The Role of Notch and Gamma-secretase Inhibition in an Ovarian Cancer Model

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Abstract. *Background: The Notch pathway is dysregulated in ovarian cancer. We sought to examine the role of Notch and gamma-secretase (GS) inhibition in ovarian cancer. Materials and Methods: Established ovarian cancer cell lines were used. Quantitative polymerase chain reaction (qPCR) was used to determine the relative expression of Notch receptor and ligands. Effects of GS inhibition on proliferation, colony formation, and downstream effectors were examined via methylthiazole tetrazolium (MTT) and Matrigel assays, and qPCR, respectively. In vivo experiments with a GS inhibitor and cisplatin were conducted on nude mice. Tumors were examined for differences in microvessel density, proliferation, and apoptosis. Results: Notch3 was the most up-regulated receptor. The ligands JAGGED1 and DELTA-LIKE4 were both up-regulated. GS inhibition did not affect cellular proliferation or anchorage-independent cell growth over placebo. The GS inhibitor Compound-E reduced microvessel density in vivo. Conclusion: GS inhibition does not directly affect cellular proliferation in ovarian carcinoma, but Notch pathway blockade may result in angiogenic alterations that may be therapeutically important.*

Ovarian cancer is the most lethal gynecological malignancy in the United States (1). Symptoms of ovarian cancer are subtle and are often missed, resulting in a high percentage of women being diagnosed with advanced-stage disease. While initially chemotherapy-responsive, disease in many women develops resistance to traditional cytotoxic therapy.

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Key Words: Ovarian cancer, Notch, gamma-secretase inhibitor, model.

The Notch pathway functions in cell fate determination and has been implicated in a number of pathological conditions (2). There are four Notch receptors (Notch 1-4) and five ligands (JAGGED1 [JAG1], JAGGED2, DELTA-LIKE [DLL] 1, 3 and 4) that have been identified in mammals (3). The receptor-ligand interaction at the cell membrane precipitates cleavage of the intracellular domain (ICD) by the gamma secretase (GS) enzyme complex (3). The Notch ICD translocates to the nucleus, binds to the transcription factor *CBF1*, *Su*(H), *Lag*-1 (CSL), and promotes expression of a number of downstream target genes. The Notch pathway is dysregulated in leukemia, lymphoma, and a variety of solid tumors, making it an attractive candidate for therapeutic intervention (4-6). Recently, The Cancer Genome Atlas Research Network published their results analyzing the most common molecular abnormalities found in a panel of 489 cases of high-grade serous ovarian cancers (7). Notably, the Notch pathway was dysregulated in 22% of the samples, further underscoring the importance of targeting this pathway in ovarian cancer. A number of investigations have also suggested that Notch signaling may play a role in ovarian carcinogenesis (8-11).

Given the potential role of Notch signaling in ovarian cancer, the pathway may be an important therapeutic target. The goal of our analysis was to determine the role of Notch signaling in ovarian cancer. More specifically, we characterized the expression of the Notch receptors and ligands in ovarian cancer cell lines and performed *in vitro* and *in vivo* experiments to determine the effect of gamma-secretase inhibition on ovarian cancer progression.

Materials and Methods

Cell culture. Seven ovarian cancer cells lines were obtained commercially (SKOV3, OVCAR3, TOV 112D, TOV 21G, OV90, CaOV3, OVCAR8) from the American Type Culture Collection (ATCC, Manassas, VA, USA). Immortalized normal ovarian surface epithelium (IOSE 364, immortalized with SV40 large T-antigen)

was a kind gift from the Canadian Cancer Bank (Vancouver, British Columbia, Canada). The commercially-obtained lines were plated in the ATCC-recommended media supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco). IOSE 364 was maintained in MCDB 105:199 supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100μg/ml streptomycin. All lines were maintained in a humidified incubator at 37˚C in 5% CO_2.

qPCR. Relative mRNA expression of the Notch receptors 1-3, the ligands JAG1 and DLL- 4, and the downstream targets HES1 and HEY1 was measured *via* qPCR using an Applied Biosystem (Carlsbad, CA, USA) 7300 RT-PCR system. Threshold cycle numbers (Ct) were obtained *via* the 7300 SDS software. Commercially available PCR primer-probes were obtained from Applied Biosystem for the above listed genes. The mean Ct of the genes of interest was calculated from triplicate measurements, then normalized using the mean Ct of the housekeeping gene human hypoxanthine ribosyltransferase (HPRT) (12). The Ct for each gene was further normalized to the respective Ct from IOSE 364.

