Combined inhibition of IL-6 and IL-8 pathways suppresses ovarian cancer cell viability and migration and tumor growth

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Abstract. Ovarian cancer is the most lethal gynecological cancer type in the United States. The success of current chemotherapies is limited by chemoresistance and side effects. Targeted therapy is a promising future direction for cancer therapy. In the present study, the efficacy of co-targeting IL-6 and IL-8 in human ovarian cancer cells by bazedoxifene (Baze) + SCH527123 (SCH) treatment was examined. ELISA, cell viability, cell proliferation, cell migration, cell invasion, western blotting and peritoneal ovarian tumor mouse model analyses were performed to analyze the expression levels of IL-6 and IL-8, tumor growth, tumor migration and invasion, and the possible pathways of human ovarian cancer cell lines (SKOV3, CAOV3 and OVCAR3) and patient-derived OV75 ovarian cancer cells. Each cell line was treated by monotherapy or combination therapy. The results demonstrated that IL-6 and IL-8 were secreted by human ovarian cancer cell lines. Compared with the DMSO control, the combination of IL-6/glycoprotein 130 inhibitor Baze and IL-8 inhibitor SCH synergistically inhibited cell viability in ovarian cancer cells. Baze + SCH also inhibited cell migration and invasion, suppressed ovarian tumor growth and inhibited STAT3 and AKT phosphorylation, as well as survivin expression. Therefore, co-targeting the IL-6 and IL-8 signaling pathways may be an effective approach for ovarian cancer treatment.

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Abbreviations: GP130, glycoprotein 130; PS, penicillin/streptomycin; CI, combination index

Key words: IL-6, IL-8, bazedoxifene, SCH527123, STAT3, AKT, ovarian cancer

Introduction

Ovarian cancer is the most lethal gynecological cancer type and the fifth leading cause of cancer-related mortality of women in the USA in 2016 (1). In 2019, there were ~22,530 new cases of ovarian cancer, resulting in 13,980 deaths (2). Since most patients with ovarian cancer are diagnosed at an advanced stage, chemotherapy is usually required before or after surgery (3). The combination of platinum and taxane is regarded as the first-line approach (3). Despite intensive research during the past 20 years, the 5-year survival rates have not sufficiently improved, due to both intrinsic and acquired chemoresistance (3). In addition, current chemotherapeutic approaches may have several side effects that may limit their application (4). Identification of novel, more effective and less toxic therapeutic targets is necessary.

Both IL-6 and IL-8 are inflammatory chemokines that have been demonstrated to serve an important role in the tumorigenesis of a variety of malignancies, including ovarian cancer (5). IL-6 and IL-8 are involved in tumor cell apoptosis and invasion, tumor growth and metastasis (6-9). IL-6 and IL-8 upregulation has been associated with chemoresistance in ovarian cancer (10). Certain clinical studies have demonstrated that the IL-6 and IL-8 concentration in the peritoneal fluid of patients with ovarian cancer is 100-1,000-fold higher than that in the serum, and is associated with advanced stage, high grade, lymph node metastasis and poor prognosis in ovarian cancer (6,7,11).

Our previous study demonstrated that the simultaneous inhibition of IL-6 and IL-8 can reduce the viability, colony formation and migration of triple-negative breast and pancreatic cancer cells; however, the potential mechanism was not elucidated (5). Therefore, the aim of the present study was not only to define the anticancer activity of IL-6/IL-8 co-inhibition in ovarian cancer but also to identify the mechanisms underlying IL-6 and IL-8 signaling. To accomplish this, two agents with demonstrated activity in pancreatic, triple-negative breast and colon cancer were used in combination to treat ovarian cancer *in vitro* and *in vivo*; Baze, a third-generation selective estrogen receptor modulator approved by the Food and Drug Administration and novel inhibitor of IL-6/glycoprotein 130 (GP130) protein-protein interactions (12-15), and

SCH, an IL-8/chemokine (CXC motif) ligand 1/CXC chemokine receptor 2 (CXCR2) antagonist (16).

Materials and methods

Materials. SCH was purchased from AdooQ Bioscience. Baze acetate was purchased from Merck KGaA. The stock concentration of SCH and Baze acetate was 20 mM reconstituted in DMSO (Merck KGaA) and stored at -20˚C. MTT was purchased from Sigma-Aldrich; Merck KGaA, and a 5-mM stock concentration was prepared in ddH_2O and stored at -20˚C. All primary and secondary antibodies were purchased from Cell Signaling Technology, Inc.

Cell lines. SKOV3, CAOV3, OVCAR3 and A2780 human ovarian cancer cell lines were purchased from American Type Culture Collection. OV75 primary ovarian cancer cells were provided by Dr Jocelyn Reader and Dr Dana Roque, who were the original suppliers (Division of Gynecologic Oncology, University of Maryland School of Medicine, Baltimore, MD, USA). OV75 cells are primary ovarian cancer cells available for research labs from the University of Maryland. The SKOV3, CAOV3 and A2780 cells were cultured in DMEM (Corning, Inc.) supplemented with 10% FBS (Sigma-Aldrich; Merck KGaA) and 1% penicillin/streptomycin (PS). OVCAR3 cells were cultured in RPMI 1640 medium (Corning, Inc.) with 10% FBS and 1% PS. OV75 cells were cultured in HOSE Media [1:1 mixture of MCDB 105 (Sigma-Aldrich; Merck KGaA) and Medium 199 (Thermo Fisher Scientific, Inc.)], 10% FBS, 1% L-glutamine, 1% non-essential amino acids, 1% PS, sodium bicarbonate at pH 7.4) as described previously (17). All cell lines were grown in a humidified 37˚C incubator with 5% CO_2/95% air, and the medium was replaced twice a week.

Measurement of IL-6 and -8. Immunoreactive IL-6 and IL-8 were measured using an Quantikine ELISA kit (Human CXCL8/IL-8, cat. no. D8000C; human IL-6, cat. no. D6050; R&D Systems, Inc.). Ovarian cancer cells were seeded in 6-well plates at a confluency of 70%. The cell-free culture supernatant of every well was collected and incubated, and optical density (OD) was read at 450 nm. Each assay detects cytokines as low as 5±7 pg/ml.

