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Preclinical Development

Molecular Cancer Therapeutics

Smoothened Antagonists Reverse Taxane Resistance in Ovarian Cancer

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Abstract

The hedgehog pathway has been implicated in the formation and maintenance of a variety of malignancies, including ovarian cancer; however, it is unknown whether hedgehog signaling is involved in ovarian cancer chemoresistance. The goal of this study was to determine the effects of antagonizing the hedgehog receptor, Smoothened (Smo), on chemotherapy response in ovarian cancer. Expression of hedgehog pathway members was assessed in three pairs of parental and chemotherapy-resistant ovarian cancer cell lines (A2780ip2/A2780cp20, SKOV3ip1/SKOV3TRip2, HeyA8/HeyA8MDR) using quantitative PCR and Western blot analysis. Cell lines were exposed to increasing concentrations of two different Smo antagonists (cyclopamine, LDE225) alone and in combination with carboplatin or paclitaxel. Selective knockdown of Smo, Gli1, or Gli2 was achieved using siRNA constructs. Cell viability was assessed by MTT assay. A2780cp20 and SKOV3TRip2 orthotopic xenografts were treated with vehicle, LDE225, paclitaxel, or combination therapy. Chemoresistant cell lines showed higher expression (>2-fold, $P < 0.05$) of hedgehog signaling components compared with their respective parental lines. Smo antagonists sensitized chemotherapy-resistant cell lines to paclitaxel, but not to carboplatin. LDE225 treatment also increased sensitivity of ALDH-positive cells to paclitaxel. A2780cp20 and SKOV3TRip2 xenografts treated with combined LDE225 and paclitaxel had significantly less tumor burden than those treated with vehicle or either agent alone. Increased taxane sensitivity seems to be mediated by a decrease in P-glycoprotein (MDR1) expression. Selective knockdown of Smo, Gli1, or Gli2 all increased taxane sensitivity. Smo antagonists reverse taxane resistance in chemoresistant ovarian cancer models, suggesting combined anti-hedgehog and chemotherapies could provide a useful therapeutic strategy for ovarian cancer. *Mol Cancer Ther; 11(7); 1587–97. ©2012 AACR.*

Introduction

Ovarian cancer is the leading cause of death from a gynecologic malignancy. Although ovarian cancer is among the most chemosensitive malignancies at the time of initial treatment (surgery and taxane/platinum-based chemotherapy), most patients will develop tumor recurrence and succumb to chemoresistant disease (1). Evaluation of multiple chemotherapy agents in several combinations in the last 20 years has yielded modest improvements in progression-free survival, but no increase in durable cures. This clinical course suggests that a population of tumor cells has either inherent or acquired resistance to chemotherapy that allows survival with initial therapy and ultimately leads to recurrence. Targeting the cellular pathways involved in this resistance may provide new treatment modalities for ovarian cancer.

The Hedgehog pathway plays an important role in cell growth and differentiation during embryonic development (2). There are 3 known mammalian hedgehog ligands—Sonic, Indian, and Desert. These ligands are secreted peptides that bind to the transmembrane Patched (Ptch) receptor. In the absence of hedgehog ligand, Ptch serves as a negative regulator of Smoothened (Smo), a G-protein–coupled receptor. In the presence of hedgehog ligand, Ptch repression of Smo is abolished, leading to downstream activation of the Gli family of transcription factors (Gli1; refs. 2, 3). Gli transcription factors translocate from the cytoplasm to the nucleus, where they bind DNA and activate transcription of hedgehog target genes, including *PTCH1* and *GLI1*, the expression of which are frequently measured to evaluate the presence or absence of hedgehog pathway activity (3, 4). Gli homologues have distinct, but overlapping functions; Gli1 serves only as a transcriptional activator, whereas Gli2 and Gli3 are capable of both activating and repressing hedgehog gene transcription.

Recent reports have implicated hedgehog signaling in multiple malignancies (5, 6), including ovarian cancer (7–9), and suggest this pathway may be especially important in maintaining the subpopulation of cancer cells

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with stem cell properties (10, 11) as well as conferring resistance to chemotherapies (12, 13). Inhibition of the hedgehog signaling pathway, therefore, has become a desirable therapeutic strategy for the treatment of various cancers. Cyclopamine, a steroidal alkaloid derived from the lily plant *Veratrum californicum*, was the first compound identified that inactivates hedgehog signaling by antagonizing Smo function (14–16). Since this discovery, pharmaceutical companies have synthesized more selective Smo antagonists, including NVP-LDE225 (17), which is currently being investigated in clinical trials (11).

The effects of Smo antagonists, both alone and in combination with chemotherapies, remains an active area of study in cancer research. Examination of combination effects is potentially important, given the hypothesized role of stem cell pathways in chemoresistance. However, the mechanisms by which hedgehog inhibition might sensitize cells to chemotherapy, and whether such an approach would be effective in ovarian cancer, are not known. In our study, we sought to determine the effects of Smo antagonists on the viability of ovarian cancer cells, both alone and in combination with chemotherapy. We show that Smo antagonists have activity alone, but more dramatically can reverse taxane resistance in ovarian cancer, both *in vitro* and *in vivo*, through modulation of the multidrug resistance mediator, P-glycoprotein (MDR1). These findings provide new insight into hedgehog signaling, its contribution to an aggressive subpopulation of cells, and new opportunities for clinical development.

Materials and Methods

Reagents and cell culture

Cyclopamine was purchased from Toronto Research Chemicals and dissolved in 95% ethanol to create a 10 mmol/L stock solution. NVP-LDE225 (LDE225) was kindly provided by Novartis Pharma AG and dissolved in dimethyl sulfoxide (DMSO) to create a 10 mmol/L stock solution. The ovarian cancer cell lines A2780ip2, A2780cp20, HeyA8, HeyA8MDR, SKOV3ip1, and SKOV3TRip2 (18–23) were maintained in RPMI-1640 medium supplemented with 10% FBS (Hyclone). A2780cp20 (platinum- and taxane-resistant), HeyA8MDR (taxane-resistant), and SKOV3TRip2 (taxane-resistant, a kind gift of Dr. Michael Seiden; ref. 24) were generated by sequential exposure to increasing concentrations of chemotherapy (25). HeyA8MDR and SKOV3TRip2 were maintained with the addition of 150 ng/mL of paclitaxel. All cell lines were routinely screened for *Mycoplasma* species (GenProbe Detection Kit; Fisher) with experiments done at 70% to 80% confluent cultures. Purity of cell lines was confirmed with STR genomic analysis, and only cells less than 20 passages from stocks were used in experiments.

