

# Signaling pathway inhibitors target breast cancer stem cells in triple-negative breast cancer

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**Abstract.** The present study aimed to investigate the efficacy of five signaling pathway inhibitors, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester, vismodegib, salinomycin, ruxolitinib and stattic, as novel therapeutic agents that target breast cancer stem cells (BCSCs) in triple-negative breast cancer (TNBC). The *in vitro* anti-proliferative, anti-invasive, pro-apoptotic and inhibitory effects on BCSC self-renewal of these signaling pathway inhibitors on the TNBC stem cell line HCC38 were examined by MTT assays, Matrigel invasion assays, flow cytometry and suspension mammosphere assays, respectively. For the *in vivo* study, another TNBC stem cell line, HCC1806, pretreated with these signaling pathway inhibitors, was inoculated into female nonobese diabetic/severe combined immunodeficient mice, and the tumor volumes were measured following tumor formation. Treatment of HCC38 cells with each signaling pathway inhibitor significantly decreased TNBC cell proliferation, cell invasion and mammosphere formation

while inducing cell apoptosis by inhibiting the protein expression or phosphorylation of downstream signaling molecules. In the xenograft mouse models, tumor formation and growth of HCC1806 cells pretreated with each signaling pathway inhibitor were effectively suppressed. Treatment with these signaling pathway inhibitors may provide a novel therapeutic strategy against TNBC by targeting BCSCs, thus providing promising insight for clinical applications in patients with TNBC.

## Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-associated mortality in women worldwide (1). It accounts for 30% all novel cancer diagnoses in women (1). Triple-negative breast cancer (TNBC), characterized by the absence of estrogen receptor and progesterone receptor in addition to a lack of overexpression of human epidermal growth factor receptor 2 (HER2), accounts for 15% breast cancer cases (2,3). As a distinct subtype of breast cancer, TNBC does not respond to the standard endocrine therapies, including tamoxifen (an anti-estrogen agent against the estrogen receptor) and trastuzumab (a monoclonal antibody against HER2), thus presenting a clinical challenge, as it is associated with a higher incidence of visceral metastases, poorer prognosis, shorter survival and higher risk of distant recurrence compared with other types of breast cancer (2). Identifying novel potential targets and novel therapeutic options are urgently required to manage this aggressive type of breast cancer.

Increasing evidence suggested that the aggressiveness of TNBC and its resistance to standard drug therapies may be partially due to the presence of breast cancer stem cells (BCSCs) within TNBC tumors in addition to the normal tissue adjacent to TNBC tumors (4-6). In human cancer, including breast cancer, there is a small population of cancer stem cells, which are capable of self-renewal, differentiation, and tumor initiation and development (7). In breast cancer, a subpopulation of breast cancer cells [CD44 antigen (CD44<sup>+</sup>)/ signal transducer (CD24) CD24<sup>-low</sup>] was isolated and defined as BCSCs, which have unique stem cell-like properties that may contribute to chemotherapy and/or radiotherapy resistance (8).

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**Abbreviations:** ALDH1, aldehyde dehydrogenase 1; BCSCs, breast cancer stem cells; bFGF, basic fibroblast growth factor; cl-Notch1, cleaved Notch1 intracellular domain; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; FBS, fetal bovine serum; GDC-0449, vismodegib; HER2, human epidermal growth factor receptor 2; JAK, Janus kinase; NOD/SCID, non-obese diabetic/severe combined immunodeficient; PTCH, Patched; SMO, smoothened; STAT3, signal transducer and activator of transcription 3; TBST, Tris-buffered saline containing Tween-20; TNBC, triple-negative breast cancer; Wnt, wingless-type MMTV integration site family

**Key words:** TNBC, BCSCs, signaling pathway inhibitors, ALDH1, Notch, Hedgehog, JAK

In addition to the expression of CD44 and CD24, an alternate cell surface marker, aldehyde dehydrogenase 1 (ALDH1), has been used to identify BCSCs (8-11). Ginestier *et al* (11) identified that only ALDH1<sup>+</sup> cells may develop tumors in mice, albeit in small numbers, whereas CD44<sup>+</sup>/CD24<sup>-</sup> is not able to. ALDH1 is additionally considered a predictor of prognosis in patients with breast cancer (12-15). Therefore, ALDH1 was used as a BCSC marker in the present study.

In BCSCs, the stem-like properties, including self-renewal, treatment-resistance and aggressiveness, are coordinated by a network of cellular signaling pathways, including the Notch, Hedgehog, wntless-type MMTV integration site family (Wnt)/ $\beta$ -catenin, and Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) signaling pathways (16). Aberrations in one or more of these signaling pathways have been identified in cancer stem cells, including BCSCs (16). Therefore, targeting these signaling pathways in BCSCs is an attractive strategy for TNBC therapy (17).

In the present study, using triple-negative, ALDH1<sup>+</sup> BCSC lines HCC38 and HCC1806, *in vitro* and *in vivo* studies were conducted to investigate the anti-tumor effects of five signaling pathway inhibitors, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT; Notch pathway inhibitor), vismodegib (GDC-0449; Hedgehog pathway inhibitor), salinomycin (Wnt/ $\beta$ -catenin pathway inhibitor), ruxolitinib and stattic (JAK/STAT3 pathway inhibitors; Table I), on BCSCs in TNBC.

