dose SGI-110 treatment on self-renewal, the same assay was performed on the A2780_CR5 ALDH\(^+\) cells. Treatment with SGI-110 alone had no effect on ALDH\(^+\) ovarian cancer cell growth rate (Supplementary Fig. S2), eliminating growth rate as a major contributing factor to the sphere or colony formation capability of ALDH\(^+\) cells. ALDH\(^+\) formed a greater \( (P < 0.05) \) number of spheroids compared with ALDH\(^-\) cells (87 ± 52 vs. 212 ± 97) (Fig. 2D, Supplementary Fig. S4), but no difference in colony formation was observed for ALDH\(^+\) vs. ALDH\(^-\) (Fig. 2E). In addition, low-dose SGI-110 treatment inhibited \( (P < 0.05) \) sphere-forming capability of ALDH\(^+\) cells (212 ± 97 vs. 112 ± 50; Fig. 2D) and reduced \( (P < 0.001) \) clonogenicity of ALDH\(^+\) (113 ± 25 vs. 23 ± 10) and ALDH\(^-\) (90 ± 17 vs. 18 ± 5) cells (Fig. 2E). Moreover, serial passaging indicated that ALDH\(^+\) cells maintained their sphere-forming ability over multiple passages, whereas the number of cell aggregates formed by ALDH\(^-\) cells was reduced from passage 2 to 3 (7.7 ± 1.4 vs. 40 ± 1.4; Supplementary Fig. S5). Although ALDH\(^+\) ovarian cancer cells were incapable of long-term survival in stem cell culture conditions, the cells demonstrated limited survival in anchorage-independent conditions by grouping together to form loosely adhesive cell clusters (30). Those clusters were unable to undergo serial passage and, therefore, were not considered true "spheroids." Thus, the initial 3-day low-dose SGI-110 treatment inhibited ALDH\(^+\) sphere-forming ability over three passages \( (P < 0.05); \) Supplementary Fig. S5).

As A2780_CR5 ALDH\(^+\) cells generated a greater \( (P < 0.05) \) number of ALDH\(^+\) cells in DMEM than in the RPMI-1640 medium, and SGI-110 treatment decreased \( (P < 0.01) \) the ALDH\(^+\) subpopulation under either culture condition (Fig. 2F). Although A2780_CR5 ALDH\(^+\) cells persisted and maintained stemness properties during the initial culture period, the proportion of ALDH\(^+\) cells declined \( (P < 0.05) \) by 42 days in culture (Fig. 2F), indicating that A2780_CR5 ALDH\(^+\) cells were able to repopulate ALDH\(^+\) and ALDH\(^-\) and low-dose SGI-110 therapy–induced ALDH\(^+\) differentiation (Supplementary Fig. S6).

Low-dose SGI-110 blunts tumorigenicity of ovarian CSCs by targeting ALDH\(^+\) cells