RNA extraction and reverse transcription

Total RNA was isolated from ovarian cancer cell lines using TRIzol reagent (Invitrogen) per manufacturer's instructions. RNA was then DNase treated and purified using the RNeasy Mini Kit (QIAGEN). RNA was eluted in 50 μL of RNase-free water and stored at −80°C. The concentration of all RNA samples was quantified by spectrophotometric absorbance at 260/280 nm using an Eppendorf BioPhotometer plus. Before cDNA synthesis, all RNA samples were diluted to 20 ng/μL using RNase-free water. cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNA samples were analyzed using quantitative PCR (qPCR).

Quantitative PCR

Primer and probe sets for *Desert HH* (Hs0036806_m1), *GLI1* (Hs00171790_m1), *GLI2* (Hs00257977_m1), *Indian HH* (Hs00745531_s1), *MDR1* (Hs00184500_m1), *PTCH1* (Hs00181117_m1), *SMO* (Hs00170665_m1), *Sonic HH* (Hs), and *RPLP0* (Hs99999902_m1; housekeeping gene) were obtained from Applied Biosystems and used according to manufacturer's instructions. PCR amplification was conducted on an ABI Prism 7900HT sequence detection system and gene expression was calculated using the comparative C_T method as previously described (26). Briefly, this technique uses the formula $2^{-\Delta\Delta C}{}_T$ to calculate the expression of target genes normalized to a calibrator. The cycling threshold (C_T) indicates the cycle number at which the amount of amplified target reaches a fixed threshold. C_T values range from 0 to 40 (the latter representing the default upper limit PCR cycle number that defines failure to detect a signal).

Western blot analysis

Cultured cell lysates were collected in modified radioimmunoprecipitation assay lysis buffer with protease inhibitor cocktail (Roche) and subjected to immunoblot analysis by standard techniques (25) using anti-Gli1 antibody (Cell Signaling Technology) at 1:1,000 dilution overnight at 4°C, anti-Smo antibody (LifeSpan Biosciences) at 1:1,000 dilution overnight at 4°C, or anti-β-actin antibody (AC-15, Sigma-Aldrich) at 1:20,000 dilution for 1 hour at room temperature, which was used to monitor equal sample loading. After washing, blots were incubated with goat anti-rabbit (for Gli1 and Smo) or goat anti-mouse (for β-actin) secondary antibodies (Bio-Rad) conjugated with horseradish peroxidase. Visualization was done by the enhanced chemiluminescence method (Pierce Thermo Scientific).

siRNA transfection

To examine downregulation of Smo, Gli1, or Gli2 individually with siRNA, cells were exposed to control siRNA (target sequence: 5′-UUCUCCGAACGUGUCACGU-3′, Sigma-Aldrich), one of 2 tested Smo-targeting constructs (siRNA1: 5′-GAGGAGUCAUGACUCUGUUCUCCAU-3′ or siRNA2: 5′-UGACCUCAAUGAGCCCUCAGCUGAU-3′; Invitrogen), one of 2 tested Gli1-targeting constructs (siRNA1: 5′-CUACUGAUA-

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CUCUGGGAUA-3′ or siRNA2: 5′-GCAAAUAGGGCUUCACAUA-3′; Sigma-Aldrich), or one of 2 tested Gli2-targeting constructs (siRNA1: 5′-GACAUGAGCUCCAUGCUCA-3′ or siRNA2: 5′-CGAUUGACAUGCGACACCA-3′; Sigma-Aldrich) at a 1:3 siRNA (μg) to Lipofectamine 2000 (μL) ratio. Lipofectamine and siRNA were incubated for 20 minutes at room temperature, added to cells in serum-free RPMI to incubate for up to 8 hours, followed by 10% FBS/RMPI thereafter. Transfected cells were grown at 37°C for 48 to 72 hours and then harvested for qPCR or Western blot analysis.

Assessment of cell viability and cell-cycle analysis

To a 96-well plate, 2,000 cells/well were exposed to increasing concentrations of cyclopamine or LDE225, alone or in combination with carboplatin or paclitaxel, in triplicate. Viability was assessed with 0.15% MTT (Sigma-Aldrich). For effects of siRNA-mediated downregulation on paclitaxel IC_{50}, cells were first transfected with siRNA (5 μg) for 24 hours in 6-well plates, then trypsinized and replated at 2,000 cells per well, followed by addition of chemotherapy after attachment. IC_{50} of the agent of interest was determined by finding the dose at which the drug had 50% of its effect, calculated by the equation $[(OD450_{MAX} - OD450_{MIN})/2) + OD450_{MIN}]$. For cell-cycle analysis, cells were treated with vehicle alone, paclitaxel alone, LDE225 alone, or combined LDE225 and paclitaxel for 72 hours, trypsinized, and fixed in 100% ethanol overnight. Cells were then centrifuged, washed in PBS, and resuspended in PBS containing 0.1% Triton X-100 (v/v), 200 μg/mL DNase-free RNase A, and 20 μg/mL propidium iodide (PI). PI fluorescence was assessed by sflow cytometry and the percentage of cells in sub-G_0, G_0–G_1, S-, and G_2–M phases was calculated by the cell-cycle analysis module for Flow Cytometry Analysis Software (FlowJo v.7.6.1).

ALDEFLUOR assay

Active aldehyde dehydrogenase (ALDH) was identified with the ALDEFLUOR assay according to manufacturer's instructions (StemCell Technologies). The ALDH-positive population was defined by cells with increased FITC signal absent in DEAB-treated cells, as previously described (27). ALDEFLUOR-positive and -negative populations from SKOV3Trip2 cells were sorted with a FACS Aria II flow cytometer (BD Biosciences), and collected cells were seeded onto a 96-well plate at a concentration of 2,000 cells/well. After overnight attachment, cells were then exposed to either DMSO or 5 μmol/L LDE225, alone or in combination with increasing concentrations of paclitaxel. Viability was assessed with 0.15% MTT (Sigma-Aldrich).

Orthotopic ovarian cancer model

For orthotopic therapy experiments using ovarian cancer cell lines, female athymic nude mice (NCr-nu) were purchased from the National Cancer Institute (Frederick, MD, USA) after Institution Animal Care and Use Committee approval of protocols, and cared for in accordance with guidelines of the American Association for Accreditation of Laboratory Animal Care. For all *in vivo* experiments, trypsinized cells were resuspended in 10% FBS-containing RPMI, washed with PBS, and suspended in serum-free HBSS at a concentration of 5×10^6 cells/mL, and 1×10^6 cells (A2780cp20 or SKOV3TRip2) were injected IP in 200 μL into 40 mice per experiment. After 1 week, mice ($n = 10$ per group) were randomized to treatment with (a) vehicle alone (0.5% methyl cellulose/0.5% Tween 80 in sterile water), (b) vehicle plus paclitaxel 75 μg, (c) LDE225 alone (60 mg/kg), or (d) combined LDE225 and paclitaxel. Vehicle and LDE225 were administered by gavage once daily and paclitaxel was administered i.p. weekly. Mice were treated for 4 weeks (A2780cp20) or 6 weeks (SKOV3TRip2, which grow more slowly) before sacrifice and tumor collection. All tumors were excised and weighed in total.