Proliferation assays. Each cell line was plated at a density of 5×10^4 cells/ml in a 96-well plate and allowed to attach overnight. Four experimental treatments were examined: gamma secretase inhibitor (GSI) (compound-E) (Seoul, Korea), cisplatin (Sigma, St. Louis, MO, USA), compound-E plus cisplatin, and placebo. Compound-E was serially diluted from 1 μM to 25 nM in media. Cisplatin was serially diluted from 128 μM to 2 μM in media. The combination treatment arm employed serial dilutions of both compound-E and cisplatin from 1 μM to 25 nM and 128 μM to 4 μM, respectively. The placebo arm included corresponding volumes of DMSO (Sigma) (placebo for compound-E) and saline (placebo for cisplatin) in media. Each treatment arm had a negative control without the addition of drug or placebo. Treatments were added 24 h after plating. Plates were maintained at 37˚C in 5% CO_2 for 48 h. The media were then removed and replaced with media combined with CK-8 dye (Dojindo Molecular Technologies, Rockville, MD, USA) at a 1:10 concentration. The plates were incubated for 45 min, then the media was transferred to a clean 96-well plate and the absorbance read with a Bio-Rad (Philadelphia, PA, USA) microplate reader at 450nm. The mean absorbance was calculated from triplicate measurements. This same protocol was followed using N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) and dibenzazepine (DBZ) (both obtained from Tocris Bioscience, Minneapolis, MN, USA), two other commercially available GSIs.

Matrigel assay. Preliminary studies revealed that two lines, TOV 112D and TOV 21G, formed colonies in Matrigel (BD Biosciences, San Jose, CA, USA). These lines were plated in duplicate in Matrigel with 200 nM compound E or an equal volume of DMSO. Plates were incubated for 72 h at 37˚C in 5% CO_2. Subsequently, 200 μL of MTT dye were added to each well. Plates were incubated for 3 h, then colonies were counted with a Nikon inverted microscope (Melville, NY, USA).

Drug efficacy assay. A total of 9×10^5 cells of each cancer line were plated in a 10-cm tissue culture dish in duplicate. Cells were allowed to attach overnight; the media were then replaced in both plates. Compound-E at 200 nM was added to one plate and an equal volume of DMSO was added to the second plate. These were incubated at 37˚C in 5% CO_2 for 48 h, then harvested for RNA isolation. RNA was extracted from these cells and made into cDNA. qPCR was performed on these cDNA samples to assess efficacy of GSI-induced down-regulation Notch downstream targets *HES1* and *HEY1*.

In vivo experiment. 6-week-old nude female mice (average weight 20 g) were obtained from Taconic Farms (Germantown, NY, USA) and were allowed to acclimatize for one week. They were then injected with 10^6 OVCAR3 cells in the right flank. Tumors were allowed to develop for one week. Mice were then randomized into four groups of six mice each. Mice in group one received daily weight-based compound-E at 30 μmol/kg intraperitoneally. Compound-E was dissolved first in DMSO and then further diluted in 0.5% methylcellulose/0.1% Tween 80. Mice in group two received 160 μg cisplatin intraperitoneally once weekly. Mice in group three received both compound-E daily and cisplatin weekly as described above. Mice in group four received DMSO diluted in vehicle daily and saline weekly as placebo treatments. The total treatment time was four weeks, after which 100 μg of bromodeoxyuridine [BrdU] (Roche Applied Bioscience, Mannheim, Germany) was injected into all mice intraperitoneally one hour prior to sacrifice. After sacrifice, tumors were harvested from the flank and weighed, then submerged in 4% paraformaldehyde for preservation and paraffin embedding.

Immunohistochemistry. Immunohistochemistry (IHC) was performed on serial sections of the tumors. Cluster of differentiation-31 (CD31) staining was performed per standard protocol for paraffin embedded slides (CD31 antibody 1:200, Angio-Proteomie [Boston, MA, USA) (13). Slides were stained for BrdU (Roche Applied Bioscience) and terminal deoxynucleotidyl transferase mediated dUTP nick- end labeling (TUNEL) (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Slides were examined using a Nikon Eclipse E800 microscope and 10 representative images from each section were taken at 20× magnification with a Nikon digital camera (DXM 1200). Microvessel density was calculated by counting the number of vessels contained in each field, then averaging those values for each specimen. The same method was used to quantify the number of BrdU-positive and TUNEL-positive cells.