To examine the effect of SGI-110 on OCSC tumor formation in vivo, untreated and SGI-110–treated ALDH\(^+\)/ALDH\(^-\) cells derived from the A2780_CR5 ovarian cancer cell line and primary patient tumors were evaluated in a xenograft model (Fig. 3A). ALDH\(^+\) cells from human tumors (1,500 cells per mouse; Fig. 3B) or A2780_CR5-derived ALDH\(^+\) (20,000 cells per mouse; Fig. 3C) displayed robust \( (P < 0.05) \) tumor-initiating capacity compared with ALDH\(^-\) cells. Importantly, untreated or SGI-110 pretreated primary tumor-derived ALDH\(^-\) cells were nontumorigenic under these conditions (Fig. 3B), whereas 20,000 A2780_CR5-derived ALDH\(^+\) cells exhibited reduced \( (P < 0.05) \) tumor formation ability compared with A2780_CR5-derived ALDH\(^+\) cells [area under the curve (AUC), 83 ± 12 vs. 1259 ± 44; Fig. 3C]. Furthermore, increasing the number of ALDH\(^+\)/ALDH\(^-\) cells used in xenograft assays accelerated tumor growth and shortened tumor-initiation time for ALDH\(^+\) and ALDH\(^-\) cells (Supplementary Table S3). Treatment with low-dose SGI-110 for 3 days in vitro (before injection into mice) prolonged \( (P < 0.05) \) the time to tumor initiation and reduced tumor volume in ALDH\(^+\) xenografts (Supplementary Table S3). To examine the effects of SGI-110 on OCSCs, the percentage of ALDH\(^+\) cells in untreated versus SGI-110–treated A2780_CR5-derived ALDH\(^+\)/ALDH\(^-\) xenograft tumors was assessed by FACS. ALDH\(^+\) cells were highly enriched \( (P < 0.05) \) in the A2780_CR5-derived ALDH\(^+\) xenografts compared with ALDH\(^+\) tumors (71.2% ± 8.7% vs. 1.3%), supporting that ALDH\(^-\) cells were unable to dedifferentiate into ALDH\(^+\) cells. SGI-110 reduced \( (P < 0.05) \) ALDH\(^+\) subpopulation (71.2% ± 8.6% to 55.8% ± 0.9%) in xenografts (Fig. 3D).

ALDH\(^+\) cells overexpress stemness-associated genes

To confirm the stem-like properties of the ALDH\(^+\) ovarian cancer cells, we examined the mRNA levels of several known stem cell–associated genes (4, 11, 30). Compared with nOSE, HGSOCs \((n = 5)\) displayed increased \( (P < 0.05) \) expression of stem cell–associated genes, NOTCH3, OCT4, and ALDH1A1 (Fig. 4A). Importantly, increased \( (P < 0.05) \) expression levels of ALDH1A1 (162.4 ± 28.8-fold), BMI1 (88.8 ± 0.2-fold), NANOG (9.5 ± 0.8-fold), NOTCH3 (1.9 ± 0.6-fold), and OCT4 (71.4 ± 1.7-fold) and decreased \( (P < 0.05) \) expression levels of the differentiation-related gene HOXA10 (4.1 ± 0.7-fold) and HOXAI1 (2.4 ± 0.8-fold) were observed in A2780_CR5- derived ALDH\(^+\) compared with ALDH\(^-\) cells (Fig. 4B and Supplementary Fig. S7).

Low-dose SGI-110 induces differentiation of ALDH\(^+\)

Methytransferases DNMT1, 3A and 3B are the main effectors of DNA methylation. Deregulated levels of DNMTs have been reported in cancer (31) and in ovarian cancer (32, 33), and in association with platinum resistance. Therefore, DNMT1, 3A, and 3B expression levels were measured in platinum-sensitive (A2780) and –resistant (A2780_CR5) sublines and in A2780_CR5-derived ALDH\(^+\) and ALDH\(^-\) cells. DNMT1 was \( (P < 0.05) \) significantly upregulated in cisplatin-resistant A2780_CR5 and ALDH\(^+\) cells, and DNMT3A and 3B were overexpressed in ALDH\(^-\) cells, suggesting that aberrant...
Figure 3. SGI-110 decreases tumorigenesis by targeting ALDH+ cells. A, schematic diagram of the approach used to study in vivo tumorigenesis of low-dose SGI-110–untreated and -treated ALDH+/– cells. B, primary xenograft tumor growth curve of 1,500 patient-derived ALDH+ or ALDH– cells pretreated with SGI-110 (100 nmol/L) for 72 hours or DMSO in mice (n = 3 for each group) during 8 weeks. Average of AUC was calculated and shown in the histogram. ALDH+ cells isolated from three high-grade serous human tumors (1,500 cells per mouse). C, xenograft tumor growth curve of 20,000 A2780_CR5–derived ALDH+ or ALDH– cells pretreated with SGI-110 (100 nmol/L) for 72 hours or DMSO in mice in 7 weeks (n = 3 for each group). AUC was calculated and shown in the histogram. D, average number of ALDH+ population present in untreated or SGI-110 (100 nmol/L)-pretreated A2780_CR5–derived ALDH+ or ALDH– xenograft tumors. Mean values ± SD of three independent experiments are shown (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
methylations patterns may be associated with increased or altered DNMT activity in OCSCs (Fig. 4B). We then assessed stem-cell, differentiation-related genes, and DNMT isoforms expression levels in response to treatment with low-dose SGI-110 in a time-course experiment (Fig. 4C). In ALDH− cells derived from A2780_CR5, SGI-110 suppressed (P < 0.05) the expression of stemness genes BMI, NANOG, NOTCH3, and OCT4 (Fig. 4D), and induced upregulation (P < 0.05) of the differentiation gene HOXA10 (Fig. 4E). The latter was accompanied by HOXA10 promoter Cpg island demethylation (Fig. 4E), consistent with