Statistical analysis

Comparisons of gene expression, cell viability, PI fluorescence, and mean tumor weight were analyzed using a 2-tailed Student *t* test, if assumptions of data normality were met. Those represented by alternate distribution were examined using a nonparametric Mann–Whitney *U* test. Differences between groups were considered statistically significant at $P < 0.05$. Error bars represent standard deviation unless otherwise stated. Number of mice per group ($n = 10$) was chosen as directed by a power analysis to detect a 50% decrease in tumor growth with β error of 0.2.

Results

Expression of hedgehog pathway members in chemosensitive and chemoresistant ovarian cancer cell lines

We first examined mRNA expression of hedgehog ligands [Sonic (*SHH*), Indian (*IHH*), Desert (*DHH*)], receptors (*PTCH1*, *SMO*), and transcription factors (*GLI1*, *GLI2*) in 3 pairs of parental and chemoresistant ovarian cancer cell lines: A2780ip2/A2780cp20 (20-fold increased cisplatin resistance and 10-fold increased taxane resistance), HeyA8/HeyA8MDR (500-fold taxane resistant), and SKOV3ip1/SKOV3TRip2 (1000-fold taxane resistant). As shown in Fig. 1A, mRNA levels of *SHH* were significantly higher in A2780cp20 (17.4-fold, $P < 0.05$) and SKOV3-TRip2 (2.4-fold, $P < 0.05$) cells compared with parental. *IHH* was also higher (3.5-fold, $P < 0.05$) in SKOV3TRip2 cells with *DHH* expression remaining unchanged or decreased in chemoresistant cell lines compared with parental. mRNA levels of *PTCH1* were significantly higher (2.1-fold, $P < 0.05$) in SKOV3TRip2 compared with parental SKOV3ip1 cells; however, no significant changes in *SMO* expression were observed between chemoresistant and chemosensitive cell lines (Fig. 1B). Protein expression of Smo was confirmed in all cell lines tested and did not always correlate with expression at the mRNA

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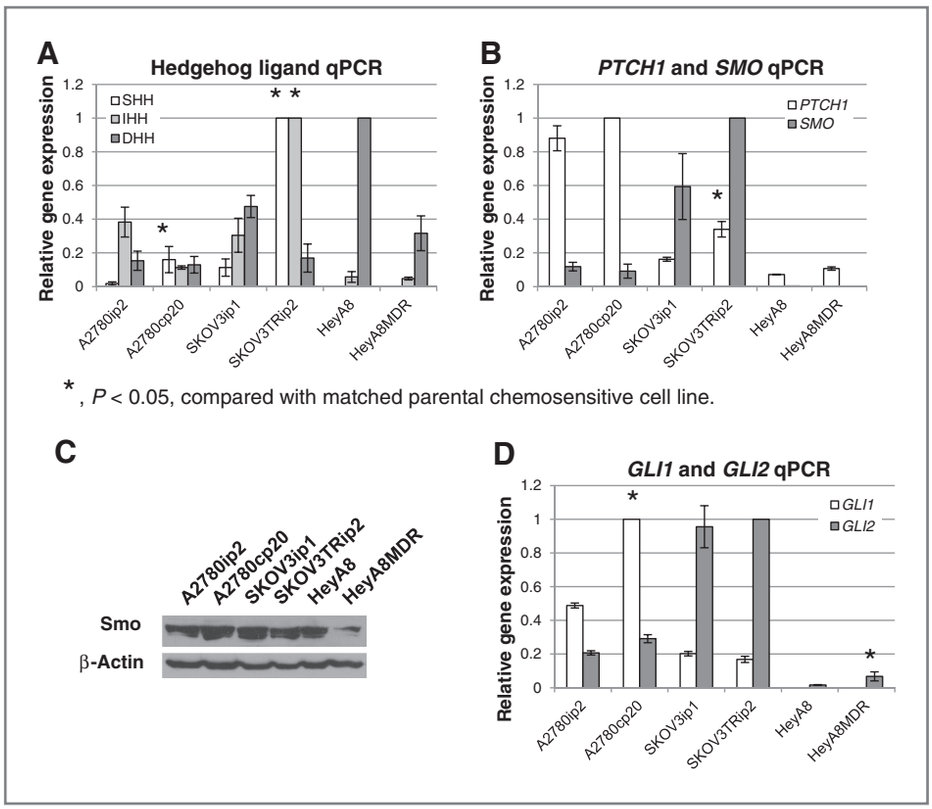

Figure 1. Expression of hedgehog signaling components in chemosensitive and chemoresistant ovarian cancer cell lines. Gene expression was calculated relative to the sample/cell line with the highest expression of a particular gene. A, mRNA expression of hedgehog ligands, Sonic (*SHH*), Indian (*IHH*), and Desert (*DHH*). HH, hedgehog. B, mRNA expression of hedgehog receptors, *PTCH1* and *SMO*. C, protein expression of Smo was also measured using Western blot analysis. β-Actin was used as a loading control. D, mRNA expression of hedgehog transcription factors, *GLI1* and *GLI2*. Data are representative of 3 independent experiments. *, $P < 0.05$, compared with parental chemosensitive cell line.

level (Fig. 1C). *GLI1* mRNA expression was significantly higher (2.0-fold, $P < 0.05$) in A2780cp20 compared with parental A2780ip2 cells and *GLI2* mRNA expression was significantly higher (4.1-fold, $P < 0.05$) in HeyA8MDR compared with parental HeyA8 cells, although at very low levels in both (Fig. 1D). These results show that hedgehog signaling is often higher in chemoresistant matched ovarian cancer cell lines.