## Materials and methods

**Reagents and cell culture.** DAPT, salinomycin, MTT, hydrocortisone and insulin were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). GDC-0449, ruxolitinib and stattic were obtained from Selleck Chemicals (Houston, TX, USA). RPMI-1640, B27, penicillin and streptomycin were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were provided by Prospec-Tany TechnoGene, Ltd. (East Brunswick, NJ, USA). The HCC38 breast cancer cell line was obtained from The American Type Culture Collection (Manassas, VA, USA). The HCC1806 breast cancer cell line was provided by Dr Shibo Fu at the Transform Medical College of The First Hospital of Jilin University (Changchun, China). The two cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.; cat. no. 16000-044), penicillin (6.25  $\mu$ g/ml) and streptomycin (100  $\mu$ g/ml) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The two cell lines were tested and validated by the Department of Cell Biology, Institute of Basic Medical Science, Chinese Academy of Medical Science (Beijing, China) and the Cell Resource Center of the Shanghai Institute of Biological Science (Shanghai, China).

**MTT cell proliferation assay.** HCC38 breast cancer cells were seeded in 96-well plates at a density of 2x10<sup>3</sup> cells/well in serum-free RPMI-1640. On the following day, cells were treated at 37°C with DAPT (10, 20 and 40  $\mu$ M), GDC-0449 (10, 20 and 40  $\mu$ M), salinomycin (10, 20 and 40  $\mu$ M) for 24 h, or ruxolitinib (1, 10 and 20  $\mu$ M) and stattic (1, 10 and 20  $\mu$ M) for 72 h. Dimethyl sulfoxide (DMSO) was used as a vehicle

Table I. Signaling pathways and inhibitors.

Signaling pathway inhibitors	Signaling pathways	Targets
DAPT	Notch	$\gamma$ -secretase
GDC-0449	Hedgehog	SMO
Salinomycin	Wnt/ $\beta$ -catenin	$\beta$ -catenin
Ruxolitinib	JAK/STAT	JAK
Stattic	JAK/STAT	STAT3

DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; SMO, smoothened; JAK, janus kinase; STAT, signal transducer and activator of transcription; GDC-0449, vismodegib.

control. MTT reagent was added and incubated for 1 h at 37°C. The absorbance was measured at 570 nm using a SynergyHT microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Data were analyzed using Excel 12.0 (Microsoft Corporation, Redmond, WA, USA).

**Apoptosis assay by flow cytometry.** HCC38 cells were treated with vehicle or each signaling pathway inhibitor for the indicated time, and 1x10<sup>6</sup> cells were subsequently trypsinized to obtain a single-cell suspension. Apoptosis analysis was performed by flow cytometry using an Annexin V Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. Cells were stained with Annexin V-fluorescein isothiocyanate and propidium iodide on ice for 20 min prior to analysis. Data acquisition was performed on an LSR-II flow cytometer (BD Biosciences) with FACSDiva 8.0.1 software (BD Biosciences).

**Mammosphere formation assay.** For primary mammosphere culture, HCC38 cells were harvested from monolayer culture and resuspended by gentle aspiration to obtain a single-cell suspension. The cells were subsequently seeded at a density of 1x10<sup>5</sup> cells/well in ultra-low attachment 6-well plates (Costar; Corning, Inc., Corning, NY, USA), and grown in serum-free Dulbecco's modified Eagle's medium/F12 (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 2% B27, 20 ng/ml EGF, 20 ng/ml bFGF, 6.25  $\mu$ g/ml penicillin, 100  $\mu$ g/ml streptomycin, 1 ng/ml hydrocortisone and 10 mg/ml insulin. Cells were divided into two groups; one group was pretreated with DMSO or signaling pathway inhibitors on the following day; and the other was treated immediately following mammosphere formation. After incubation for 7 or 14 days following the treatments, mammospheres >50  $\mu$ m in diameter were counted and imaged under an inverted light microscope (Olympus IX51; Olympus Corporation, Tokyo, Japan; magnification, x20).

**Matrigel invasion assay.** A cell invasion assay was performed using 24-well BD biocoat Matrigel invasion chambers with an 8.0- $\mu$ m pore size (BD Biosciences) according to the manufacturer's protocol. In total, 4x10<sup>4</sup> HCC38 cells were loaded into the Matrigel-coated upper chamber filled with 500  $\mu$ l serum-free RPMI containing DMSO or a signaling pathway

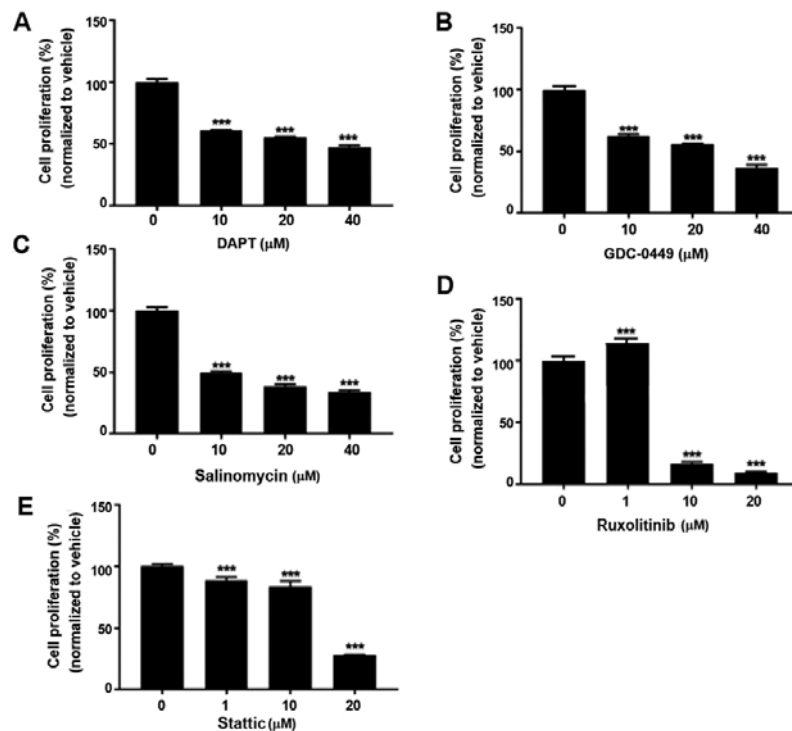


Figure 1. Anti-proliferative effects of the signaling pathway inhibitors on HCC38 cells. Cell proliferation was measured using an MTT assay following cell treatment with (A) DAPT, (B) GDC-0449, (C) salinomycin, (D) ruxolitinib and (E) stattic. Data are presented as the mean  $\pm$  standard deviation.  $n=4$ . \*\*\* $P<0.001$  vs. respective 0  $\mu\text{M}$ . DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester; GDC-0449, vismodegib.