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<th>Time (days)</th>
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<tr>
<td>0</td>
<td>-2.0</td>
</tr>
<tr>
<td>4</td>
<td>-1.5</td>
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<tr>
<td>7</td>
<td>-1.0</td>
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<td>14</td>
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**Figure 4.** SGI-110 decreases expression of pluripotency genes and induces differentiation-associated genes in ovarian cancer (OC). A, average expression of stemness genes (ALDH1A1, BMI, NOTCH3, and OCT4) was measured in five primary high-grade serous ovarian epithelial cancers from patients compared with normal ovarian epithelial cells. B, average expression of stemness genes (ALDH1A1, BMI, NANOG, NOTCH3, and OCT4). DNMT isoforms (DNMT1, DNMT3A, and DNMT3B) and differentiation-associated genes (HOXA10 and NOTCH3) were measured by qRT-PCR in A2780_CR5 (platinum-resistant), ALDH+ and ALDH− cells derived from parental A2780 cells. C, scheme of low-dose SGI-110 time-course treatment. D, average expression of stemness genes (BMI, NANOG, NOTCH3, and OCT4) were measured by qRT-PCR in A2780_CR5 compared with untreated ALDH+ cells. E, average expression of the differentiation-associated gene HOXA10 mRNA measured by qRT-PCR in A2780_CR5-derived ALDH+ cells over SGI-110 (100 nmol/L) treatment compared with untreated ALDH+ cells. F, average expression of the differentiation-associated gene HOXA10 mRNA measured by qRT-PCR in A2780_CR5-derived ALDH+ cells at indicated time points over SGI-110 (100 nmol/L) treatment compared with untreated ALDH+ cells. Three independent experiments were performed and mean values ± SD are calculated (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
Figure 5. In vivo effects of carboplatin on xenograft growth and ovarian CSCs. A, effects of carboplatin on weights, volumes, and metastases sites of xenograft tumors derived from A2780 cells. Bars represent average measurements ±SD; ***; \( P < 0.001 \) (\( n = 6 \) per group). B, percentage of ALDH\(^+\) cells in control or carboplatin-treated xenografts. Cells were isolated by mechanical and enzymatic digestion and ALDH\(^+\) cells were detected by FACS. Bars represent average of four measurements ±SD; ***; \( P < 0.001 \) (left). Representative FACS histograms are shown on the right. C, spheroid formation by cells dissociated from control or carboplatin-treated xenografts; bars represent average of three measurements ±SD; **; \( P < 0.01 \) (left). Phase microscopy shows morphology of spheres formed by cells dissociated from control or carboplatin-treated xenografts (×100 magnification, right).
Injection A2780 cells
Carboplatin SGI-110 Vehicle
Day 0 4 11 18 21 28 35
Tumor collection
A

B

C

D

E

F

Effects of SGI-110 on DNA methylation profiles
Gene networks affected by SGI-110

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Wang et al.