Smo antagonists diminish cell viability and hedgehog gene expression in ovarian cancer cell lines

Having observed Smo expression (both mRNA and protein) in both chemosensitive and chemoresistant ovarian cancer cell lines, we next examined response to the Smo antagonists cyclopamine and LDE225 among these cell lines. The chemical structure of LDE225 is shown in Fig. 2A. As shown in Table 1, cyclopamine IC_{50}s varied from 7.5 μmol/L (A2780ip2) to 19 μmol/L (SKOV3TRip2) and LDE225 IC_{50}s varied from 7.5 μmol/L (A2780cp20) to 24 μmol/L (SKOV3ip1). Interestingly, chemoresistant cell lines were more sensitive (up to 2.25-fold, $P < 0.05$) to LDE225 compared with their chemosensitive counterparts. Chemoresistant cell lines were also more sensitive to LDE225 than cyclopamine. To confirm that decreased cell viability was associated with diminished hedgehog pathway activity, A2780cp20 cells were exposed to increasing concentrations of LDE225 (1, 5, and 10 μmol/L) for 72 hours and gene expression of hedgehog target genes *PTCH1*, *GLI1*, and *GLI2* was analyzed by qPCR. A dose-dependent decrease in the expression of all 3 genes was observed with a maximum reduction of 39%, 43%, and 32% ($P < 0.05$), respectively, after exposure to 10 μmol/L LDE225 (Fig. 2B). Protein expression of the hedgehog transcriptional activator Gli1 was also reduced in a dose-dependent manner after LDE225 treatment (Fig. 2C). Taken together, these data show the efficacy and hedgehog-specific activity of LDE225 in multiple chemoresistant cell lines.

Smo antagonism reverses taxane resistance in chemoresistant ovarian cancer cell lines both *in vitro* and *in vivo*

Having observed increased expression of hedgehog signaling components and response to Smo antagonists in chemoresistant ovarian cancer cell lines, we sought to determine whether targeting the hedgehog pathway could increase sensitivity to carboplatin and paclitaxel, chemotherapy agents most commonly used in the treatment of ovarian cancer. Neither cyclopamine nor LDE225 affected response to carboplatin among the chemoresistant cell lines examined (data not shown). However, as shown in Table 1, both Smo antagonists significantly increased the sensitivity of all 3 chemoresistant cell lines to paclitaxel (by up to 27- and 20-fold, respectively; $P < 0.05$). Increased sensitivity to paclitaxel after combination with cyclopamine or LDE225 even occurred at low

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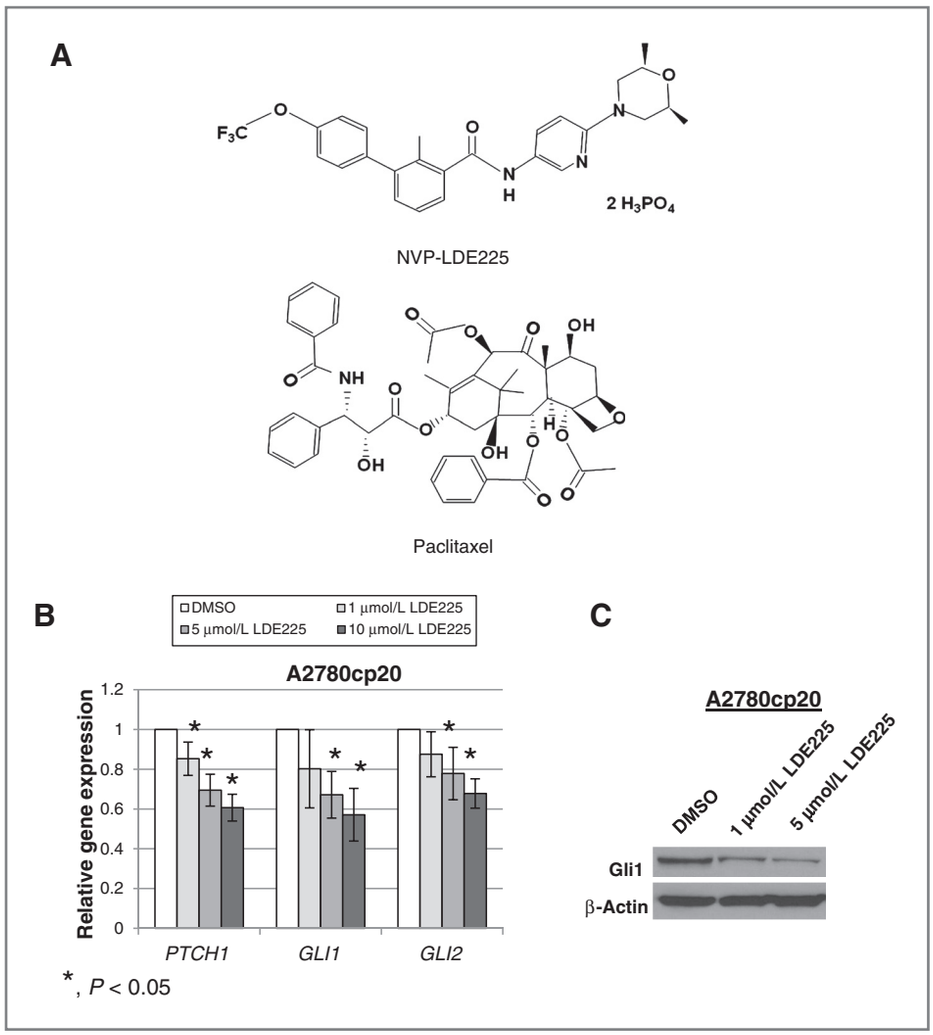

Figure 2. LDE225 reduces hedgehog pathway activity in chemoresistant ovarian cancer cells. A, chemical structures of NVP-LDE225 and paclitaxel. B, gene expression of *PTCH1*, *GLI1*, and *GLI2* was examined in A2780cp20 cells after exposure to increasing concentrations of LDE225 using qPCR. *, $P < 0.05$, compared with DMSO vehicle control. C, protein expression of Gli1 in A2780cp20 cells after exposure to increasing concentrations of LDE225 was measured using Western blot analysis to confirm mRNA results. β-Actin was used as a loading control. Data are representative of 3 independent experiments.

doses that were not effective alone (5 μmol/L cyclopamine, Fig. 3A and 1 μmol/L LDE225, Fig. 3B). To determine the mechanism by which Smo antagonism combined with paclitaxel affects cell growth, we carried out cell-cycle analysis on A2780cp20 cells that were treated with DMSO alone (vehicle control), paclitaxel alone (30 nmol/L), LDE225 alone (5 μmol/L), or combined paclitaxel and LDE225 for 72 hours. As shown in Fig. 3C, combination treatment resulted in a greater accumulation of cells in the sub-G_0/apoptotic, S-, and G_2–M

Table 1. Ovarian cancer cell line response to Smo antagonists, alone and in combination with paclitaxel

	Mean IC_{50}, μmol/L		Mean paclitaxel IC_{50}, nmol/L			
Cell line	**Cyclopamine**	**LDE225**	**Control**	**w/Cyclopamine (5 μmol/L)**	**w/LDE225 (5 μmol/L)**	***P***
A2780ip2	7.5	12	4	1.5	2.6	NS
A2780cp20	10	7.5	30	1.3	1.5	<0.05
SKOV3ip1	14	24	6	3	5.5	NS
SKOV3TRip2	19	12	400	15	120	<0.05
HeyA8	12	18	7	4.2	6.5	NS
HeyA8MDR	13	8	650	50	115	<0.05

Abbreviation: NS, not significant.