inhibitor. To induce cell invasion, 10% FBS-containing RPMI was loaded into the lower chamber. Following incubation overnight, non-invading cells remaining in the upper chamber were removed with a cotton swab. The invading cells that were adhered to the lower surface were fixed for 20 min in 4% para-formaldehyde at 4°C and stained in 0.1% crystal violet solution at 25°C for 15 min using Diff-Quik (Siemens AG, Munich, Germany). The stained cells were counted in five randomly selected fields under an inverted light microscope (Olympus IX51; Olympus Corporation; magnification,  $\times 20$ ).

**Western blot analysis.** HCC38 cells were treated with DMSO or different concentrations of signaling pathway inhibitors for 24 h and lysed with lysis buffer (Cell Signaling Technology, Danvers, MA, USA; cat. no. 9803) on ice. Cell lysates were collected by centrifugation at  $13,800 \times g$  at 4°C for 10 min. The protein concentration was measured using a bicinchoninic acid protein assay kit with bovine serum albumin, according to the manufacturer's protocol (Beyotime Institute of Biotechnology, Haimen, China; cat. no. P0010). Subsequently, protein samples were heated at 95°C for 5 min in loading buffer. In total, 30  $\mu\text{g}$  protein was loaded in each lane and separated by 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane and blocked for 1 h with 5% non-fat milk in Tris-buffered saline containing Tween-20 (TBST) at room temperature. Subsequently, the membranes were incubated overnight with primary antibodies against cleaved Notch1 (cat. no. 4147; 1:1,000), zinc finger protein GLI1 (cat. no. 3538; 1:1,000),  $\beta$ -catenin (cat. no. 8480; 1:1,000; all Cell Signaling Technology, Inc.), STAT3 (cat. no. ab68153; 1:1,000), phospho-STAT3 (cat. no. ab76315; 1:2,000), JAK1 (cat. no. ab133666; 1:1,000), JAK2 (cat. no. ab108596; 1:2,000) and  $\beta$ -actin (cat.

no. ab8226; 1:1,000; all Abcam, Cambridge, UK) at 4°C. The membranes were washed with TBST three times, subsequently incubated with horseradish peroxidase-conjugated secondary antibodies [cat. no. 7076, anti-mouse immunoglobulin G (IgG), 1:2,000; cat. no. 7074, anti-rabbit IgG, 1:2,000; both Cell Signaling Technology, Inc.] for 1 h at room temperature, and washed with TBST. An enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.; cat. no. 32106) was used and the signal was detected using the ChemiScope 5300 chemiluminescence system (Clinx Science Instruments Co. Ltd., Shanghai, China) and quantified using Quantity One software (v4.6.6; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Mouse xenograft and orthotopic tumor models.** In total, 54 female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (National Institute of Food and Drug Control of China, Beijing, China), 5-6 weeks old and weighing 15-20 g were bred and maintained under specific pathogen-free conditions (temperature at 18-29°C; air changes 10-20/h; air velocity  $<0.18$  m/sec; 12 h light/dark cycle; and free access to food and water) at The Animal Experiment Center, Basic Medical College of Jilin University (Changchun, China). All the animal care details and procedures described in the present study were approved by the Ethics Committee of The First Hospital of Jilin University. All animal experiments were performed in accordance with guidelines for proper conduct of animal experiments (Jilin University). The MTT assay determined that 10  $\mu\text{M}$  was the value close to the half maximal inhibitory concentration ( $\text{IC}_{50}$ ) of the majority of the inhibitors in the present study. The cells were more sensitive to ruxolitinib compared with the other inhibitors; 3  $\mu\text{M}$  was the value close to the  $\text{IC}_{50}$  of ruxolitinib. Triple-negative and  $\text{ALDH1}^+$