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the SGI-110–hypomethylating effect, SGI-110 also reduced (P < 0.05) the mRNA expression levels of DNMT1 (Fig. 4F), DNMT3A, and 3B (Supplementary Fig. S8A and S8B) and ALDH1A1 (Fig. 4F) in ALDH+ cells up to 14 days (P < 0.05). Three-day low-dose SGI-110 treatment also resulted in decreased DNMT1 and ALDH1A1 protein levels in A2780, A2780_CR5, and SKOV3 cells (Supplementary Fig. S8C–S8E). These data support that low doses of SGI-110 promote differentiation of ALDH+ ovarian cancer cells and suppress their stem-like properties.

Enrichment in ALDH+ cells after platinum in ovarian cancer xenografts

To test the hypothesis that CSCs persist in ovarian tumors after platinum-based chemotherapy, we used an intraperitoneal xenograft model derived from parental (platinum-sensitive) A2780 ovarian cancer cells treated with carboplatin or vehicle (control). Tumor volume, weight, and number of metastases were significantly decreased (P < 0.001) by weekly treatment with carboplatin at 50 mg/kg (Fig. 5A). Vehicle and carboplatin-treated tumors were dissociated to single-cell suspension at the end of treatment and cells were analyzed for Aldefluor positivity, and for ability to form spheres in anchorage-independent conditions. The percentage of ALDH+ cells was increased (P < 0.001) approximately 20-fold in tumors residual after carboplatin compared with vehicle-treated tumors (Fig. 5B). Cells dissociated from carboplatin-treated tumors formed increased (P < 0.001) numbers and size of spheres compared with cells dissociated from control tumors (Fig. 5C), consistent with an OCSC phenotype. In all, carboplatin significantly decreased tumor growth in vivo but also contributed to enriching the OCSC population in residual tumors.

DNA demethylation induced by SGI-110 delayed recurrence of ovarian cancer xenografts

To determine whether DNA hypomethylation induced by SGI-110 prevents tumor recurrence after maximal response to platinum therapy, mice bearing intraperitoneal A2780-derived xenografts and treated with carboplatin were randomized to a 2-week treatment with SGI-110 or vehicle (Fig. 6A, n = 12 mice per group). Treatment with SGI-110 decreased (P < 0.05) the total tumor weight and volume compared with control-treated mice (Fig. 6B). Cells dissociated from SGI-110–treated tumors significantly reduced spheroid formation capability in vitro (Fig. 6C, P < 0.05), consistent with inhibition of stem cell properties. To demonstrate that SGI-110 induced global DNA hypomethylation consistent with its DNMT inhibitory properties, Illumina Infinium HumanMethylation450 arrays were used to quantify DNA methylation in control and SGI-110–treated ovarian xenografts. More than 62,000 methylation sites and 10,000 CpG islands were found to be significantly hypomethylated in SGI-110–treated tumors compared with controls (Table 1). The substantial global DNA hypomethylation induced by SGI-110 in vivo was also demonstrated by a decrease of 6% in β-values across all CpG islands (P < 0.0001, Fig. 6D) and through unsupervised hierarchical clustering analysis of methylation sites in control and SGI-110–treated xenografts (Fig. 6E). To understand biologic processes represented by the genes whose promoter CpG islands were significantly hypomethylated in response to SGI-110, we grouped those genes into well-defined functional gene ontology (GO) categories, using DAVID (Database for Annotation, Visualization, and Integrated Discovery; ref. 34). Of the 84 genes meeting the criteria (described in Materials and Methods, Supplementary Table S4), 65 had well-defined GO categories, and 40 could be grouped into ten functional categories containing at least three assigned genes. These categories represent important biologic processes, including metabolism, apoptosis, proteolysis, cell development, morphogenesis, cell adhesion, transport, signaling, transcriptional regulation, and GTPase regulation (Fig. 6F), suggesting that hypomethylation induced by SGI-110 alters critical pathways in cancer. Genes included in these networks include PCDH10, a gene known to be downregulated in cancer through DNA methylation (35), miR-203 that is epigenetically silenced in myeloma and involved in apoptosis control (36), PTK6, involved in epithelial–mesenchymal transition (37), and others.