Western blot analysis. Cells were seeded in 10-cm plates at 70% confluency and treated with DMSO, Baze, SCH or Baze + SCH (CAOV3, Baze 10 μM, SCH 50 μM; SKOV3, Baze 5 μM, SCH 50 μM) at 37˚C overnight before being harvested, lysed in cold Cell Lysis Buffer (0.5% 0.2 M PMSF, 0.5% 0.2 M NaF, 0.5% 0.2 M NaPP, 0.5% 0.1 M Na_3VO_4 and 4% 25X CP1; Cell Signaling Technology, Inc.) to collect the protein for western blot analysis. The protein concentration was determined using a Microplate BCA Protein Assay kit (cat. no. 23252; Thermo Fisher Scientific, Inc.). The proteins (30 μg/lane) were separated by 10% SDS-PAGE (30 μg per lane), transferred to a PVDF membrane at 350 mA for 110 min, blocked with 5% milk/TBS-8% Tween-20 (TBST) for 1 h at room temperature and incubated with primary antibodies overnight at 4˚C. The membranes were washed with TBST three times (15 min each), blotted with the secondary antibody for 1.5 h at room temperature. The visualization reagent (Western Lighting Plus-ECL; PerkinElmer, Inc.) was added and membranes were scanned using the Amersham Imager 600 (GE Healthcare, version 2.0.0).

The following primary antibodies were purchased from Cell Signaling Technology, Inc. and diluted at 1:1,000 in 5% milk: Phosphorylated (p)-STAT3 (Y705; rabbit mAb; cat. no. 9131S), STAT3 (rabbit mAb; cat. no. 4904S), p-S6 (rabbit mAb; cat. no. 4858S), S6 (rabbit mAb; cat. no. 2217S), p-AKT (rabbit mAb; cat. no. 4060S), AKT (rabbit mAb; cat. no. 4691S), survivin (rabbit mAb; cat. no. 2808S) and GAPDH (rabbit mAb; cat. no. 2118S). The secondary antibody was diluted at 1:10,000 in 5% milk (anti-rabbit IgG HRP-linked antibody; cat. no. 7074; Cell Signaling Technology, Inc.).

Cell proliferation assay. Ovarian cancer cells were seeded in 24-well plates at the same cell density, depending on the growth ability of each cell line (SKOV3, $2x10^3$ cells per well; CAOV3, $3x10^3$ cells per well; OVCAR3, $3x10^3$ cells per well), cultured overnight at 37˚C and treated with DMSO, Baze, SCH or Baze + SCH at 37˚C until the end of the assay (CAOV3, Baze 5 μM, SCH 15 μM; SKOV3, Baze 2.5 μM, SCH 15 μM; OVCAR3, Baze 2.5 μM, SCH 15 μM). The cell number of each well was then counted every 2 days after treatment (on days 2, 4, 6 and 8) to generate the growth curves. The cell density of cell growth assay is much lower than that of other assays, so the doses of Baze were less than those of other assays (CAOV3, Baze 5 μM; SKOV3, Baze 2.5 μM; OVCAR3, Baze 2.5 μM). However, the effects of different concentrations of SCH (such as 10 or 15 μM) were similar. Therefore, the SCH concentration remained constant in a number of experiments, including the MTT and wound healing/cell migration assays (the concentration of SCH here is 15 μM for each cell line).

MTT assay. A total of 3,000 cells in 100 μl medium per well were seeded into 96-well microtiter plates and treated overnight with DMSO, Baze, SCH or Baze + SCH (CAOV3, Baze 5 μM, SCH 15 μM; SKOV3, Baze 5 μM, SCH 15 μM; OVCAR3, Baze 5 μM, SCH 15 μM; OV75, Baze 10 μM, SCH 15 μM), followed by incubation at 37˚C for 72 h. Each well was treated with 20 μl MTT, incubated for ~4 h and then combined with 150 μl N,N-dimethylformamide solution, followed by further incubation overnight at room temperature protected from the light. Cell viability was assessed by measuring absorbance at 595 nm for each well. The cell viability of DMSO control cells was set at 100% and the cell viability of drug-treated cells was compared with that of DMSO-treated cells. The combination index (CI) was then determined using CompuSyn software (www.combosyn.com; ComboSyn, Inc.). CI values of 1 indicate an additive effect, values of >1 an antagonistic effect and values of <1 a synergistic effect, based on the theorem of Chou (18).

Wound healing/cell migration assay. SKOV3 cells were seeded in 6-well plates and incubated at 37˚C overnight until they reached 100% confluence. A 100-μl pipette tip was used to scrape the monolayer of each well, each well was washed twice with PBS, serum-free culture medium containing different drugs (DMSO, Baze, SCH and their combination)

was added (SKOV3, Baze 5 μM, SCH 15 μM) and images were captured under the light microscope.

Cells were incubated at 37˚C and imaged again when the wound in the DMSO control was well healed (17 h). Migration inhibition was measured using ImageJ software 1.53e (National Institutes of Health) and calculated using the following formula: Percentage of wound healing = 100-[(final area/initial area) x 100%] (19).

In vitro invasion assay using Matrigel®. This assay was performed as described previously (20). Matrigel was diluted at 1:8 with DMEM (without FBS and PS), added on the top of the Transwell membrane and allowed to polymerize for a minimum of 30 min at 37˚C. Next, $5x10^4$ cells were added in 200 μl culture medium without FBS on the top of the Matrigel and 600 μl normal culture medium (DMEM or 1640 with 10% FBS) was added to the bottom of the lower chamber in a 24-well plate. The cells were treated with different drugs: DMSO, Baze, SCH or Baze + SCH for 24 h at 37˚C (CAOV3, Baze 5 μM, SCH 15 μM; OVCAR3, Baze 10 μM, SCH 15 μM). The Transwell® insert (permeable cell culture inserts; CELLTREAT Scientific Products) was then removed from the plate and the media and the remaining cells were carefully removed from the top of the membrane without damaging it using a cotton-tipped applicator. The Transwell insert was placed into 70% ethanol for 10 min and then stained with 0.2% crystal violet for 5-10 min at room temperature, rinsed, dried and imaged under a light microscope. Finally, the Transwell membrane was decolorized with 3% acetic acid to completely elute the crystal violet, and 100 μl/well eluent was added into a 96-well plate to measure the OD value (570 nm) on a microplate reader.