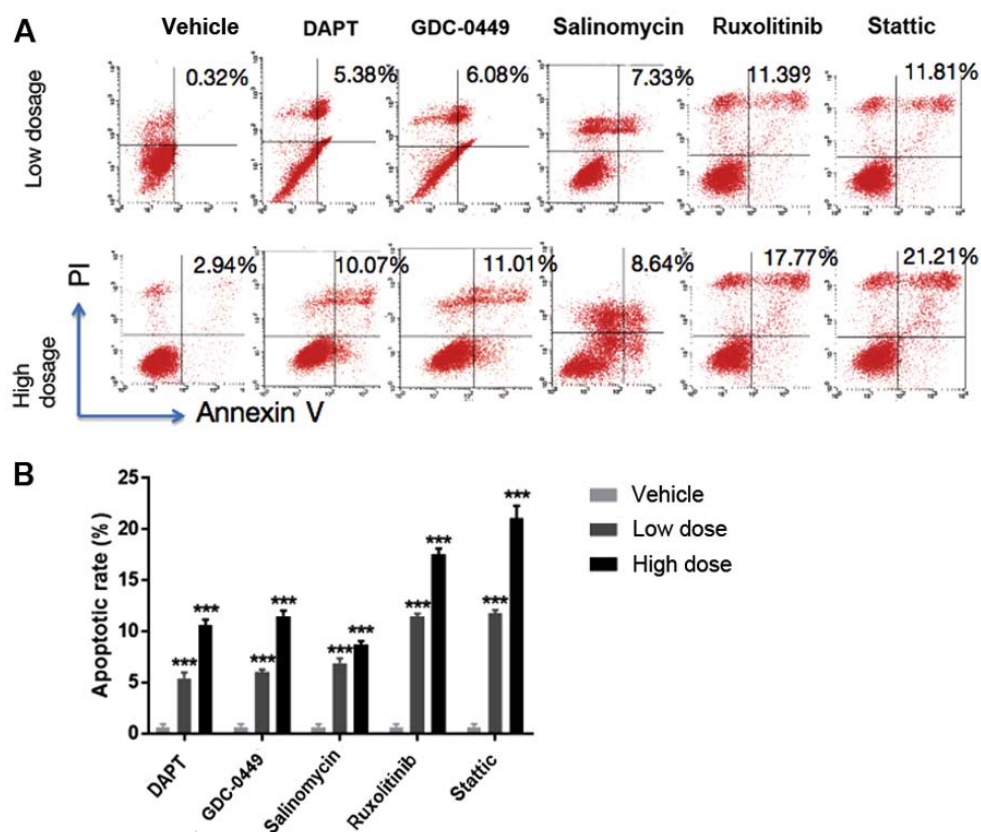


Figure 2. Pro-apoptotic effects of signaling pathway inhibitors on HCC38 cells. (A) Apoptotic rates of HCC38 cells treated with vehicle, DAPT, GDC-0449, salinomycin, ruxolitinib and stattic. (B) Quantification of the apoptotic rates. Data are presented as the mean  $\pm$  standard deviation. All experiments were repeated at least three times.  $n=3$ . \*\*\* $P<0.001$  vs. respective vehicle. DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester; GDC-0449, vismodegib; PI, propidium iodide.

HCC1806 cells were pretreated *in vitro* with DMSO, DAPT (10  $\mu$ M), GDC-0449 (10  $\mu$ M), salinomycin (10  $\mu$ M), ruxolitinib (3  $\mu$ M) or stattic (10  $\mu$ M) for 4 h prior to mixing with an equal volume of Matrigel (BD Biosciences). The mice were randomly divided into six groups ( $n=9$ /group) and injected with a mixture of pretreated HCC1806 cells and Matrigel into the mammary fat pad, as previously described (18). Following the initial appearance, tumors were measured every 2 days using a caliper. Tumor volumes were calculated using the formula (length  $\times$  width<sup>2</sup>)/2 (19). The tumor-free survival rate of mice was analyzed using the Kaplan-Meier method and the log rank test.

**Statistical analysis.** All experiments were repeated at least three times. Data are presented as the mean  $\pm$  standard deviation. Statistical significance was assessed using Student's *t*-test or one-way analysis of variance to compare multiple groups followed by Tukey's or Welch's *t*-test (variances are not equal) to conduct multiple comparisons between the groups with SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA).  $P<0.05$  was considered to indicate a statistically significant difference.

## Results

**Signaling pathway inhibitors suppress BCSC proliferation.** To determine if various signaling pathway inhibitors have effects on BCSC proliferation, an MTT assay was conducted

on HCC38 cells. As presented in Fig. 1, the inhibitors generally suppressed the proliferation of HCC38 cells in a dose-dependent manner, suggesting the anti-proliferative roles of these inhibitors in BCSCs.

**Signaling pathway inhibitors induce apoptosis of BCSCs.** To further investigate if the five signaling pathway inhibitors induce BCSC apoptosis, which is another important cellular event in breast cancer therapy in addition to cell proliferation, a flow cytometry assay was performed in HCC38 cells. As presented in Fig. 2, treatment with DAPT, GDC-0449, salinomycin, ruxolitinib and stattic resulted in a significantly increased apoptotic percentage  $\leq 10.07$ , 11.01, 8.64, 17.77 and 21.21%, respectively, compared with the vehicle-treated cells ( $P<0.001$ ), suggesting the pro-apoptotic roles of these inhibitors in BCSCs.

**Signaling pathway inhibitors suppress invasion of BCSCs.** Cell invasion is a key process in cancer metastasis (20). To investigate the potential effects of the signaling pathway inhibitors on the capacity of BCSC invasion, invasion assays were performed. As presented in Fig. 3, a significantly lower number of invading HCC38 cells was observed with treatment with DAPT, GDC-0449, salinomycin, ruxolitinib and stattic, compared with the vehicle-treated group ( $P<0.001$ ), suggesting that the signaling pathway inhibitor-mediated suppression of BCSC invasion is a possible mechanism in metastatic breast cancer therapy.