Statistical analysis. qPCR results for the Notch receptors and ligands are reported as relative expression levels compared to normal ovarian surface epithelium. All data are reported descriptively. Mean mouse weights, tumor weights, and results from IHC staining from the *in vivo* studies were compared across groups using Student's *t*-tests. A *p*-value of <0.05 was considered statistically significant.

Results

We utilized qPCR analysis to evaluate the expression of the Notch receptors and ligands in ovarian cancer lines (SKOV3, OVCAR 3, TOV 112D, CAOV3, OV90, SKOV3 IP, TOV 21G, OVCAR8) and compared that expression to the one found in IOSE 364. Notch3 was up-regulated in all of the ovarian cancer lines by at least a factor of 2 (OV90) and was up to 238-times greater (OVCAR3) than IOSE 364. Notch1

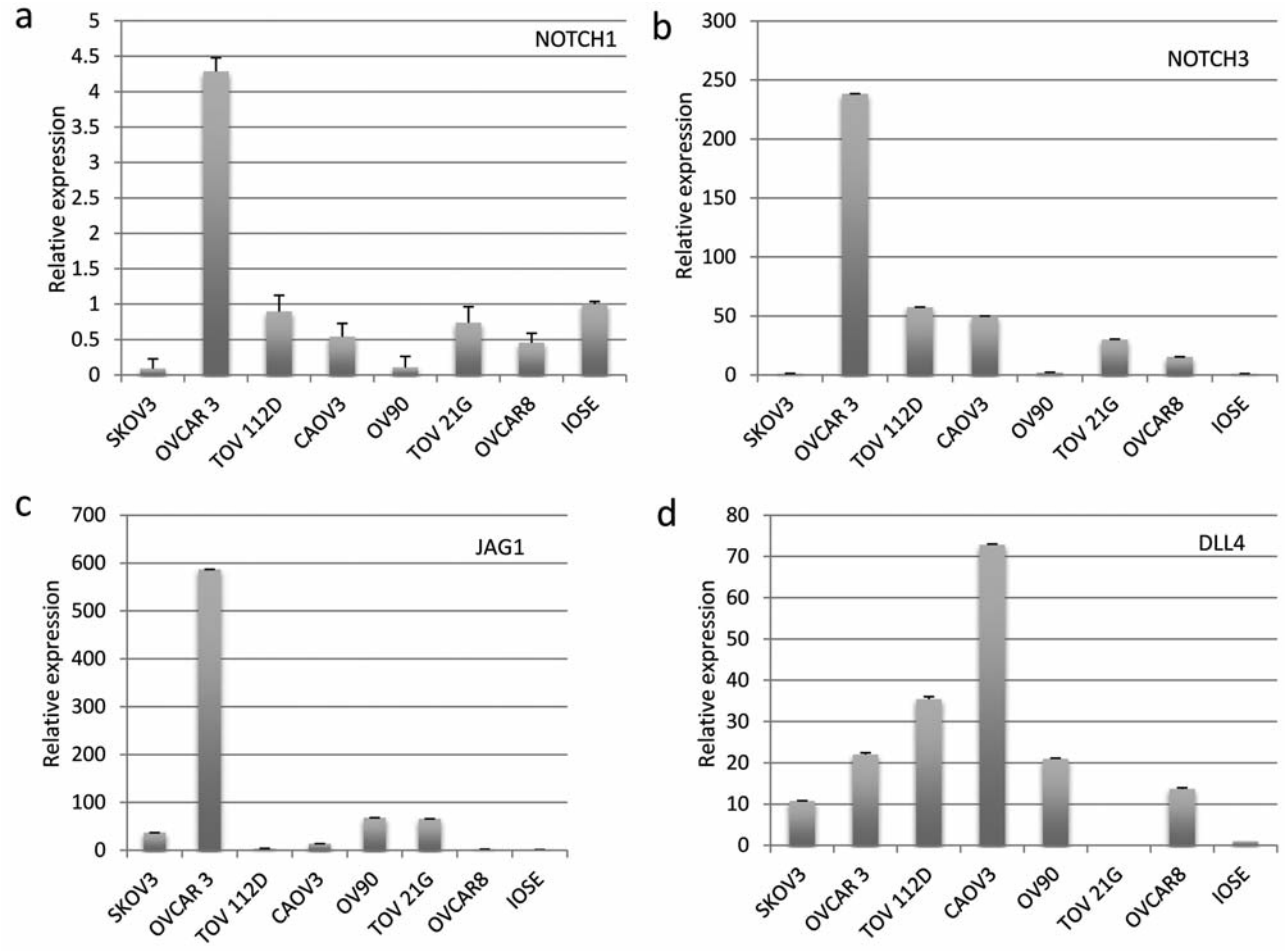

Figure 1. *Expression levels of different Notch genes in ovarian cancer cell lines normalized to the expression found in IOSE cells. a: Notch1, b: Notch3, c: JAG1, and d: DLL4. Notch3 is the most up-regulated receptor.*