Discussion

Our results demonstrate that ALDH+ ovarian cancer cells possessing stem cell characteristics are enriched in platinum-resistant ovarian cancer cell lines, human tumors, and xenografts residual after platinum therapy. The novel DNMT1 SGI-110 inhibits ALDH+ cell viability, sphere formation, and tumor-initiating capacity, represses stem cell–associated gene transcription, and resensitizes platinum-resistant ovarian cancer cells to platinum. In vivo, maintenance treatment with SGI-110 after carboplatin induces profound global hypomethylation and delays tumor progression. Collectively, our data suggest that a strategy targeting DNA methylation in ovarian cancer exerts potent antitumor activity by allowing elimination of ALDH+ cells enriched in residual, platinum-resistant tumors. Our data have several implications.

First, we assert that ALDH1A1 expression and activity characterizes ovarian cancer cells with stem cell properties,

Figure 6. In vivo effects of SGI-110 as maintenance therapy following carboplatin treatment. A, diagram illustrating the experimental design including the carboplatin treatment phase followed by randomization to either SGI-110 (2 mg/kg twice weekly) or diluent. B, effects of SGI-110 on tumor weight and volume. Bars represent average measurements ±SD; *, P < 0.05 (n = 12 per group). C, spheroid formation by cells dissociated from control or SGI-110–treated xenografts. Bars represent average of three measurements ±SD; **, P < 0.05. D, mean β-value calculated across all CpG sites measured using Infinium 450 human methylation arrays in control and SGI-110–treated xenografts (β, P < 0.001). E, hierarchical clustering displays differential DNA methylation profiles of SGI-110 or control treated xenografts (n = 3 replicates) measured using Infinium 450 human methylation arrays. Columns represent individual samples and rows represent methylation sites. Each cell corresponds to the level of methylation at a specific site in a given sample. A visual dual color code is used with red and blue indicating high and low methylation levels, respectively. The scale of color saturation, which reflects the methylation levels, is included. F, functional relationships between genes significantly hypomethylated by SGI-110 treatment were determined by using GeneMANIA and visualized by Cytoscape, as described.
in agreement with reports from other groups (6, 30, 38). Aldefluor positivity detectable by FACS identifies the enzymatic activity of ALDH1, a member of the ALDH family that metabolizes reactive aldehydes (39). Although ALDH positivity has been recognized as a stem cell marker in various tissues, the role of the enzyme in the functions of CSC remains elusive. A potential function relates to its regulatory role in the synthesis of retinoids, which play a critical role in cellular differentiation. Whether the enzyme has other functions important to the maintenance of CSCs remains not known. Here, we show that ALDH⁺ cells derived from ovarian cancer cell lines and from primary ovarian tumors are more resistant to platinum, express stem cell restricted transcription factors, and are able to generate spheres and tumors in vivo.

Second, we demonstrate that ovarian xenografts residual after treatment with platinum are enriched in ALDH⁺ cells, suggesting that cells with stem cell characteristics escape traditional cytotoxic treatments. Our model is consistent with the proposed concept that stem cells elude the effects of traditional anticancer strategies and can reconstitute recurrent tumors that become recalcitrant to chemotherapy (12). We use A2780, a tumorigenic and one of the few available platinum-sensitive ovarian cancer cell lines, to recapitulate the clinical evolution of ovarian cancer, with massive initial response to chemotherapy, followed by inevitable resurgence of resistant tumors. Although it has been suggested that the genomic signature of A2780 does not fully match that of HGSOC (40, 41), previous studies demonstrated aggressive in vivo growth of A2780 cells (26, 42) and response to platinum, resembling the human disease. Our model supports that tumor recurrence could be attributed to the persistence of platinum refractory stem cells at the end of initial treatment. We propose a novel strategy to target these resistant cells through epigenomic reprogramming by using a novel DNMTI. We show that SGI-110 suppressed the viability of ALDH⁺ cells, their ability to form spheres in vitro, and their tumorigenic potential in vivo. Importantly, treatment with SGI-110 re-sensitized platinum-resistant ALDH⁺ cells to platinum, providing proof-of-concept for further investigating hypomethylating strategies as means to re-sensitize tumors to chemotherapy.