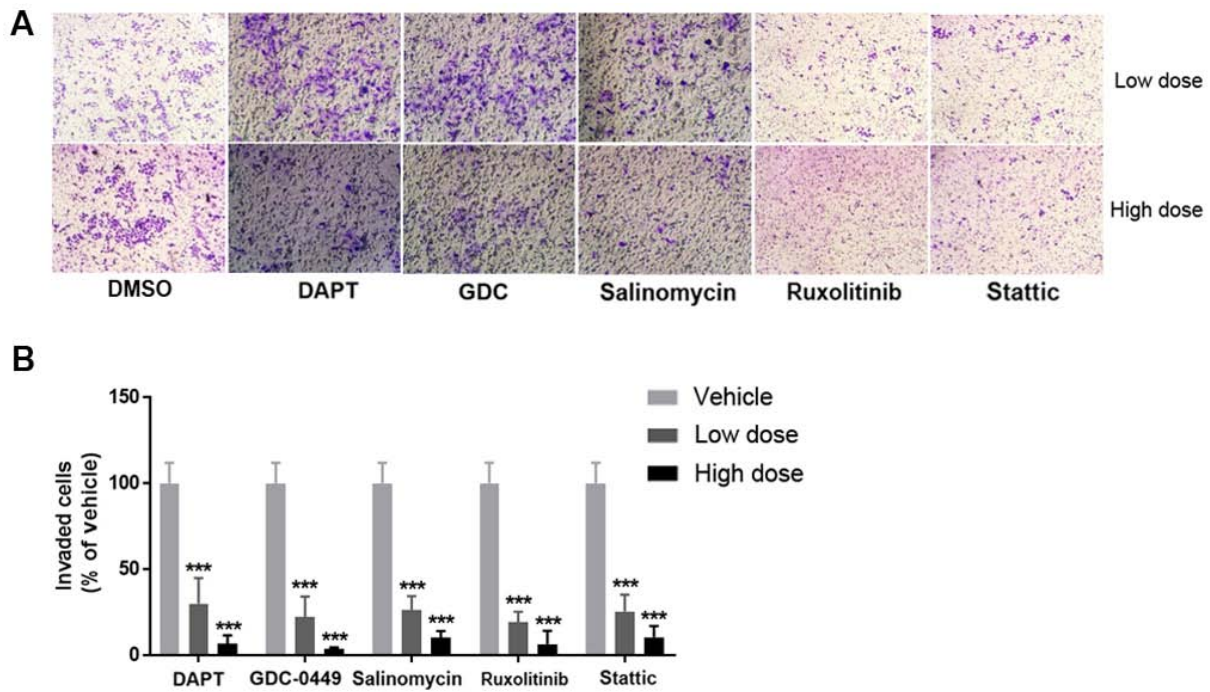


Figure 3. Signaling pathway inhibitors suppress invasion of HCC38 cells. (A) Representative images of invading cells in the Transwell assay are presented. Magnification, x20. (B) Quantification of the number of invaded cells. All experiments were repeated at least three times. n=6. Data are presented as the mean  $\pm$  standard deviation. \*\*\*P<0.001 vs. respective vehicle. DMSO, dimethyl sulfoxide; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester; GDC, GDC-0449/vismodegib.

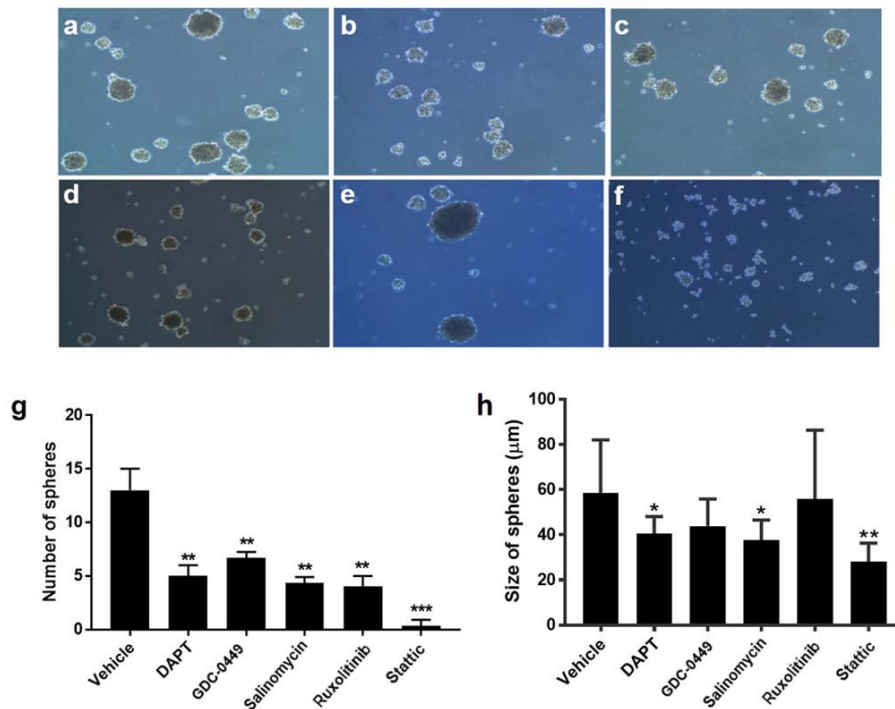


Figure 4. Signaling pathway inhibitors suppress mammosphere formation of HCC38 cells. Morphology of HCC38 cell-formed mammospheres after a 7-day pretreatment with (a) vehicle, (b) 20  $\mu$ M DAPT, (c) 20  $\mu$ M GDC-0449, (d) 40  $\mu$ M salinomycin, (e) 10  $\mu$ M ruxolitinib or (f) 5  $\mu$ M stattic under a microscope. Magnification, x20. (g) Quantification of the number of mammospheres formed after a 7-day incubation with the inhibitors. (h) Quantification of the size of mammospheres formed after a 7-day incubation with the inhibitors. All experiments were repeated at least three times. n=3. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 vs. vehicle. DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester; GDC-0449, vismodegib.