and -2 were down-regulated, except for the OVCAR3 line, in comparison to IOSE 364. JAG1 expression was up to 587-times greater in ovarian cancer lines than IOSE 364, and DLL-4 expression was at least 13-times greater in the cancer lines as compared to IOSE 364 (Figure 1).

The effect of GSIs on cell proliferation was then examined. Cells were grown in the presence of different concentrations of compound-E, cisplatin, a combination of compound-E and cisplatin, or placebo. To ensure the activity of compound-E in inhibition of Notch signaling, we measured the relative expression levels of the downstream Notch targets *HES1* and *HEY1*. Cells treated with compound-E had significantly lower levels of *HES1* (p=0.04), suggesting that compound-E indeed inhibited Notch signaling (Figure 2a). HEY1 expression was lower although this did not reach significance (p=0.24). Notch inhibition with GSI had no apparent effect on cell proliferation (Figure 2b). Cells treated with compound E and cisplatin showed decreased proliferation that was similar to that noted with cisplatin-alone. The experiment was repeated with OVCAR 3, OVCAR 8 and SKOV3 using the GSIs DAPT and DBZ (Figure 2c). The results of treatment with DBZ and DAPT were similar to the ones obtained with compound-E, suggesting that a wide range of GSIs have little direct effect on cellular proliferation.

To determine the effect of Notch inhibition on anchorage-independent growth we examined the effect of GSI treatment on cells grown in suspension. TOV 112D and TOV 21G cells were grown in Matrigel with compound-E or placebo. TOV 21G and TOV 112D cells formed colonies in small numbers in the presence of both placebo and compound-E. This confirmed our findings that GSIs did not inhibit cell growth or colony formation *in vitro*.

The effect of Notch inhibition *in vivo* was then examined. A dose-finding toxicity experiment was conducted; results from that preliminary study suggested that 30 μmol/kg were