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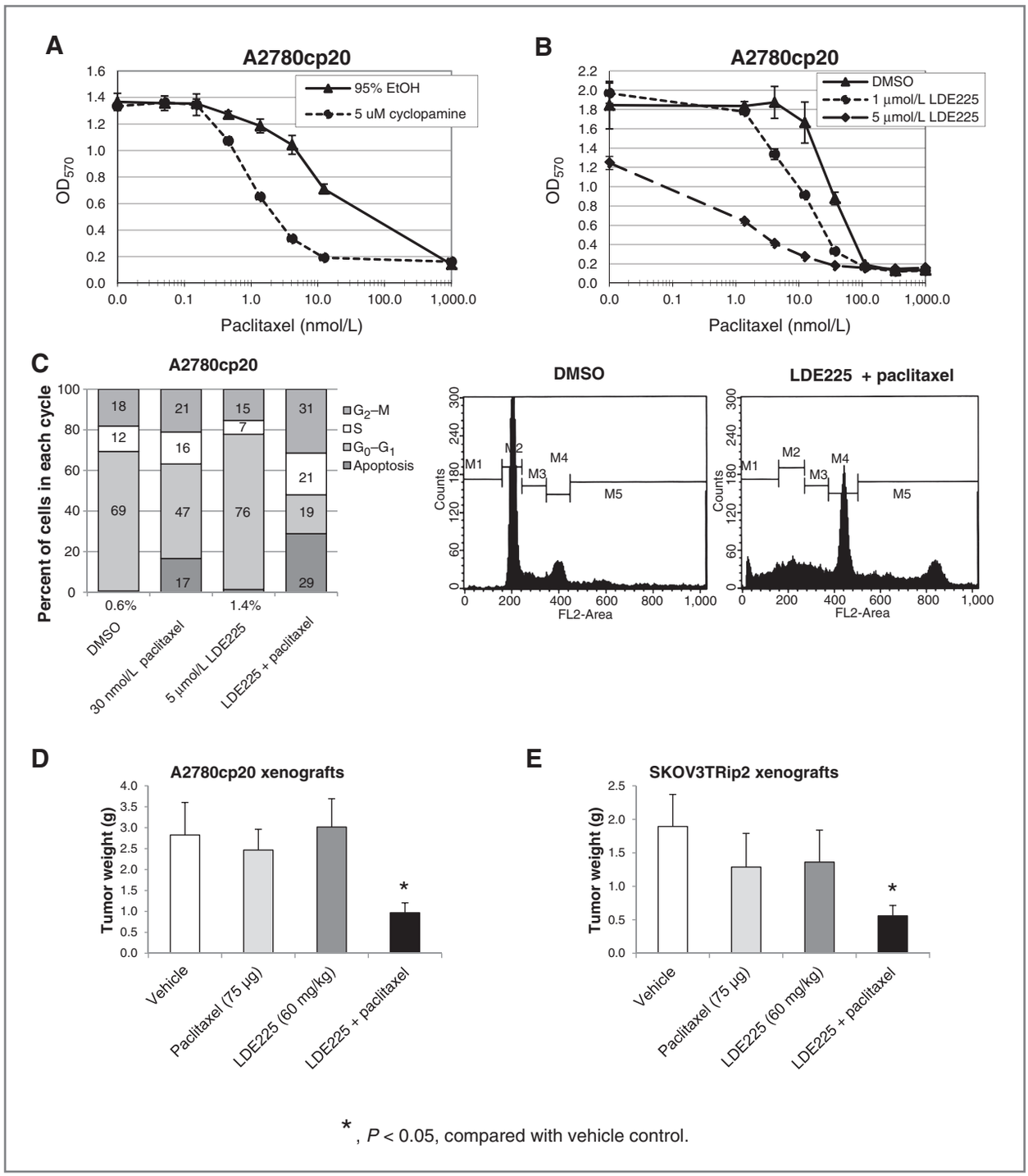

Figure 3. Smo antagonism reverses taxane resistance in chemoresistant ovarian cancer cell lines both *in vitro* and *in vivo*. A, A2780cp20 cells were exposed to either 95% ethanol (EtOH, vehicle control) or cyclopamine (5 μmol/L) in combination with increasing concentrations of paclitaxel. Cell viability was determined by MTT assay. B, A2780cp20 cells were exposed to either DMSO (vehicle control) or LDE225 (1 and 5 μmol/L) in combination with increasing concentrations of paclitaxel. Cell viability was determined by MTT assay. C, cell-cycle analysis was conducted on A2780cp20 cells treated with DMSO alone, paclitaxel alone, LDE225 alone, or combined paclitaxel and LDE225 using propidium iodide staining. Representative histograms of DMSO- and combination-treated cells are shown on the right. Data are representative of 3 independent experiments. D, mice injected intraperitoneally with A2780cp20 cells were treated with vehicle alone, paclitaxel alone, LDE225 alone, or combined paclitaxel + LDE225. E, mice injected intraperitoneally with SKOV3TRip2 cells were treated with either vehicle alone, paclitaxel alone, LDE225 alone, or combined paclitaxel + LDE225. For both xenograft models, mice treated with the combination paclitaxel + LDE225 showed a significant reduction in tumor weight compared with treatment with vehicle alone. Mean tumor weights with standard error are presented. *, $P < 0.05$, compared with vehicle control.

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phases compared with control or either treatment alone. These data suggest that LDE225 enhances cell-cycle arrest and cell death induced by the microtubule-stabilizing effects of paclitaxel.

To determine if LDE225 can similarly reverse taxane resistance *in vivo*, an orthotopic mouse model using chemoresistant cell lines was used. Nude mice were injected intraperitoneally with either A2780cp20 or SKOV3TRip2 cells and randomized to 4 treatment groups: (a) vehicle alone, (b) paclitaxel alone (75 μg weekly), (c) LDE225 alone (60 mg/kg daily), or (d) combined paclitaxel and LDE225. When control mice started to become moribund with tumor burden, all mice were sacrificed and total tumor weights recorded. In the A2780cp20 model (Fig. 3D), there was no significant reduction in tumor growth with either paclitaxel or LDE225 alone. However, the combination of paclitaxel and LDE225 resulted in significantly reduced tumor weight, by 65.7% compared with vehicle alone ($P = 0.028$). This represented a 60.7% reduction compared with paclitaxel alone ($P = 0.014$) and a 68% reduction compared with LDE225 alone ($P = 0.010$), again showing synergy of paclitaxel and LDE225. Similar results were observed in SKOV3TRip2 xenografts (Fig. 3E). Neither paclitaxel nor LDE225 alone had a statistically significant impact on tumor growth, whereas combination treatment significantly reduced tumor weight, by 70.4% compared with vehicle alone ($P = 0.015$). This represented a 56.6% reduction compared with paclitaxel alone ($P = 0.18$) and a 58.8% reduction compared with LDE225 alone ($P = 0.13$), although neither was statistically significant.