**Signaling pathway inhibitors suppress BCSC self-renewal.** As stem cell self-renewal serves a critical role in stem cell proliferation and differentiation, which are closely associated

with cancer development (21,22), it was investigated whether the five inhibitors affect BCSC self-renewal. A suspension mammosphere assay, which is commonly used for measuring

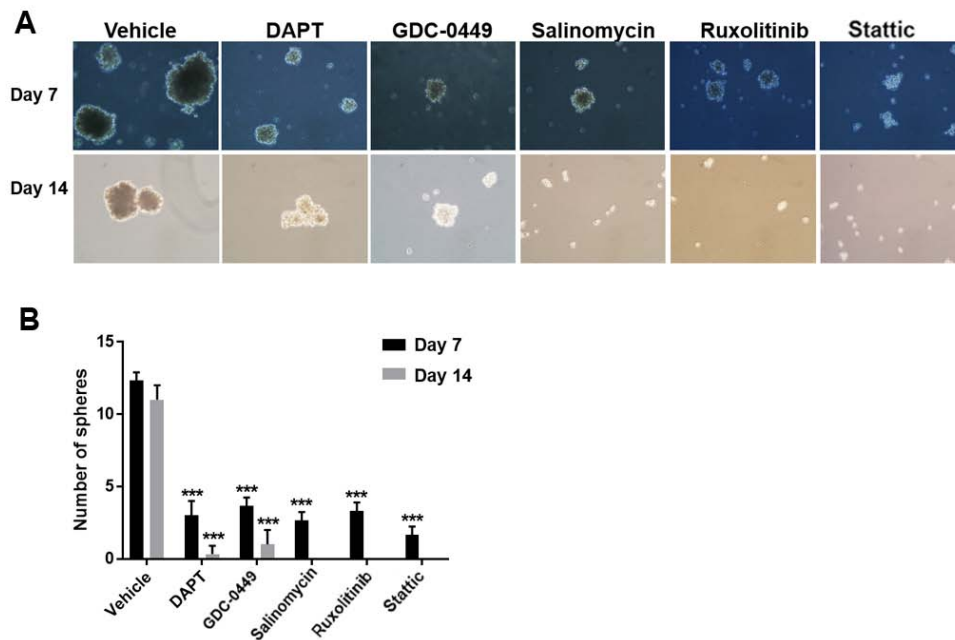


Figure 5. Signaling pathway inhibitors promote the disassembly of HCC38 cell-formed mammospheres. (A) Morphology of HCC38-formed mammospheres treated with signaling pathway inhibitors immediately following mammosphere formation. Magnification, x20. (B) Quantification of the number of mammospheres formed after 7-day and 14-day incubations following the treatments. All experiments were repeated at least three times.  $n=3$ . \*\*\* $P<0.001$  vs. respective vehicle. DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanine]-*S*-phenylglycine *t*-butyl ester; GDC-0449, vismodegib.

stem cell activity and *in vitro* stem/progenitor cell frequency, was performed on HCC38 cells. The results demonstrated that pretreatment with signaling pathway inhibitors prior to mammosphere formation markedly decreased the sphere size and the number of HCC38 cells, compared with the vehicle-treated control (Fig. 4). Among the inhibitors, stattic was the most potent one, as demonstrated by the lack of any mammospheres (Fig. 4f-h).

Similarly, treatment with the signaling pathway inhibitors immediately following mammosphere formation failed to maintain the structure of the formed mammospheres (Fig. 5). Following prolonged incubation, treatment with salinomycin, ruxolitinib and stattic even led to further disassembly of mammospheres (Fig. 5). These results demonstrated that the signaling pathway inhibitors diminished the tumor-sphere-forming ability of BCSCs and the maintenance of BCSC-formed mammospheres, suggesting the negative roles of these inhibitors in the BCSC self-renewal process.

**Signaling pathway inhibitors suppress expression and phosphorylation of downstream targets.** As the inhibitors block signal transduction, they were predicted to inhibit the activity of their downstream target molecules. In general, these inhibitors markedly decreased the expression or phosphorylation of their corresponding downstream signaling molecules in a dose-dependent manner, as demonstrated in Fig. 6.

**Signaling pathway inhibitors suppress the tumor-forming ability of TNBC.** To determine the effects of the signaling pathway inhibitors on the breast tumor-forming ability *in vivo*, HCC1806 cells pretreated with vehicle or inhibitors were injected into NOD/SCID mice. It was observed that all mice injected with vehicle-treated HCC1806 cells developed mammary tumors at 7 days following injection (Fig. 7A). In

contrast, mice injected with inhibitor-treated HCC1806 cells exhibited a delay in tumor formation and a decrease in tumor incidence (Fig. 7A). At 21 days after injection, all the mice in the treatment groups exhibited a significant decreased tumor volume compared with the control group (Fig. 7B and C;  $P<0.05$ ), suggesting that these inhibitors suppress the growth of BSBC-derived tumors *in vivo*.

## Discussion

The present study aimed to determine if inhibitors of the Notch, Hedgehog, Wnt and JAK/STAT signaling pathways may be used as potential therapeutic agents targeting BCSCs in TNBC. It was demonstrated that the five signaling pathway inhibitors, DAPT, GDC-0449, salinomycin, ruxolitinib and stattic, individually suppressed the proliferation and promoted the apoptosis of HCC38 cells, a TNBC cell line with stem cell-like characteristics ( $CD44^{+}/CD24^{low/-}$  and  $ALDH^{+}$ ) that exhibits the features of BCSCs. The invasion assay demonstrated that the five inhibitors significantly decreased HCC38 invasion compared with the control group, suggesting their suppressive effects on the breast cancer invasive capacity. In addition, these inhibitors blocked the BCSC mammosphere process by preventing mammosphere formation and promoting mammosphere disassembly, suggesting that these inhibitors may simultaneously inhibit the proliferation and differentiation of cancer stem cells. However, it was observed that GDC-0449 and ruxolitinib had no significant inhibitory effects on the mammosphere size. A possible explanation for this discrepancy is that the mammospheres were no longer sensitive to the present drug concentrations when they grew to a given size ( $40\ \mu\text{m}$ ). The mammospheres  $>40\ \mu\text{m}$  had limited drug contact with interior HCC38 cells due to their large size. Therefore, higher concentrations may be required to increase