Ovarian peritoneal tumor growth. A peritoneal ovarian tumor mouse model was used to evaluate the efficacy of Baze + SCH compared with monotherapy in suppressing ovarian tumor growth. CAOV3-luciferase ovarian cancer cells ($1x10^7$) in DMEM without FBS were injected intraperitoneally into a total of 20 female athymic nude mice aged 6-8 weeks purchased from Jackson Laboratory (21,22). Mice were housed at ~37˚C with a 12/12-h light/dark cycle and *ad libitum* access to food and water. The development and growth of the tumors were monitored 1-2 times a week using an IVIS™ instrument (bioluminescent imaging; PerkinElmer, Inc.) (23). After the tumors had been measured, the mice were divided into four different groups with 5 mice/group: i) Vehicle, DMSO; ii) Baze, 8.8 mg/kg/mouse (24,25); iii) SCH, 25 mg/kg/mouse (16); and iv) Baze + SCH. Both drugs were administered daily by intraperitoneal injection. All mice were monitored using an IVIS instrument. Once experiments had been completed at 2 months after injection, tumors were harvested, weighed and snap-frozen in liquid nitrogen, and stored at -80˚C. Tumor tissue was ground into powder, lysed and separated by SDS-PAGE to examine the expression of the downstream targets of IL-6 and IL-8 in different mouse groups, as described in the western blot analysis subsection. The method of euthanasia used for the mice was CO_2 asphyxiation followed by cervical dislocation (CO_2 was introduced into the chamber at a rate of 30-70% of the chamber volume per min to minimize distress). The maximum tumor diameter was 14.5 mm and the volume obtained was 988.2675 mm^3. The use of mice was approved on June 11, 2019 by the Institutional Animal Care & Use Committee of the University of Maryland (Baltimore, MD, USA).

Bioluminescent imaging. The development and growth of tumors were monitored by detecting the bioluminescence through an IVIS™ Imaging System, which has a high-sensitivity cooled charge-coupled camera mounted in a light-tight box (21,23). The images were collected and analyzed using Living Image™ software (Version:4.5.2.18424; PerkinElmer, Inc.). The mice were anaesthetized and injected intraperitoneally with d-luciferin potassium salt solution (150 mg/kg), and then placed into the imaging chamber. Bioluminescent images were collected per week (days 7, 14, 22, 29, 36, 44 and 62) throughout the duration of the study.

Anesthesia. Mice were anesthetized using pharmaceutical grade isoflurane dispensed from a precision vaporizer in 100% O_2 delivery gas at 3.5-4.5% to an induction chamber with charcoal scavenger attached (scavenger was weighed before use and weight recorded on side of cannister to determine disposal point by weight gain). For Xenogen Imaging, there was a dedicated XGI8 gas inhalation anesthesia apparatus next to the Xenogen IVIS-200 imager. Once recumbent 20-30 sec and respiratory rate was noted to slow, isoflurane was stopped, the chamber was flushed with 100% O_2 (to reduce technician exposure to anesthetic when chamber is opened) and the animal was removed to continue anesthesia with isoflurane at 1.5-2.5% via face mask inside the imaging chamber. After the imaging was performed, mice were moved to a clean cage with bedding. Mice were monitored until able to ambulate normally prior to return to their assigned husbandry rooms.

Statistical analysis. The significance of associations was determined using GraphPad Prism 7 software (GraphPad Software, Inc.). Data for experiments performed in triplicate are presented as the mean ± standard error of the mean. One-way ANOVA and Tukey's post hoc test were used to analyze the statistical differences among groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

IL-6 and IL-8 levels in human ovarian cancer cells. The secretion of IL-6 and IL-8 was examined in human ovarian cancer cell lines. The present results demonstrated that OVCAR3, SKOV3 and CAOV3 human ovarian cancer cell lines, and OV75 primary human ovarian cancer cells, secreted both IL-6 and IL-8 at moderate to high levels [IL-6 (pg/ml): OVCAR3, 58.75±2.859; SKOV3, 191.4±7.469; OV75, 4,113±110.7; CAOV3, 12,517±11.15; IL-8 (pg/ml): OVCAR3, 112.4±6.138; SKOV3, 2,215±68.58; OV75, 18,757±233.3; CAOV3, 4,776±53.34; Fig. 1]. As a negative control, A2780 non-serous human ovarian endometroid adenocarcinoma cells did not secrete detectable IL-6 and IL-8. In general, endometrioid carcinoma is associated with an improved prognosis compared with serous ovarian cancer (26). These results supported the current hypothesis that IL-6 and IL-8 signaling pathways may serve as potential therapeutic targets for the most aggressive human ovarian cancer types.

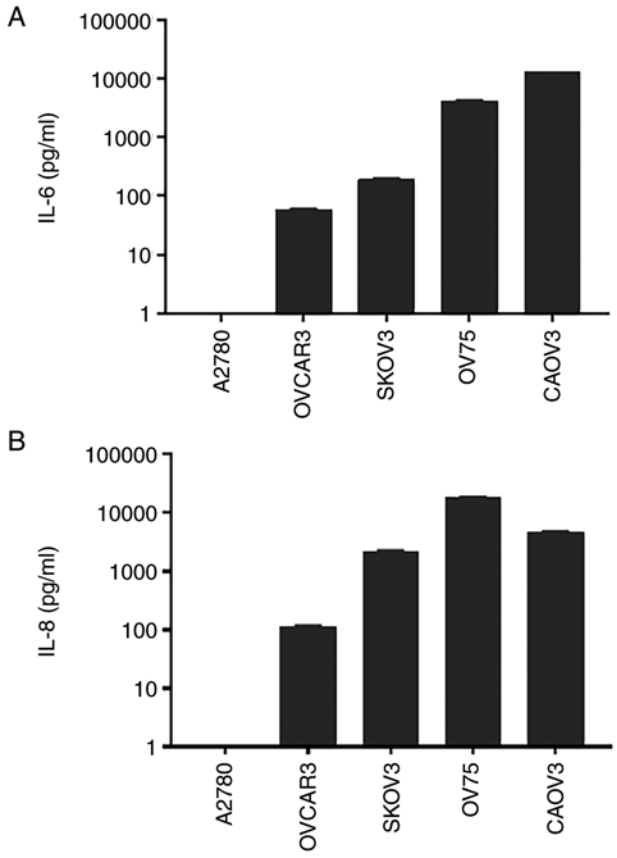

Figure 1. Secretion of (A) IL-6 and (B) IL-8 in human ovarian cancer cells.