LDE225 sensitizes chemoresistant ovarian cancer cells to paclitaxel by downregulating MDR1 expression and sensitizes both ALDH-negative and -positive ovarian cancer cells to paclitaxel

The primary mediator of taxane resistance in general, and in the chemoresistant cell lines examined in this study (27), is the expression of the drug efflux protein, P-glycoprotein (ABCB1/MDR1). To identify the mechanism underlying taxane sensitization after Smo antagonism, we next examined whether LDE225 could modulate *MDR1* gene expression. In A2780cp20 cells exposed to LDE225 alone, paclitaxel alone, and combined LDE225 + paclitaxel for 72 hours, it was observed that LDE225 decreased *MDR1* expression (by up to 49.2%, $P < 0.05$), whereas paclitaxel actually led to a compensatory *increase* in *MDR1* expression (2.88-fold, $P < 0.05$) compared with vehicle control (Fig. 4A). This compensatory increase in *MDR1* was alleviated by LDE225 in a dose-dependent manner (up to a 59.9% decrease, $P < 0.05$), showing that this compound increases sensitivity to paclitaxel, at least in part, by downregulating *MDR1*. Similar results were observed in SKOV3TRip2 cells (Fig. 4B); LDE225 decreased *MDR1* expression both alone (by up to 36.4%, $P < 0.05$ compared with vehicle control) and in combination with paclitaxel (by up to 50.8%, $P < 0.05$ compared with paclitaxel alone). In this cell line, a compensatory increase in *MDR1* was not observed with paclitaxel alone, likely because *MDR1* is already expressed at extremely high levels (140-fold more than in A2780cp20) in this 1,000-fold taxane-resistant cell line (27). To determine if similar modulation of *MDR1* occurs *in vivo*, RNA isolated from A2780cp20 tumors (from Fig. 3D) was examined. In agreement with the *in vitro* data, LDE225 alone significantly reduced *MDR1* expression (by 35.2%, $P < 0.05$) and paclitaxel alone significantly increased *MDR1* expression (2.55-fold, $P < 0.05$) compared with vehicle control (Fig. 4C). In addition, combination treatment significantly reduced *MDR1* expression compared with paclitaxel alone (by 48.8%, $P < 0.05$), blunting this compensatory rise.

In addition to our examination of MDR1 expression after LDE225 treatment, we also examined βIII-tubulin and stathmin, proteins that have been associated with microtubule regulation and resistance to taxanes (28). It was found that neither of these proteins was affected by LDE225 treatment *in vitro* (as determined by Western blot analysis, data not shown). Taken together, these data support a mechanism whereby LDE225 causes the downregulation of *MDR1* expression, which then leads to increased uptake of paclitaxel within chemoresistant cells, rather than potentiating the microtubule stabilizing effect of this compound.

We have previously shown that ALDH activity is associated with enhanced tumorigenicity and chemoresistance in ovarian cancer, and may define one of potentially many cancer cell populations with stem cell-like features (27, 29). To determine whether cancer stem cells (CSC) might play a role in taxane sensitization after LDE225 treatment, we collected ALDH-negative and -positive cell populations from the SKOV3TRip2 cell line, and exposed them to combined LDE225 and paclitaxel. As shown in Fig. 4D, it was found that ALDH-negative and -positive SKOV3TRip2 cells showed a similar decrease in viability after LDE225 treatment alone (21.4% vs. 16.8%, respectively), compared with DMSO control. In addition, sensitivity to paclitaxel (as determined by IC_{50}) was similarly increased after combination treatment in ALDH-negative and -positive cells (5.1-fold vs. 4.0-fold change in IC_{50}, respectively). These results indicate that the more tumorigenic ALDH-positive cells are just as susceptible to LDE225 treatment as ALDH-negative cells, and that hedgehog inhibition can sensitize both populations to taxane therapy. Whether other putative CSC populations such as CD133, CD44, and the side population, with which there is some (but not complete) crossover with the ALDH population (30), can also be sensitized to taxanes will be the subject of future investigations.

Knockdown of Smo diminishes hedgehog pathway activity, reduces viability, and reverses taxane resistance in ovarian cancer cells

To determine whether LDE225 reverses taxane resistance through inhibition of Smo alone or off-target effects, we selectively targeted hedgehog pathway members

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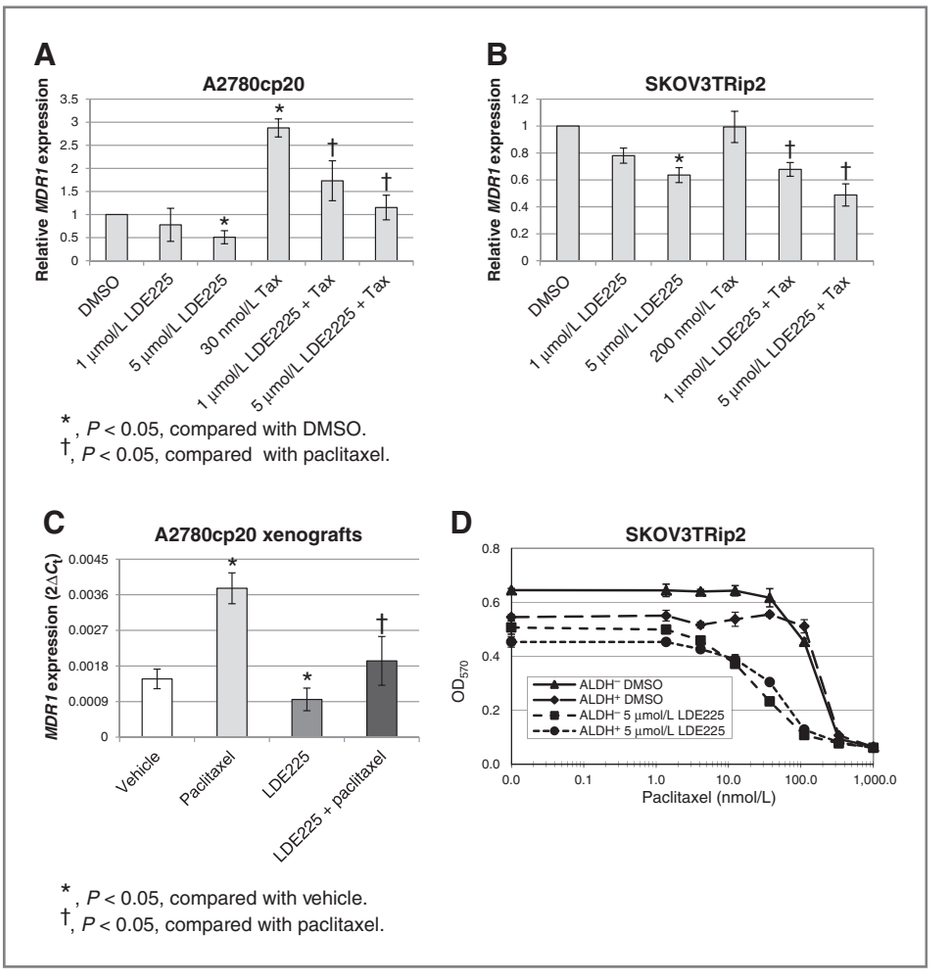

