Synthesis and Evaluation of Naphthalene Derivatives as Potent STAT3 inhibitors and Agents Against Triple-Negative Breast Cancer Growth and Metastasis

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Research Article

Keywords: TNBC, STAT3, SMY002, Cyclin D1, MMP9

Posted Date: September 27th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1238979/v2>

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Abstract

Purpose

Triple-negative breast cancer (TNBC) represents the worst prognostic subtype of breast cancer and lacks targeted therapeutic drugs. Signal transducer and activator of transcription 3 (STAT3) is overexpressed and constitutively activated in TNBCs and associated with poor patient outcomes. However, no agents targeting STAT3 have been successfully developed and marketed. Selective Estrogen Receptor Modulators (SERMs) have been reported as potential inhibitors of the IL-6/STAT3 signaling pathway. Naphthalene compounds have good pharmacological activity and significant anti-cancer activity. In this study, we synthesized a new series of naphthalene derivatives with the general structure of SERM and evaluated their effects on TNBC and STAT3 signals.

Methods

A new series of compounds based on the scaffold of SERMs and an amino group were designed and screened based on the structure-activity relationship by MTT assay. The binding activity of SMY002 to STAT3 was predicted and validated by docking and SPR. The STAT3 signaling target and anti-cancer effects of SMY002 were evaluated with three TNBC cell lines and the mice transplanted tumor model.

Results

Among the compounds, SMY002 displayed the most potent activity, which could directly interact with STAT3 SH2-domain, and strongly inhibit the phosphorylation, dimerization, nuclear distribution, transcriptional activity, and target genes expression of STAT3. Furthermore, SMY002 markedly suppressed migration, invasion, survival, growth, and metastasis of TNBC cells in vitro and in vivo via down-regulating the expression of Cyclin D1 and MMP9.

Conclusions

SMY002 can significantly inhibit the growth and metastasis of TNBC cells by targeting the STAT3 signal.

Introduction

Breast cancer (BC) has become the most prevalent cancer and the leading cause of cancer-related mortality worldwide^[1]. About 10–15% of BC patients are triple-negative breast cancers (TNBCs)^[2]. Due to the lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), TNBC patients are insensitive to endocrine therapy and HER2-targeted drugs^[3]. Moreover, TNBCs are prone to relapse or metastasis in the early stage because of their

substantial heterogeneity and invasiveness. The poor outcome highlights the necessity to exploit more efficacious agents for TNBC therapy.

Signal transducer and activator of transcription 3 (STAT3) plays essential roles in multiple signaling pathways. A prominent function of STAT3 is to transmit signals from the cell membrane to the nucleus and activate the transcription of its targeted genes. Many cytokines and growth factors can activate Janus kinases (JAKs) by binding to their receptors (such as IL-6/gp130)^[4]. STAT3 can be phosphorylated at tyrosine 705 (Tyr705) by JAKs, Src, or BCR–ABL1 enzymes and form dimmers through the Src homology domain 2 (SH2) of STAT3. The STAT3 dimmers can enter the nucleus^[5–7]. Then, STAT3 directly binding to the promoters of its target genes and regulates the transcription of these genes, such as CCND1, BCL-XL, SURVIVIN, and matrix metalloproteases (MMPs)^[8–11]. High expression of these genes is associated with cancer proliferation, survival, angiogenesis, metastasis, and immune evasion. Extensive studies certified that STAT3 is overexpressed or constitutively activated in most human cancers, including TNBCs, and is closely related to malignant progression^[12–14].

Naphthalene compounds have been widely developed into anti-tumor drugs for their good pharmaceutical activity, chemical stability, and cellular permeability. Amonafide and Mitonafide were the most representative naphthalene compounds, showing excellent activity in leukemia cells and solid tumors^[15, 16]. Wang and coworkers synthesized a novel naphthoquinone derivative, NTDMNQ, which significantly induced apoptosis by inhibiting the phosphorylation of MAPK/AKT/STAT3 signalings in gastric cancers^[17]. These studies indicate that naphthylamine compounds offer the opportunity to be modified into STAT3 target anti-cancer drugs.