Baze + SCH treatment inhibits human ovarian cancer cell proliferation. IL-6 and IL-8 signaling can both regulate cell proliferation, adhesion, metastasis and invasion in human ovarian cancer cells (4,27-29). Therefore, cell proliferation may be decreased by IL-6 and IL-8 inhibitors (Baze and SCH). Thus, a cell proliferation assay was first performed. As shown in Fig. 2, the proliferation of the ovarian cancer cells was inhibited by each drug alone, but Baze + SCH treatment exerted a stronger inhibitory effect compared with each monotherapy.

Baze + SCH synergistically inhibit human ovarian cancer cell viability. It has been reported that IL-6 and IL-8 enhance the proliferation of ovarian cancer cells (6). Therefore, the cell viability of ovarian cancer cells may be inhibited by IL-6 and IL-8 inhibitors. MTT assays were performed using Baze + SCH in OVCAR3, SKOV3, CAOV3 and OV75 human ovarian cancer cells As shown in Fig. 3, Baze + SCH treatment exerted a stronger inhibitory effect on cell viability compared with each monotherapy. Furthermore, the CI of each of the four ovarian cancer cell lines was <1, indicating that the two drugs had a synergistic effect.

Baze + SCH treatment inhibits the migration and invasion of human ovarian cancer cells. Cell migration and invasion are important steps in tumor metastasis, which can indicate a poor prognosis (30,31). IL-6 and IL-8 are necessary and sufficient to increase tumor cell migration (7). Therefore, cell migration and invasion may be reduced by the inhibitors of IL-6 and IL-8. A cell migration/wound healing assay was performed using the SKOV3 cell line, as the monolayer phenotypes of the other two cell lines were not suitable for this assay. An *in vitro* invasion assay was also performed in OVCAR3 and CAOV3 cells using Matrigel, to detect the inhibitory effect on cell invasion. Compared with the DMSO control, cell migration (Baze, 72.73%; SCH, 71.82%; Baze + SCH, 41.82%; DMSO vs. Baze/SCH, Baze + SCH vs. Baze/SCH, P<0.0001; Fig. 4A) and invasion (Fig. 4B and C) were inhibited by Baze and SCH monotherapy (CAOV3: Baze, 68.73%; DMSO vs. Baze, P<0.0001; SCH, 76.05%, DMSO vs. SCH, P=0.0002; Baze + SCH, 54.8%, Baze + SCH vs. Baze, P<0.01; Baze + SCH vs. SCH, P<0.001; OVCAR3: Baze, 70.3%; DMSO vs. Baze, P<0.001; SCH, 87.98%, DMSO vs. SCH, P<0.05; Baze + SCH, 53.06%, Baze + SCH vs. Baze, P<0.01; Baze + SCH vs. SCH, P<0.0001). Baze + SCH treatment resulted in a greater inhibitory effect on cell migration and invasion compared with each monotherapy. These results indicated that Baze + SCH may be used to treat or prevent ovarian cancer cell invasion.

Baze + SCH synergistically inhibit the expression of targeted genes downstream of the IL-6 and IL-8 pathways in human ovarian cancer cells. Since there are several interactions between the IL-6 and IL-8 pathways (5,7), western blot analysis was performed to determine which downstream targeted genes were synergistically inhibited by these two drugs. Two ovarian cancer cell lines were seeded in 10-cm plates and treated with DMSO, a single drug or their combination. Protein expression levels were analyzed. Compared with those in cells treated with DMSO, the levels of p-STAT3, p-AKT, p-S6 and survivin were decreased by monotherapy but this was not significant in most cases (Fig. 5). The expression levels following the combination treatment were significantly lower than those of monotherapy and DMSO across all targets (p-STAT3/STAT3, p-AKT/AKT, p-S6/S6 and survivin/GAPDH). These results indicated that Baze + SCH synergistically inhibited the expression of downstream targeted genes (p-STAT3/STAT3, p-AKT/AKT, p-S6/S6 and survivin/GAPDH) of IL-6 and IL-8 pathways in human ovarian cancer cells, which may have effects on cell viability, migration and invasion.

Baze + SCH treatment suppresses ovarian cancer growth in vivo. The antitumor effect of Baze + SCH was investigated *in vivo*. CAOV3-luciferase ovarian cancer cells ($1x10^7$) were injected intraperitoneally into female athymic nude mice. The mice were divided into four different groups, with 5 mice per group: i) Vehicle, DMSO; ii) Baze, 8.8 mg/kg; iii) SCH, 25 mg/kg; iv) Baze + SCH. As shown in Fig. 6A and B, a significant reduction in tumor growth was observed in the Baze or SCH single-treatment groups. However, combination treatment with Baze + SCH exhibited a greater inhibitory effect than monotherapy. In addition, compared with the DMSO control group, combination treatment could significantly reduce the levels of p-STAT3/STAT3, p-AKT/AKT, p-S6/S6 and survivin/GAPDH in the tumor tissues (Fig. 6C).

In conclusion, the present *in vitro* and *in vivo* findings indicated that the combined inhibition of IL-6 and IL-8 has the potential to suppress the growth of ovarian cancer and prevent tumor invasion.