Downstream of the target genes over a DMSO control after treatment with the inhibitors

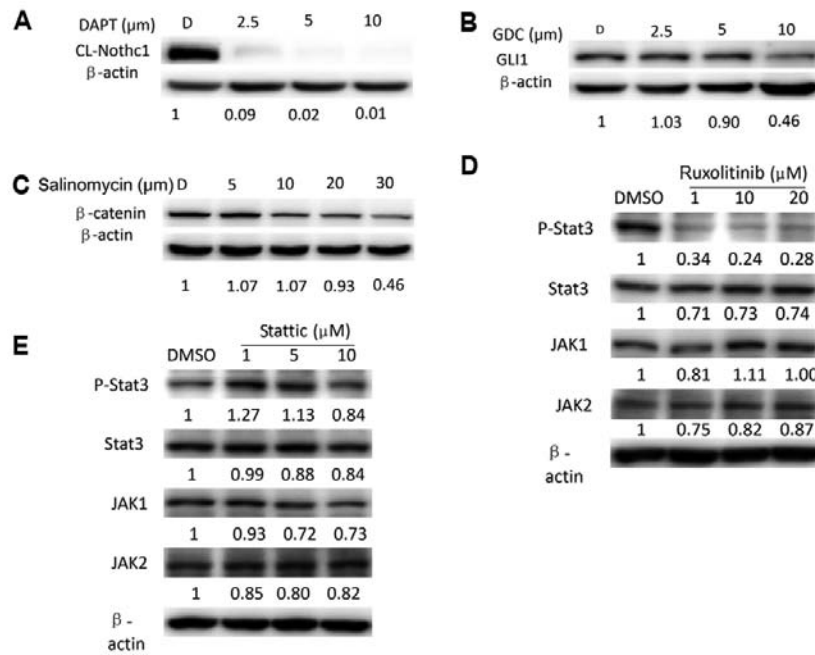


Figure 6. Signaling pathway inhibitors suppress expression or phosphorylation of downstream targets. Western blot analysis for the expression of (A) cl-Notch1, (B) GLI1, (C) β-catenin. Expression of p-STAT3, total STAT3, JAK1 and JAK2 in HCC38 cells treated with different concentrations of the (D) ruxolitinib and (E) stattic for 24 h. All experiments were repeated at least three times. STAT3, signal transducer and activator of transcription; JAK, Janus kinase; p, phosphorylated; cl, cleaved; DMSO, dimethyl sulfoxide; GLI1, zinc finger protein GLI1.

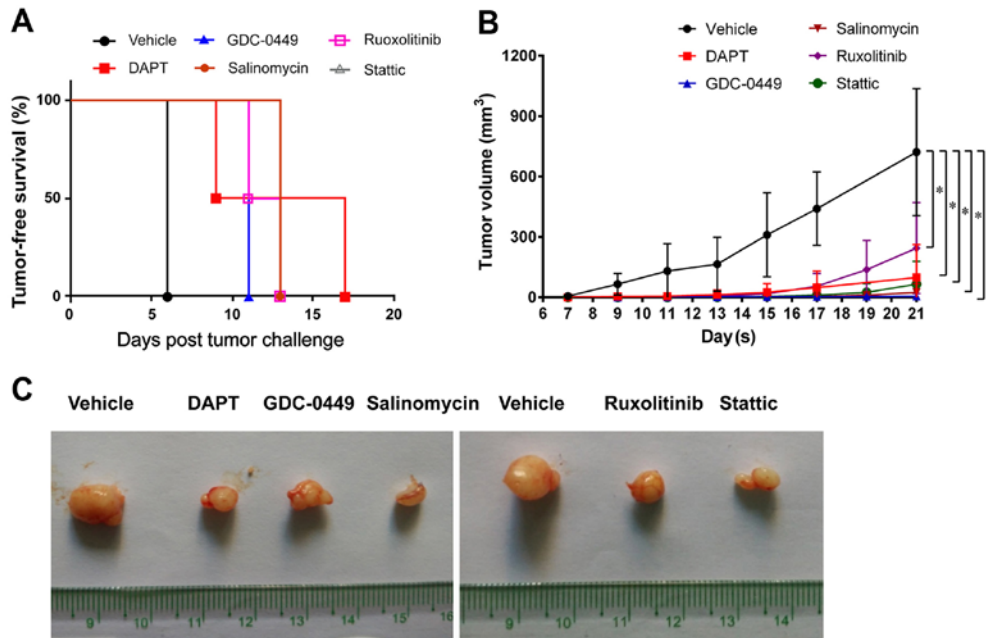


Figure 7. Signaling pathway inhibitors suppress the tumor-forming ability of HCC1806 cells. (A) Kaplan-Meier tumor-free survival curves are presented. n=3 mice/group. Mice that received subcutaneous injections developed tumors following challenge with vehicle or signaling pathway inhibitor-treated HCC1806 cells. (B) Tumor growth curves are presented. n=3 mice/group. (C) Representative images of the tumors at 21 days after tumor inoculation. n=3. \*P<0.05 vs. vehicle. DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester; GDC-0449, vismodegib.

the sensitivity of the mammospheres to these two inhibitors; further investigation in a future study is required. In the present study, only HCC38 cells (100% ALDH<sup>+</sup>) were used for the *in vitro* study as they are considered an ideal candidate cell line of BCSCs (23). To the best of the authors' knowledge, at present, no other cell line shares more characteristic features

of BCSCs than HCC38. Therefore, data from HCC38 cells are representative of an *in vitro* BCSC study.