Figure 4. LDE225 sensitizes chemoresistant ovarian cancer cells to paclitaxel by downregulating MDR1 expression and sensitizes both ALDH-negative and -positive ovarian cancer cells to paclitaxel. A, A2780cp20 cells were exposed to DMSO, LDE225 (1 or 5 μmol/L), paclitaxel (Tax, 30 nmol/L), or combined LDE225 + paclitaxel for 72 hours and examined for *MDR1* gene expression. *, $P < 0.05$, compared with DMSO; †, $P < 0.05$, compared with paclitaxel alone. B, SKOV3TRip2 cells were exposed to DMSO, LDE225 (1 or 5 μmol/L), paclitaxel (Tax, 200 nmol/L), or combined LDE225 + paclitaxel for 72 hours and examined for *MDR1* gene expression. *, $P < 0.05$, compared with DMSO; †, $P < 0.05$, compared with paclitaxel alone. Data are representative of 3 independent experiments. C, A2780cp20 xenografts ($n = 5$ per group) treated with vehicle alone, paclitaxel alone, LDE225 alone, or combined LDE225 + paclitaxel were resected after 4 weeks of therapy and examined for *MDR1* gene expression. Mean expression with SE are presented. *, $P < 0.05$, compared with vehicle; †, $P < 0.05$, compared with paclitaxel alone. D, SKOV3TRip2 cells were sorted into aldehyde dehydrogenase–negative (ALDH−) and -positive (ALDH+) populations, using the ALDEFLUOR assay, and then exposed to either DMSO or 5 μmol/L LDE225, both alone and in combination with increasing concentrations of paclitaxel. Cell viability was determined by MTT assay.

using siRNAs and observed effects on hedgehog pathway activity and paclitaxel response. As shown in Fig. 5A, knockdown of Smo was achieved both at the mRNA and protein level. As expected, this downregulation led to a significant decrease in hedgehog target genes *PTCH1* (66.6%, $P < 0.01$), *GLI1* (86.5%, $P < 0.01$), and *GLI2* (62.0%, $P < 0.01$). Individual knockdown of hedgehog mediators Smo, Gli1, or Gli2 using 2 distinct siRNA constructs for each gene led to increased sensitivity to paclitaxel (Fig. 5B–D). In particular, Smo knockdown decreased paclitaxel IC_{50} by up to 11.7-fold; Gli1 knockdown, up to 3.5-fold; and Gli2 knockdown, up to 5.9-fold. In agreement with cyclopamine and LDE225 biologic effects, knockdown of Smo, Gli1, or Gli2 alone significantly decreased cell viability (by up to 73.5%, 57.6%, and 26.5%, respectively, $P < 0.01$) compared with control siRNA. Collectively, these data suggest that hedgehog signaling promotes ovarian cancer cell survival and mediates taxane resistance.

Discussion

In this study, we found that hedgehog pathway signaling components are overexpressed in chemoresistant ovarian cancer cells. Moreover, targeting the hedgehog pathway decreased ovarian cancer cell viability and sensitized chemoresistant ovarian cancer cells to paclitaxel therapy through decreased *MDR1* expression. The participation of hedgehog signaling in ovarian cancer cell survival and chemotherapy resistance makes it an

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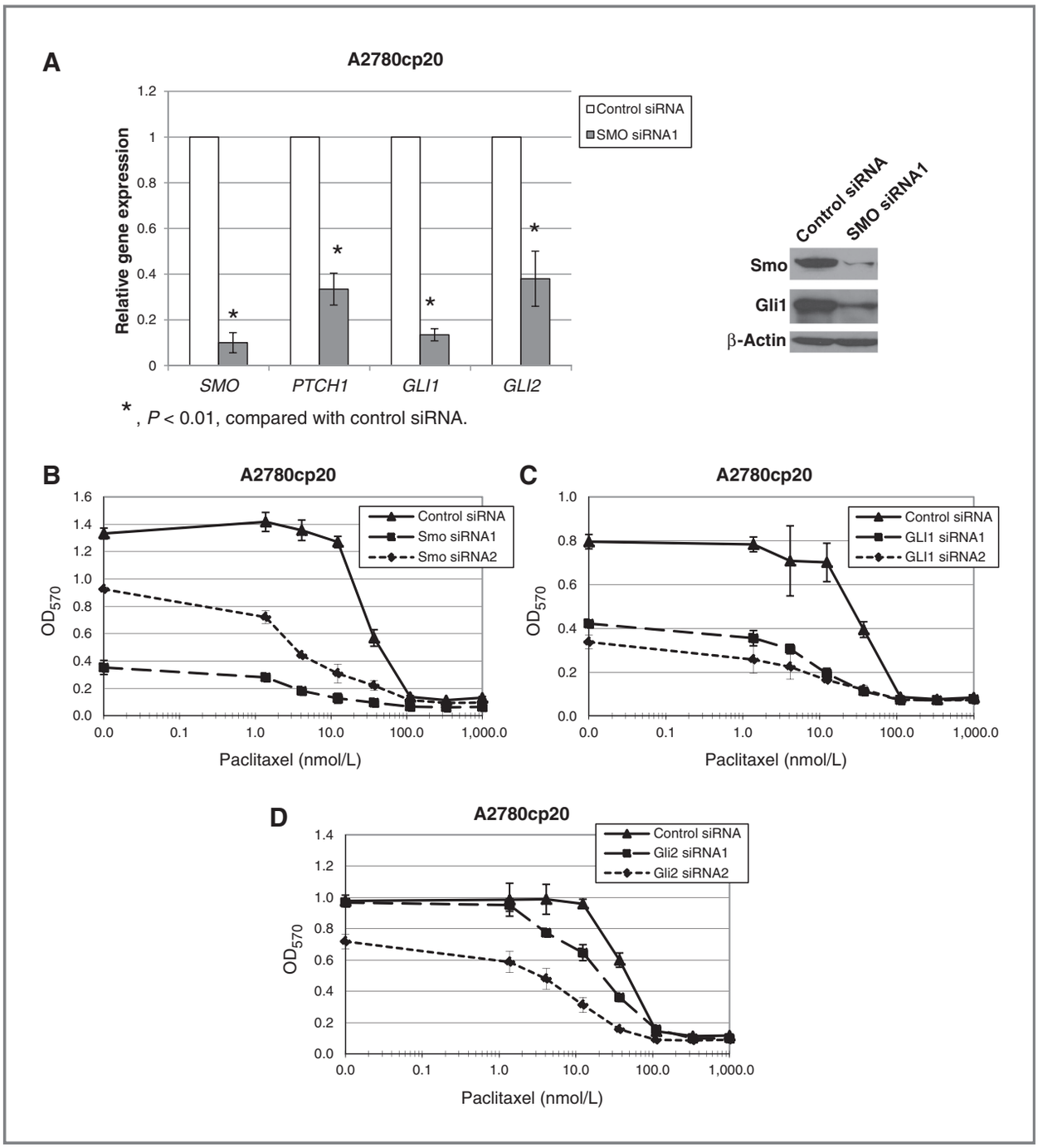