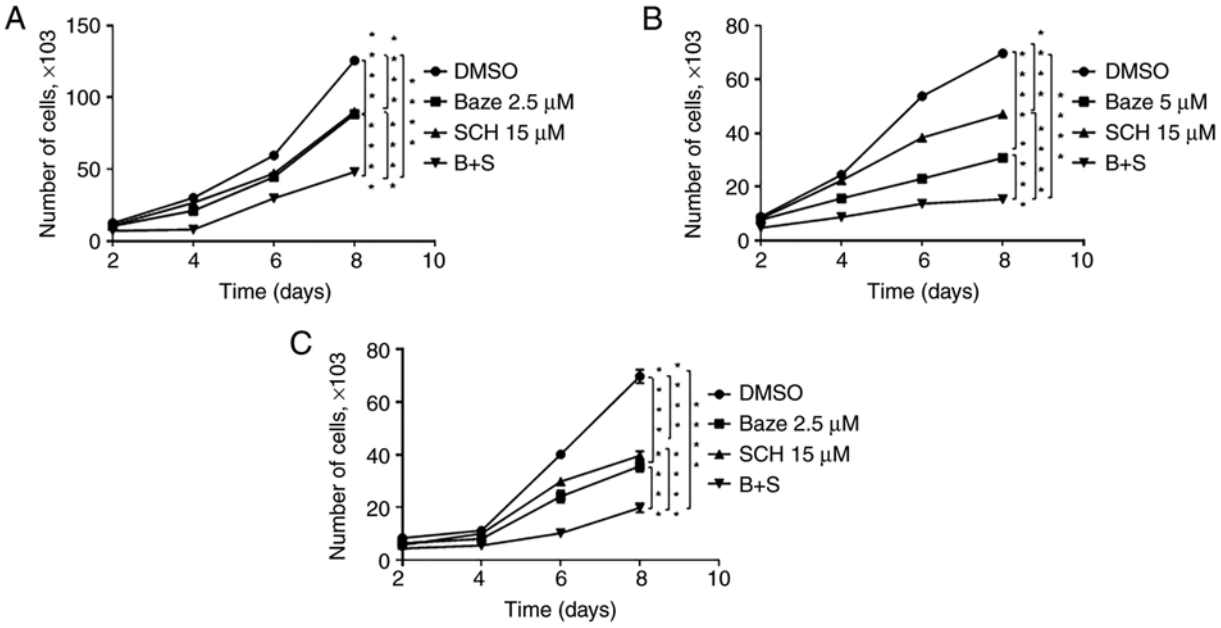

Figure 2. Effects of Baze, SCH and their combination on cell proliferation. A cell proliferation assay was performed to evaluate ovarian cancer cell proliferation. The cell proliferation of ovarian cancer cells was inhibited by Baze and SCH alone or in combination. (A) CAOV3. (B) SKOV3. (C) OVCAR3. ****P<0.0001.

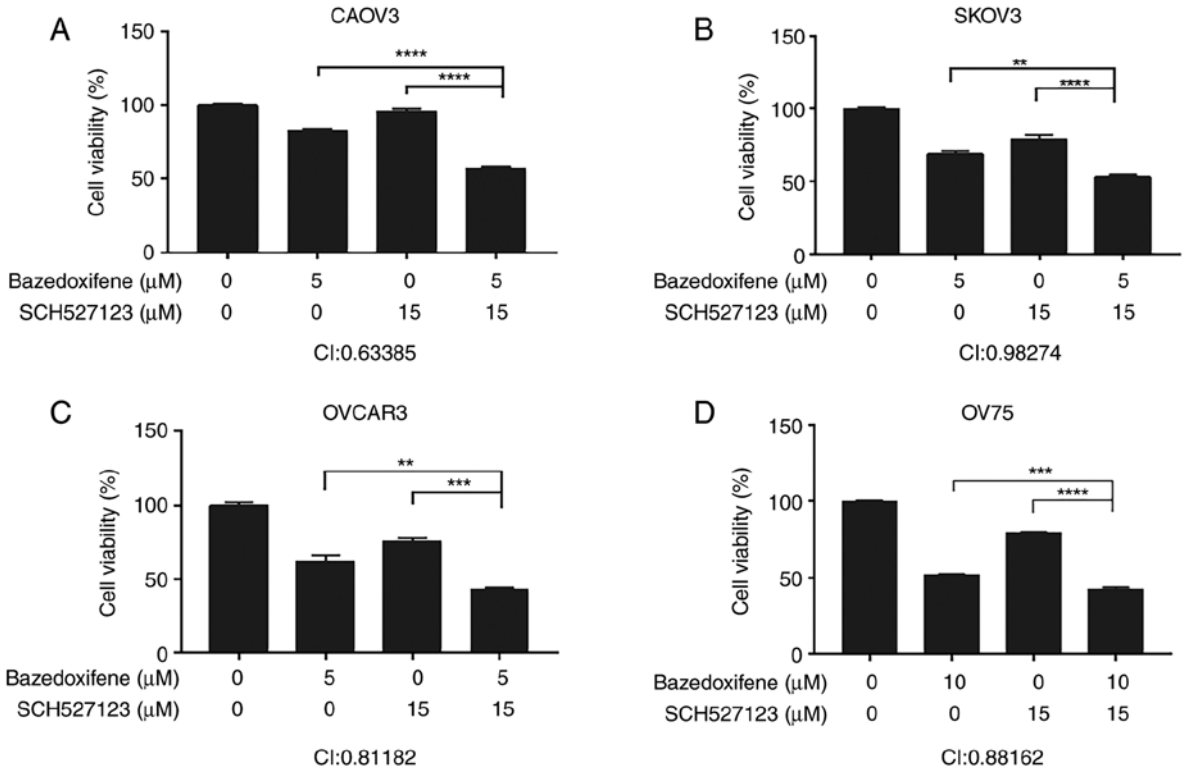

Figure 3. Effects of Baze, SCH and their combination on cell viability. The MTT assay was performed to evaluate cell viability. (A) CAOV3. (B) SKOV3. (C) OVCAR3. (D) OV75. Cell viability of ovarian cancer cells was synergistically inhibited by Baze + SCH. **P<0.01, ***P<0.001 and ****P<0.0001.