DAPT is used as a γ-secretase inhibitor to block Notch signaling (24). The present study demonstrated that DAPT downregulated the expression of cl-Notch1, the activated form of the Notch1 receptor, ≤99% in HCC38 cells, demonstrating that

DAPT prevents the final cleavage step of the Notch1 receptor by inactivating  $\gamma$ -secretase and subsequently decreasing the expression level of cl-Notch1 (25). As the Notch signaling pathway is dysregulated due to the overexpression of Notch receptors and their ligands in human breast cancer (26,27), the expression of cl-Notch1 is a notable biomarker for therapeutic efficacy of drugs or agents in breast cancer (28,29). Additionally, it was demonstrated that DAPT suppressed BCSC proliferation and tumor growth *in vitro* and *in vivo*. Therefore, DAPT may serve as a promising therapeutic agent in breast cancer by targeting the Notch signaling pathway in BCSCs.

GDC-0449 was used in the present study to target the Hedgehog signaling pathway, which is normally in a resting state; however, is activated in response to carcinogenic stimuli by hedgehog ligands binding to a transmembrane receptor called Patched (PTCH) (30). The hedgehog signaling pathway serves a significant role in cancer development and progression in various malignancies, including breast cancer (31-34). Following the hedgehog ligand-PTCH binding, smoothened (SMO) protein initiates the signaling cascade by activating GLI transcription factors, which in turn drive the expression of a variety of target genes that are associated with carcinogenesis (35,36). In the present study, treatment with 40  $\mu$ M GDC-0449 led to ~50% decrease in the GLI1 protein expression level in HCC38 cells, which is likely due to the direct inhibition of SMO by GDC-0449. Consistently, GDC-0449 inhibited BCSC proliferation, invasion and mammosphere formation, while inducing BCSC apoptosis. Based on the present *in vivo* data, it was hypothesized that SMO is undetectable in normal breast tissue however, is increased in breast cancer tissues. Consistently, SMO has been identified to be ectopically expressed in 70% ductal carcinoma *in situ* and 30% invasive breast cancer in an animal model (37), suggesting that inhibition of SMO is a valuable therapeutic strategy against BCSCs. Previously, GDC-0449 was approved by the US Food and Drug Administration for the treatment of advanced basal-cell carcinoma (38-40), which may result in the future clinical application of GDC-0449 in breast cancer therapy.

Salinomycin was originally developed as a commercial antibiotic in veterinary medicine (41). A previous study demonstrated that salinomycin may additionally be used as an effective drug against breast cancer by targeting drug-resistant BCSCs (41). Previously, Lu *et al* (42) observed that salinomycin inhibits Wnt/ $\beta$ -catenin signaling, a key signaling pathway supporting self-renewal of normal and malignant mammary stem cells (43). Enhanced Wnt signaling contributes to cell proliferation in the majority of breast cancer by downregulating the expression of secreted Frizzled-related protein 1, a negative Wnt pathway regulator (44). These results suggested that salinomycin is a promising anticancer drug by inhibiting Wnt/ $\beta$ -catenin signaling. The present results demonstrated that salinomycin, by markedly decreasing the expression of  $\beta$ -catenin, significantly suppressed BCSC proliferation and mammosphere formation *in vitro* in addition to tumor formation *in vivo*.

Ruxolitinib (Jakafi) and stattic are inhibitors of the JAK/STAT3 pathway, another important pathway in normal and cancer stem cells, which is considered a promising therapeutic target (45). JAKs phosphorylate STATs, thus subsequently activating the signaling pathway and various target genes (46). Ruxolitinib and stattic inhibit JAKs and

STATs, respectively (47,48). In the present study, ruxolitinib markedly downregulated the expression of phospho-STAT3; however, appeared to have no marked effects on JAK1 and JAK2 expression. A possible explanation for this discrepancy is that the activities of JAK1 and JAK2 are determined by their phosphorylation levels, not by their protein expression levels. However, stattic did not appear to markedly alter the expression of all four proteins. Phosphorylation levels of JAK1 and JAK2 require examination to further conform the role of stattic in suppressing breast cancer in future studies. Furthermore, further investigation is required to examine the expression pattern of SMO in the inhibitors-treated BCSCs, which may provide novel insight for the underlying mechanisms.

Although a number of previous studies demonstrated that these five signaling inhibitors exert inhibitory effects on TNBC in animal models and in clinical trials (49-53), in the present study, it was demonstrated that all five signaling inhibitors suppressed stemness of BCSCs in extensive aspects, including proliferation, invasion, apoptosis, self-renewal, mammosphere formation and tumorigenesis. In addition, the inhibition efficacy of these five inhibitors against TNBC was compared. The present *in vitro* and *in vivo* data demonstrated that these five signaling inhibitors, individually or in combination, may be applied clinically with high efficacy.

In conclusion, it was demonstrated that the signaling pathway inhibitors suppressed BCSC proliferation, invasion and mammosphere number, while inducing apoptosis. As these signaling pathway inhibitors, with the exception of stattic, have been applied clinically or in clinical trials for the intervention of disorders other than TNBC, including operable basal cell carcinoma, myelofibrosis and coccidial infection (54-60), they may provide novel therapeutic options for TNBC.

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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

WL, HY and XL performed the examination. XL and LH prepared the figures. LH and NX conducted the statistical analyses. AS analyzed and interpreted the data, and was the principal contributor in writing the manuscript. All authors read and approved the final manuscript.



## Ethics approval and consent to participate

All the animal care details and procedures described in the present study were approved by the Ethics Committee of The First Hospital of Jilin University (Changchun, China). All animal experiments were performed in accordance with guidelines for proper conduct of animal experiments (Jilin University).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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