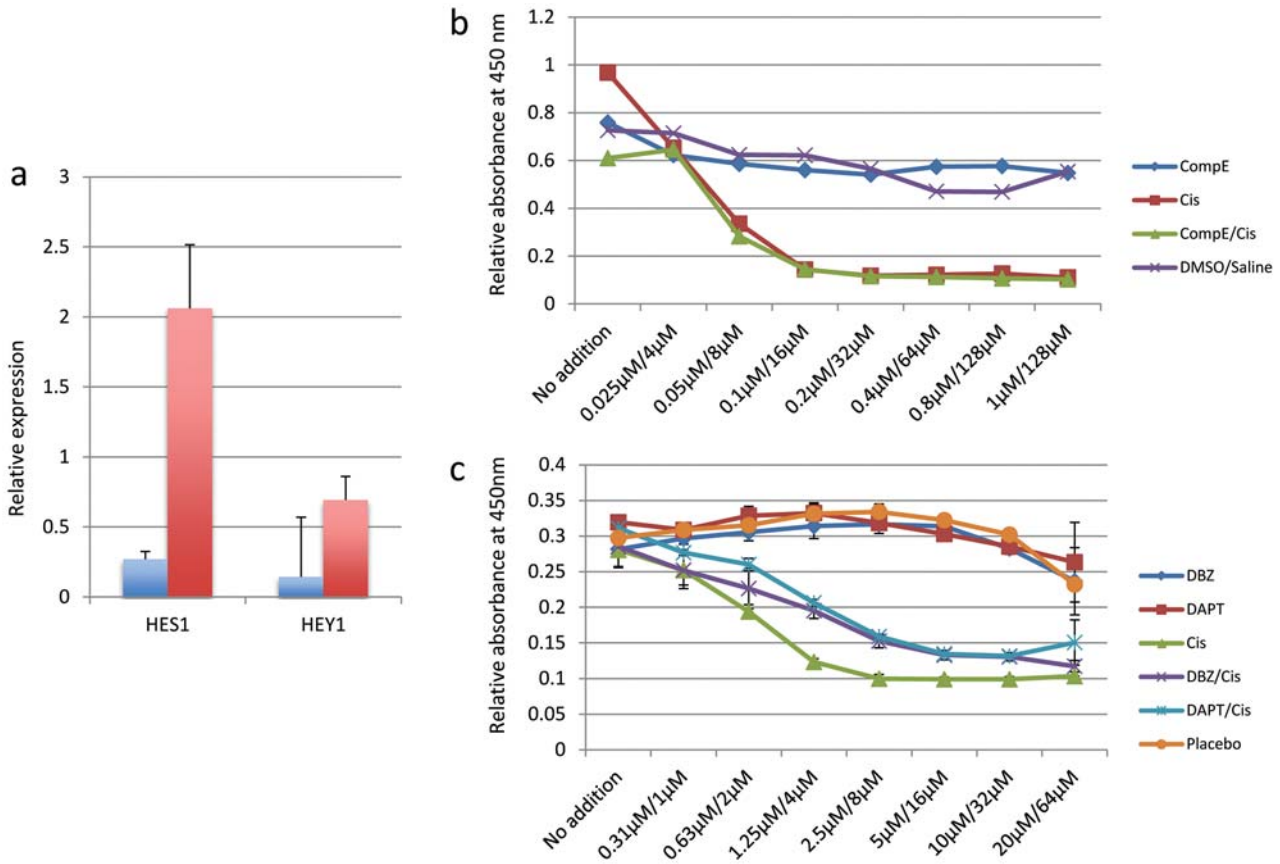

Figure 2. *a: Effect of compound-E on Hes1 and Hey1 expression via qPCR in OVCAR3 cells. b. Effect of compound-E on proliferation of OVCAR3 cells. c. Effect of DBZ and DAPT on proliferation of OVCAR3 cells. GSIs block HES1 and HEY1 expression but does not affect ovarian cancer cell proliferation.*

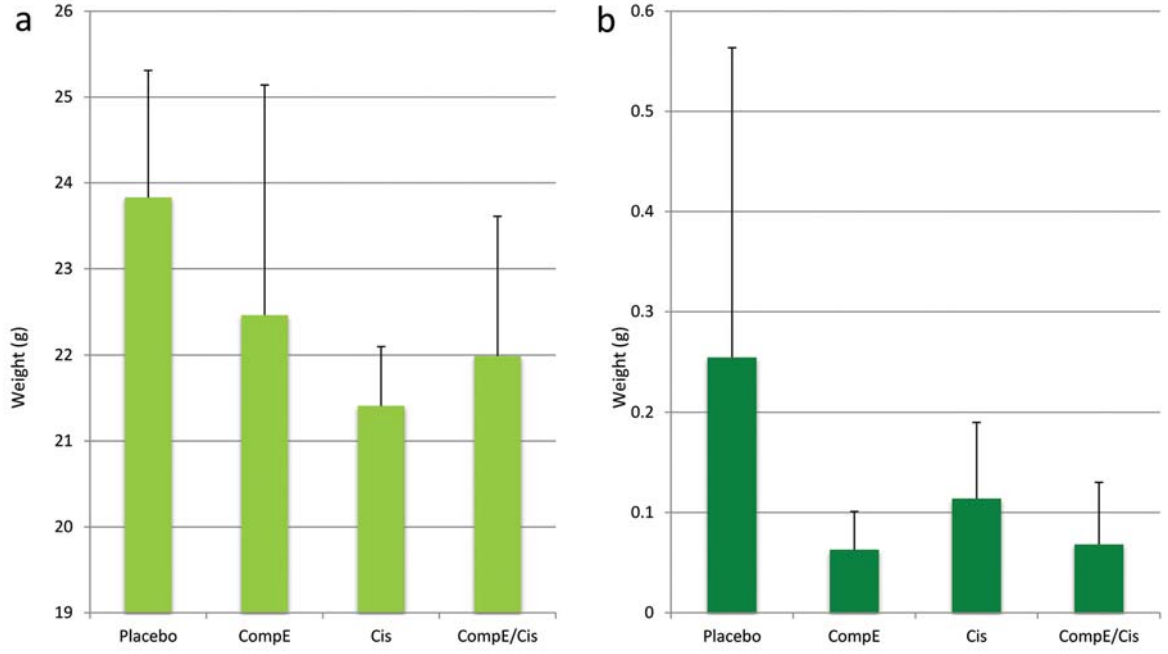

Figure 3. *Average weight of mouse (a) and tumor (b) at sacrifice in each group. Compound-E and cisplatin reduce growth of OVCAR3 ovarian cancer tumor xenografts.*