Discussion

Ovarian cancer has the highest mortality rate among gynecological malignancies (2,32). Detecting ovarian cancer early is challenging, and thus, >50% of patients are diagnosed at advanced stages (33). Secondly, ~75% of patients with ovarian cancer relapse due to intrinsic and acquired chemotherapy resistance, which leads to cancer recurrence (2,33). These are the two main reasons for the low 5-year overall survival rate, which has remained at 20-35% in the past 20 years (2). Therefore, the continuous development of novel treatment methods and drugs, such as targeted therapy, which has the advantages of precise action and fewer side effects, is crucial (33).

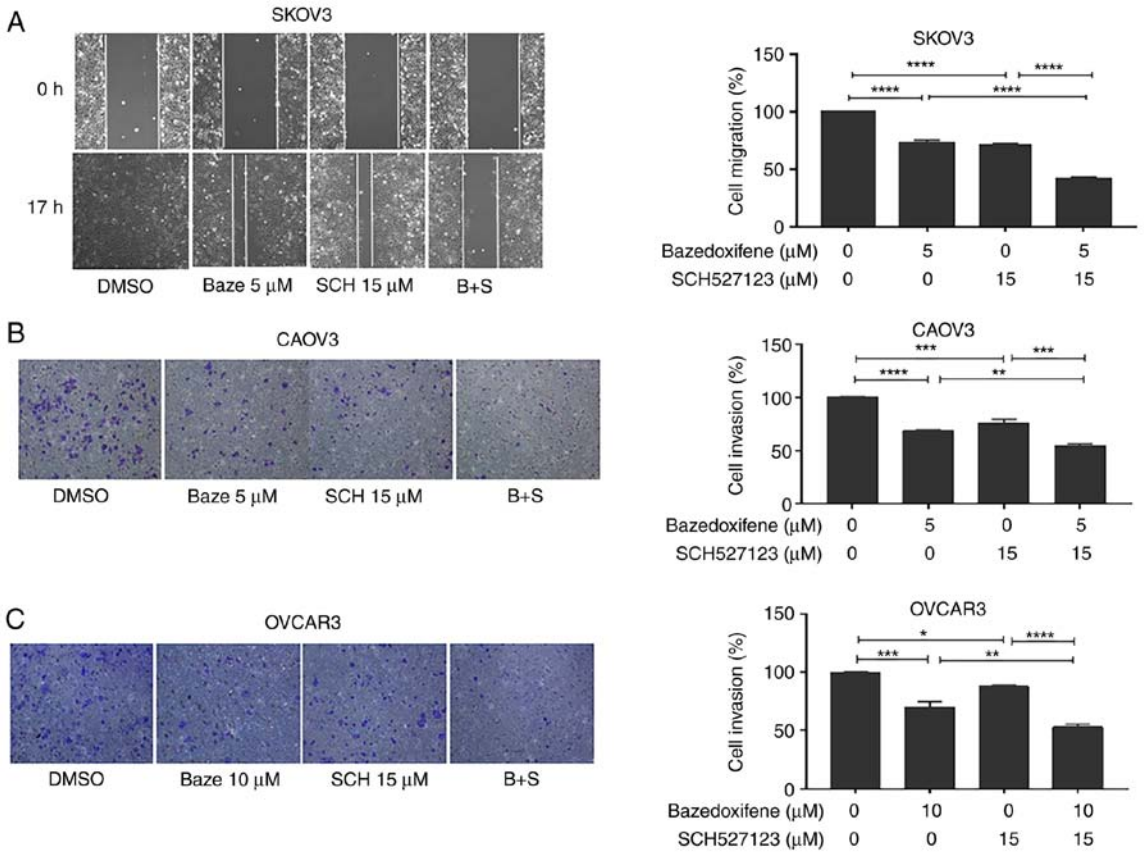

Figure 4. Effects of Baze, SCH and their combination on cell migration and invasion. (A) A wound healing assay was performed to evaluate the migration ability of SKOV3 cells. (B) CAOV3 and (C) OVCAR3. An *in vitro* invasion assay using Matrigel was performed to evaluate the invasion of OVCAR3 and CAOV3 cells. Magnification, x10. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. B+S, Baze + SCH; Baze, Baze; SCH, SCH.

IL-6 is not only an inflammatory factor but also a tumor-promoting factor, which serves an important role in tumorigenesis, cancer cell survival and tumor progression in multiple cancer types, including breast, cervical, colorectal, oesophageal, head-and-neck, ovarian, pancreatic, prostate and renal cancer (4,10,34,35). IL-6 binds to IL-6 receptor and GP130 to form a signaling complex, which then activates downstream pathways (4,10,34,35). Janus kinase/STAT3 is the main activated pathway which, in turn, leads to tyrosine phosphorylation of STAT3 (4,10,34,35). p-STAT3 translocates into the nucleus to induce the expression of target genes that promote tumor cell proliferation, migration and invasion and suppress apoptosis (4,10,34,35). In addition to the STAT3 signaling pathway, several other pathways, such as the PI3K/AKT and MEK/MAPK signaling pathways, may also be activated by IL-6 (34). IL-6 is one of the principal oncogenic mediators in ovarian cancer (35). IL-6 is either constitutively secreted directly by ovarian carcinoma cells or through secondary inflammatory and tumor-infiltrating cells, including fibroblasts, tumor-associated macrophages and T cells (36-38). Increasing evidence supports the notion that high levels of serum IL-6 are associated with poor prognosis, short survival, advanced disease and metastasis in patients with ovarian cancer (4,27,39). Higher levels of serum and peritoneal fluid IL-6 are also more commonly associated with aggressive metastatic ovarian carcinomas (27). Autocrine production of IL-6 confers resistance to chemotherapeutic agents, such as cisplatin and paclitaxel, in ovarian cancer cells (35,40). In addition, IL-6 signaling regulates anchorage-independent proliferation, adhesion and invasion in human ovarian cancer cells (4,27,28).