Figure 5. Knockdown of Smo diminishes hedgehog pathway activity, reduces viability, and reverses taxane resistance in ovarian cancer cells. A, A2780cp20 cells were exposed to either control or Smo siRNA for 72 hours and examined for mRNA expression of hedgehog pathway mediators *SMO*, *PTCH1*, *GLI1*, and *GLI2*. *, $P < 0.01$, compared with control siRNA. Protein expression of Smo and Gli (inset) was also measured using Western blot analysis to confirm mRNA results. β-Actin was used as a loading control. A2780cp20 cells were transfected with either control siRNA or 2 distinct siRNA constructs designed against Smo (B), Gli1 (C), or Gli2 (D) and exposed to increasing concentrations of paclitaxel. Cell viability was determined by MTT assay. Data are representative of 3 independent experiments.

attractive target for therapy, especially because most patients with ovarian cancer develop tumor recurrence and succumb to chemoresistant disease.

Currently, it has not been shown what role hedgehog signaling might play in mediating ovarian cancer chemoresistance, a persistent obstacle in the treatment of this disease. Although the clinical behavior of ovarian cancer suggests that most cancer cells are initially sensitive to chemotherapy, they subsequently either develop resistance or contain a population of cells that are inherently

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resistant. The latter hypothesis is consistent with what has become known as tumor initiating cells or CSCs. These CSCs are commonly believed to have enhanced tumorigenicity, differentiation capacity, and resistance to chemotherapy in comparison with non-CSCs. It is because of these features that CSCs have been examined for molecular pathways and markers that could be targeted for therapeutic purposes. Recent studies have suggested that developmental pathways, including hedgehog, play important roles in the maintenance of CSCs (10/11) and that inhibiting these pathways may provide enhanced chemosensitivity when combined with traditional chemotherapies. In our study, we sought to define a role for hedgehog signaling in ovarian cancer chemoresistance. Both *in vitro* and *in vivo*, we observed significant sensitization to paclitaxel after Smo antagonism (LDE225) in taxane-resistant ovarian cancer cells. This sensitization was also present in ALDH-positive cells, a subpopulation of cancer cells with enhanced tumorigenicity and chemoresistance. The mechanism underlying this sensitization seems to involve downregulation of P-glycoprotein (ABCB1/MDR1), a well-characterized mediator of multidrug resistance. By downregulating *MDR1* expression, uptake of paclitaxel by cancer cells would be increased, resulting in a greater response to the chemotherapeutic agent. This mechanism would explain why Smo antagonists did not sensitize chemoresistant cells to carboplatin, because this compound is not a substrate for the P-glycoprotein drug efflux pump. In addition, this model of hedgehog inhibition and chemosensitization agrees with a previous study done by Sims-Mourtada and colleagues, in which it was showed that cyclopamine sensitized prostate cancer cells to a variety of chemotherapy agents *in vitro* (including the taxane docetaxel), through modulation of MDR1 expression (12). The observation that Smo antagonism did not sensitize cells to platinum therapy highlights the specificity of this effect.

Previous studies have showed aberrant expression of the hedgehog pathway in primary specimens of ovarian cancer compared with normal ovarian epithelium (7–9), including a study that found elevated Gli1 expression is associated with decreased survival (9). These studies have also showed decreased ovarian cancer cell growth/viability after treatment with the Smo antagonist cyclopamine, results that our study supports. We have previously shown that *GLI1* and *GLI2* mRNA levels were significantly higher in cancer cells isolated from persistent/chemoresistant tumors compared with those isolated from matched primary tumors (29). Smo expression was also increased (3.7-fold) in persistent tumors; however, this increase was not statistically significant. Patients from whom persistent tumors were obtained had failed both taxane and platinum chemotherapies, making it difficult to determine whether this increase in hedgehog pathway genes is a taxane-specific effect. The *in vitro* data presented in this study, however, would suggest that Smo, as well as Gli1 and Gli2, are associated with taxane resistance. In our initial experiments examining the effects of targeting hedgehog alone, either with Smo antagonists or RNAi, ovarian cancer cell viability was significantly decreased *in vitro*, indicating that the hedgehog pathway is important for ovarian cancer survival. However, this effect did not seem to translate to our xenograft models, in which the Smo antagonist LDE225 had no significant impact on tumor growth when used alone, even in models with relatively high Gli1 expression. These findings suggest that survival pathways are activated in the murine tumor microenvironment that allows resistance to hedgehog antagonist monotherapy. Given the recognized importance of crosstalk between the tumor stromal cells and malignant cells in the hedgehog pathway (6), and the failure of this model to target both murine and human compartments, more efficacy may be noted with monotherapy in humans.

Collectively, the data presented in this study show that increased expression of hedgehog signaling components is associated with taxane resistance, which can be overcome by targeting multiple effectors of the hedgehog signaling pathway. With the ability to identify subsets of patients with cancer with hedgehog pathway overexpression, antagonism of hedgehog signaling in combination with taxane therapy could ultimately provide a useful therapeutic strategy for recurrent, chemoresistant ovarian cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A.D. Steg, C.N. Landen
Development of methodology: A.D. Steg, C.N. Landen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.D. Steg, A.A. Katre, K.S. Bevis, A. Ziebarth, Z. C. Dobbin, M.M. Shah, C.N. Landen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.D. Steg, C.N. Landen
Writing, review, and/or revision of the manuscript: A.D. Steg, A. Ziebarth, R.D. Alvarez, C.N. Landen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.A. Katre, Z.C. Dobbin, C.N. Landen
Study supervision: C.N. Landen

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