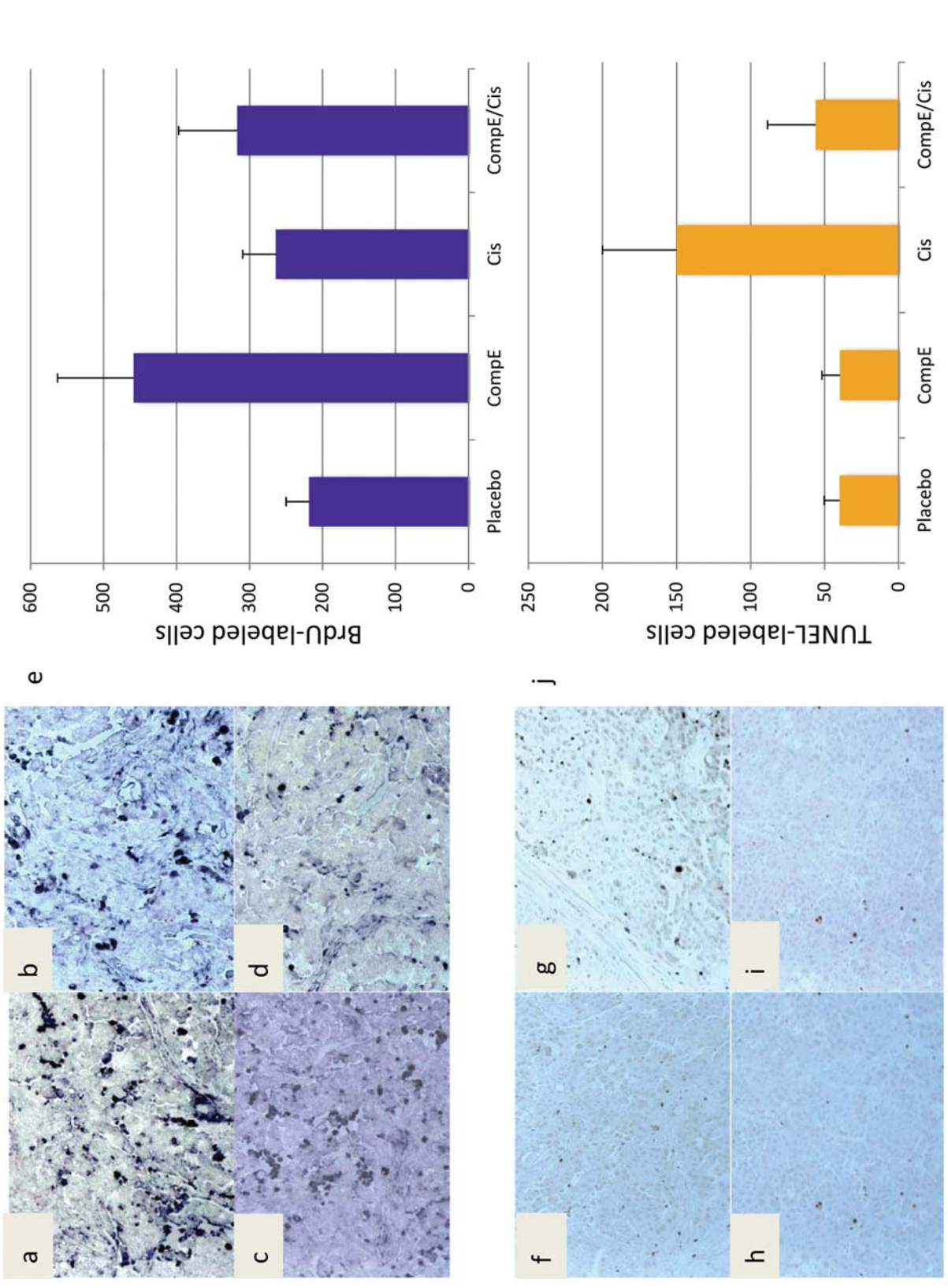

Figure 4. *In vivo examination of proliferation with BrdU staining (panels a-e), and apoptosis with TUNEL staining (panels f-j). All micrographs 20x magnification. a: Placebo; b: compound E; c: cisplatin; d: compound E and cisplatin; e: Mean BrdU counts of the four treatment groups; f: Placebo; g: compound E; h: cisplatin; i: compound E and cisplatin; j: Mean TUNEL counts of the four treatment groups. GSI and cisplatin treatment do not significantly alter cell proliferation in xenografted OVCAR3 cells.*