IL-8, also known as C-X-C motif chemokine ligand 8, is a chemokine produced by macrophages, epithelial cancer cells and other cells. IL-8 gene silencing decreases tumor growth through antiangiogenic mechanisms (29). IL-8 is upregulated in various human solid tumors (41) and is associated with advanced tumor stage (P=0.019), high tumor grade (P=0.031) and poor survival (29). The biological effects of IL-8 are mediated by the binding of IL-8 to its receptors on the surface of the cell membrane, two cell-surface G protein-coupled receptors termed C-X-C motif chemokine receptor 1 (CXCR1) and CXCR2 (42,43). Following binding, PI3K/AKT, STAT3 and other signaling pathways may be activated, leading to tumor progression through cell proliferation, migration, invasion and epithelial-mesenchymal transition in several cancer types, including ovarian cancer (8,9,29,44-48). Therefore, in addition to the IL-6 signaling pathway, the IL-8 signaling pathway is also an important potential therapeutic target in ovarian cancer.

Since IL-6/GP130-targeted small molecule drugs are still not available for clinical cancer therapy, IL-6/GP130 small molecule inhibitors have been developed using multiple ligand simultaneous docking and drug repurposing (49). In our previous study, Baze was identified as a novel inhibitor of the IL-6/GP130 interaction using a search of virtual hits on a drug database (49). Baze has also been approved as a third-generation selective estrogen receptor modulator, as well as a novel inhibitor of IL-6/GP130 (12-15,50). The direct binding of

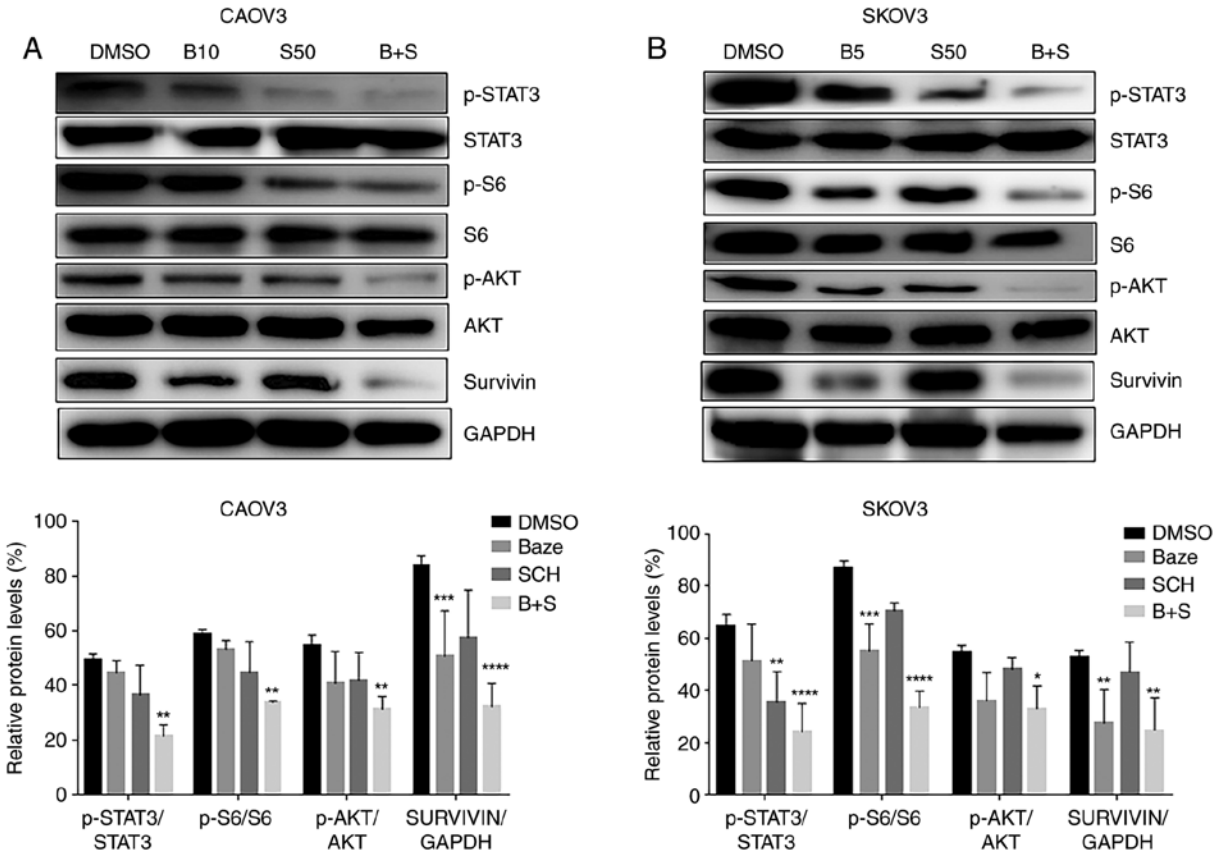

Figure 5. Baze and SCH inhibit the expression of target genes downstream of the IL-6 and IL-8 pathways in human ovarian cancer cells. SKOV3 and CAOV3 cells were treated with DMSO, a single drug or their combination. The levels of p-STAT3, p-AKT, p-S6 and survivin were determined by western blot analysis. (A) CAOV3. (B) SKOV3. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 (DMSO vs. Baze, SCH or Baze + SCH). B+S, Baze + SCH; B10, BAZE 10 μM; B5, BAZE 5 μM; BAZE, Baze; S50, SCH 50 μM; p-phosphorylated; SCH, SCH.