Third, it has been recognized that embryonic and CSCs harbor distinct DNA methylation profiles (43, 44) that enable tight control of cell differentiation and self-renewal capacity. Therefore, treatment of a stem cell–enriched cell population with DNA-hypomethylating agents would remove the repressive epigenetic brakes, allowing stem cells to undergo differentiation and leave the pluripotent undifferentiated state. Although this concept has been tested in leukemia models (45), it remains unexplored in solid tumors. Here, we show for the first time that the expression levels of all three DNMT isoforms is significantly increased in ALDH⁺ cells derived from platinum-resistant ovarian cancer cells and that SGI-110 is able to re-set these cells toward differentiation. As histone modifications have been associated with DNA methylation and regulation of stemness-associated genes, and an association between EZH2 and ALDH1A1 expression has been reported (46), it seems plausible that additional epigenetic regulatory mechanisms contribute to maintaining stem cell characteristics. Thus, our studies provide the first proof-of-principle that epigenomic strategies efficiently target ovarian cancer stem cells.

Our results also demonstrate that SGI-110 induces profound hypomethylation in vivo, with tens of thousands of CpG sites becoming demethylated in response to treatment. A distinctly hypomethylated DNA profile emerges, providing reassurance that the novel DNMTI hits its biologic targets in solid tumors in vivo. Future studies will strive to identify the critical genes or pathways responsible for tumor growth inhibition and chemotherapy re-sensitization in response to this DNMT inhibitor. It is likely that not a single gene, or pathway, but a complex program is reengaged by targeting the epigenome, as we show here by pathway analyses.

Finally, we demonstrate that treatment with SGI-110 after platinum decreases recurrent tumor burden in a platinum-sensitive ovarian cancer intraperitoneal xenograft model that recapitulates first the response to therapy, and second the recurrence of disease after chemical debulking using carboplatin. These data support exploring maintenance treatment with a hypomethylating agent after maximal response induced by traditional treatment. Maintenance strategies after chemotherapy have been investigated with variable level of success in ovarian cancer (47–49) and remain an area of active

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<th>Table 1. Number of DNA methylation sites and regions showing significant changes in methylation in xenografts treated with SGI-110 or vehicle</th>
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<tr>
<td><strong>Control vs. SGI-110</strong></td>
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<tr>
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<tr>
<td>CpG sites</td>
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<td>CpG regionsᵇ</td>
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**NOTE:** Methylation of CpG sites was determined using Infinium HumanMethylation450 arrays and was expressed as β-values ranging from 0 (unmethylated) to 1 (totally methylated). Significant changes: P < 0.01 (ANOVA) and FDR < 0.05.

ᵇΔβ, Difference in β-values between control and SGI-110 groups.

ᵇCpG islands plus shores and shelves.
expansion. Our study provides the first evidence that epigenome-targeting strategies decrease tumor progression by targeting and reprogramming residual CSCs, supporting further refinement of this intervention and translating these findings to the clinic.

Disclosure of Potential Conflicts of Interest
K.P. Nephew received a commercial research grant from and is a consultant/advisory board member for Astex Pharmaceuticals, Inc. D. Matei is a consultant/advisory board member for Astex Pharmaceuticals, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: Y. Wang, H. Cardenas, K.P. Nephew, D. Matei Development of methodology: Y. Wang, F. Fang, S. Condello, Y. Liu, K.P. Nephew, D. Matei Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Wang, H. Cardenas, S. Condello, K.P. Nephew, D. Matei Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Wang, H. Cardenas, S. Condello, K.P. Nephew, D. Matei

References
Epigenetic Targeting of Ovarian Cancer Stem Cells

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