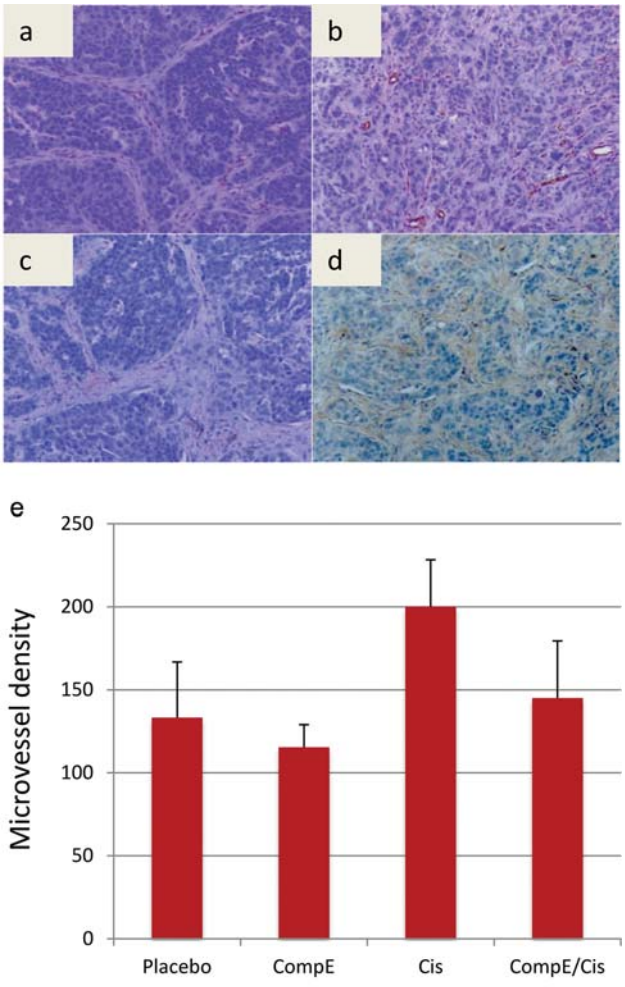

Figure 5. *In vivo examination of microvessel density with CD31 staining. All micrographs 20× magnification. a: Placebo; b: compound-E; c: cisplatin; d: compound-E and cisplatin; e: Mean microvessel density counts (average number of positive cells in four 20x fields) of the four treatment groups. Treatment with compound-E resulted in reduced microvessel density of OVCAR3 xenografts.*

tolerated well by the mice and induced goblet cell hyperplasia. This was determined by administering 10 μmol/kg, 30 μmol/kg, and 100 μmol/kg intraperitoneally, daily to three different groups of three mice each. After one week of treatment, the mice were sacrificed due to significant weight loss in the group treated with 100 μmol/kg and a segment of duodenum was harvested and preserved in formalin for IHC examination. Periodic acid Schiff (PAS) staining was used to identify mucin-containing cells in intestinal crypts. We found that 10 μmol/kg daily did not induce goblet cell hyperplasia, but 30 μmol/kg daily did without significant weight loss in the treated mice (data not shown). Twenty-four nude mice were injected with OVCAR3 and split into four groups to be treated for four weeks with placebo, compound E, cisplatin, or a combination of cisplatin and compound E. All four experimental groups tolerated treatment well, as evidenced by stable weight and activity level. After four weeks the mean tumor weights were 0.255 g for the placebo group, 0.063 g for the compound-E group, 0.114 g for the cisplatin group, and 0.0682 g for the combination group (p=0.28, Figure 3a).

To determine the effects of compound-E at the cellular level, proliferation was examined using BrdU staining and apoptosis was measured with the TUNEL assay. Tumors treated with compound-E on average had a greater number of BrdU-positive cells (average number of BrdU-positive cells per 20x field: placebo 218.3, compound-E 458.8, cisplatin 264, compound-E and cisplatin 316.8) (p=0.0006) (Figure 4). Apoptosis was greatest in the cisplatin group followed by cisplatin and the compound-E group (average number of TUNEL-positive cells per 20x field: placebo 39.8, compound-E 39.6, cisplatin 150, compound-E and cisplatin 56) (p=0.018) (Figure 4).