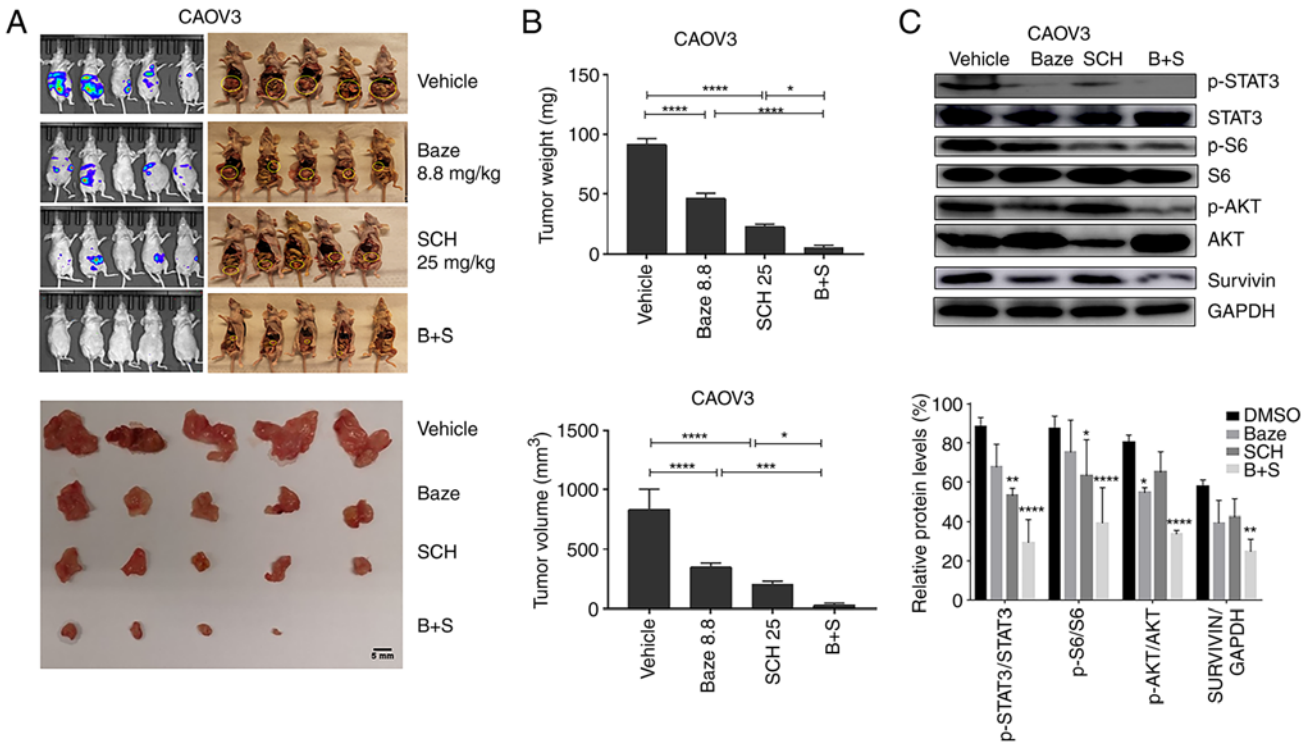

Figure 6. Baze and SCH suppress ovarian tumor growth. (A) Images of CAOV3 ovarian tumors. Scale bar, 5 mm. (B) Tumor weight and volume in different experimental groups. *P<0.05, ***P<0.001 and ****P<0.0001. (C) Western blot analysis of the levels of IL-6 and IL-8 downstream targets in representative tumors (n=12). *P<0.05, **P<0.01 and ****P<0.0001 (DMSO vs. Baze, SCH or Baze + SCH). B+S, Baze + SCH; Baze, Baze; p-, phosphorylated; Sch, SCH; Baze 8.8, Baze 8.8 mg/kg; SCH25, SCH 25 mg/kg.

Baze to GP130 was supported by our previous study (49). It was also observed in our previous study that Baze inhibits IL-6-mediated IL-6/IL-6Ra/GP130 heterotrimer in HPAC cells (data not shown), supporting the effects of bazeodxifene on blocking IL-6 signaling, which has been demonstrated to lead to growth suppression in breast (24) and colon cancer (25).

SCH is a potent allosteric CXCR1 and CXCR2 antagonist and has been shown to suppress tumor growth through the inhibition of the NF-κB/AKT/MAPK signaling pathway (16,51). SCH has been found to target IL-8/CXCR1/CXCR2 signaling in cancer cells, including in melanoma, breast and pancreatic cancer cells (19,51,52). Furthermore, SCH alone has also been tested in colon cancer cells and tumor models, and it was found to have a limited *in vivo* activity on inhibiting tumor growth and liver metastasis (16,53). SCH alone may have only a partial tumor-suppressive activity in colon cancer, mainly due to the inhibition of the IL-8 signaling pathway alone (without the simultaneous inhibition of the IL-6 pathway), which was insufficient for full tumor suppression (16,53). It may therefore be necessary to target both IL-6 and IL-8 for cancer therapy.

In our previous study, it was demonstrated that the combination of Baze and SCH for the treatment of triple-negative breast and pancreatic cancer cells had synergistic inhibitory effects (5); however, the possible mechanism was not explored. In the present study, the efficacy of Baze and SCH in ovarian cancer was examined, and it was demonstrated that cell viability, migration and invasion were inhibited. Tumor growth *in vivo* was also suppressed. Using western blot analysis, the levels of p-AKT, p-STAT3, survivin and p-S6 were found to be decreased by Baze + SCH. Baze + SCH caused a greater inhibition of p-AKT, p-S6 and survivin levels compared with monotherapy. Therefore, the dual inhibition of IL-6 and IL-8 may inhibit cross-talk between the STAT3 (survivin) and AKT (S6) signaling pathways. Notably, to the best of our knowledge, this was the first study reporting the application of Baze + SCH in ovarian cancer and its potential mechanism.

In conclusion, the present results indicated that IL-6 and IL-8 are secreted by ovarian cancer cells and may confer an aggressive phenotype. The combination of IL-6/GP130 inhibitor Baze and IL-8/CXCL2 inhibitor SCH could synergistically inhibit cell viability, and inhibited the migration and invasion of human ovarian cancer cells *in vitro*. In addition, Baze + SCH was also found to suppress ovarian cancer growth *in vivo*. Therefore, Baze + SCH treatment may be a potentially effective approach for ovarian cancer therapy and should be studied further.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

RZ and JL conceived the study. JL supervised the study. RZ carried out the experiments and wrote the manuscript with support from JL, JR and DMR. JR and DMR provided the OV75 cell line and contributed to editing the manuscript. RZ and JL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The use of mice was approved on June 11, 2019 by the Institutional Animal Care & Use Committee of the University of Maryland (approval no. 1488GCC: Collection of Tissue Samples from Women with Gynecologic Malignancies and Healthy Controls for Laboratory Research, Baltimore, MD, USA).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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