In order to examine the effect of Notch inhibition on angiogenesis we then analyzed microvessel density using CD31 staining. Mice treated with compound-E had significantly lower microvessel density (average number of microvessels per 20x field: placebo 133.3, compound-E 115.4, cisplatin 200, compound-E and cisplatin 145) (p=0.0002) (Figure 5).

Discussion

Recurrent ovarian cancer remains a challenge in gynecological oncology. Significant research effort has been made to identify therapeutic agents to address platinum-resistant cancer. The Notch pathway has been successfully manipulated with encouraging early results in breast cancer, melanoma, and lymphoma (14-16). The Notch pathway has been demonstrated to be up-regulated in ovarian cancer and may correspond to clinicopathological stage and grade (9, 17, 18). Our findings suggest that the expression of Notch receptors and ligands are up-regulated in ovarian cancer. While GSIs do not directly affect cell growth, blockade of the Notch pathway may result in alterations in angiogenesis that may be therapeutically important.

Our study confirmed that the expression of Notch receptors and ligands are up-regulated in ovarian cancer, but we found that inhibition had little direct effect on tumor growth either *in vivo* or *in vitro*. Interestingly, other groups have noted different findings when treating ovarian cancer cells with GSIs (10, 19). There are several potential explanations for these differences. There is now a growing body of evidence that the downstream effects of Notch may be triggered independently of Notch receptor activation. It has been found that aberrantly-activated IkappaB kinase causes Notch-independent *HES1* activation in colon cancer

(20) and that JAG1 is regulated by both Notch3 and Wnt/beta-catenin (21). A second possibility is that one of the Notch receptors could have been constitutively active, thus abrogating the effects of GSIs. Several authors have found mutations that confer resistance to GSIs or constitutively activate the Notch pathway in hematological malignancies (4, 22). Finally, Notch inhibition may have differential effects in different cell lines. Clearly, more work is needed to fully elucidate interactions between abnormal cellular proliferation pathways and effective blockade of these potentially druggable targets.

One well-recognized attribute of the Notch pathway is that its effects appear to be highly contextually dependent, with different effects in different tissues (6, 23). To date, the body of evidence suggests that *Notch* functions as an oncogene in ovarian cancer. Our findings that both the Notch receptors and ligands are up-regulated in ovarian cancer support this finding.

In addition to direct tumor effects, the Notch pathway plays an important role in tumor-associated angiogenesis. In ovarian cancer, angiogenesis is now recognized to play an important role in tumor growth and development. A number of therapeutic studies have focused on inhibiting angiogenesis, primarily by targeting vascular endothelial growth factor (VEGF) signaling (24-26). Our findings are interesting in that we noted that pan-Notch inhibition with a GSI markedly reduced microvessel density in our murine model of ovarian cancer. Our group has previously reported that targeted Notch1 inhibition impairs tumor growth in animal models of breast cancer and neuroblastoma (27). Inhibiting angiogenesis by blocking the Notch cascade is an appealing target as a number of studies have suggested that tumors are able to develop alternative pro-angiogenic pathways after VEGF blockade (28-30).

We acknowledge several important limitations in our study. Despite results from our dose-finding study we cannot exclude the possibility that using a higher dose of compound-E would have produced an antitumor effect. However, we did note that the use of the other GSIs also had no effect on cellular proliferation. To establish a baseline, we used an IOSE line. It is unknown what effect the immortalization process has on Notch receptor and ligand expression. While our studies of the effect of Notch inhibition are provocative, further studies to characterize the effect of Notch inhibition on ovarian cancer-associated tumor angiogenesis are necessary.

Our findings are intriguing in that *Notch* appears to be up-regulated in ovarian cancer but direct inhibition did not result in decreased tumor growth. Prior work has suggested that while some ovarian carcinomas overexpress Notch, this expression is not universal (10). These findings may imply that Notch blockade may be most effective in those tumors with identified up-regulation of the Notch pathway. Our results were also interesting in that GSI treatment resulted in marked alterations in microvessel density. These findings provide a strong rationale for studies that target multiple points in the angiogenesis pathway as well as examinations of Notch inhibition in combination with metronomic chemotherapy. Further work is underway to delineate the most effective strategy of Notch inhibition in ovarian cancer.

Conflicts of Interest

The Authors have no conflicts of interest to disclose.

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Received November 5, 2012
Revised December 17, 2012
Accepted December 18, 2012