Accumulated evidence proved that selective estrogen receptor modulators (SERMs) such as Badoxifene and Raloxifene exhibit anti-cancer effects by inhibiting IL-6/GP130/STAT3 signaling^[18, 19]. Here, we synthesized a new series of naphthylamine derivatives with the structure of 4-nitronaphthol and SERM as scaffolds. Among them, SMY002 can effectively inhibit the phosphorylation and transcriptional activity of STAT3 by interaction with the SH2 domain. Moreover, SMY002 suppressed the viability, survival, mobility, and dissemination of TNBC cells. Therefore, SMY002 holds the potential to be developed as STAT3 inhibitory tools or anti-TNBC treatment agents.

Materials And Methods

Cell Lines and Reagents

MCF-10A, MCF-7, 4T1, MDA-MB-468, and MDA-MB-231 cell lines were obtained from the American Type Culture Collection (ATCC). 4T1, MCF-7, and MDA-MB-231 were grown in Dulbecco's modified Eagle's medium (DMEM, HyClone) supplemented with 10% FBS (Biological Industries, Israel). MDA-MB-468 cells were cultured with Leibovitz's L-15 Medium (Solarbio, China) containing 10% FBS in an incubator without CO₂. MCF-10A were cultured with DMEM/F12 supplemented with 5% Horse Serum, 20 ng/ml EGF, 0.5 µg/ml Hydrocortisone, 10 µg/ml Insulin, and 1% NEAA. Recombinant human interleukin-6 (rhIL-6) was

obtained from R&D Systems. DMSO, MTT, and other conventional reagents without additional instructions were obtained from Sigma-Aldrich (US).

Computational Docking

We obtained the crystal structure of the STAT3-SH2 domain from the RCSB protein data bank (PDB code: 1BG1). We prepared its form by removing substructures, repairing the sidechains, and adding hydrogens and charges with AutoDock Vina. The results were obtained based on SCORE and the protein-compound complex analysis.

Surface Plasmon Resonance (SPR) assay

The procedure was as described previously^[20]. Briefly, phosphorylated and non-phosphorylated biotinylated EGFR-derived dodecapeptides containing the STAT3 peptide-binding motif surrounding Y1068 were immobilized to CM5 chips (GE Healthcare, Sweden)^[21]. The running buffer was prepared with Tris buffer (20 mM, pH 8.0), mercaptoethanol (2 mM), and DMSO (5%). SMY002 was diluted by running buffer to 1, 3, 10, 30, 100, 300, 1000 μ M and pre-incubated with p-STAT3 (Tyr705) peptide (500 nM, Abcam, ab179551) at 4 °C for 2 h. P-STAT3 (Tyr705) peptide's binding to EGFR-derived dodecapeptides was measured at a flow rate of 10 μ L/min for 2 min. The IC₅₀ values were analyzed by the Biacore T200 Evaluation Software Version 3.0.

Cytotoxic assay

Cells were cultured in 96-well plates with a density of 5×10^3 per well in triplicate. The next day, SMY002 was added at a concentration range from 0.01 to 100 μ M for 48 h. DMSO was utilized as the solvent. Cells exposed to SMY002-free DMSO were used as controls. The data was measured by MTT assay, compared with control cells, and converted to a percentage. The inhibitory effect was analyzed by GraphPad Prism 6.0.

Luciferase assay

Cells were cotransfected with the STAT3-Luc Reporter gene plasmid (YT455, Biolab Technology of Beijing, China) and pRL-TK vector (Promega, US) at a ratio of 1:200 (w/w) with Lipofectamine 2000 (Invitrogen, US). 48 h later, SMY002 was added to corresponding wells for another 24 h. Cells were extracted and assayed with the Dual-Luciferase Reporter Assay System (E1910, Promega, US). The luminescence values were examined by a multifunctional microplate reader (Varioskan Flash, Thermo-Fisher, US). The STAT3-dependent promoter activity was determined by the ratio of firefly luminescence value/Renilla luminescence value.

Real-Time PCR

The primers used were as follows: GCTGCGAAGTGGAACCATC and CTCCTTCTGCACACATTTGAA for CCND1; TGCATTGTTCCCATAGAGTTCCA and CCTGAATGACCACCTAGAGCCTT for BCL-XL;

TGTACCGCTATGGTTACACTCG and GGCAGGGACAGTTGCTTCT for MMP9; and CACGATGGAGGGGCCGGAATCATC and TAAAGACCTCTATGCCAACACAGT for β -actin. Real-time PCR was analyzed using SYBR Green PCR Master Mix as recommended by the manufacturer (Thermo-Fisher, US). The cycle threshold (CT) values of target genes were standardized by that of β -actin. All the data were analyzed with the comparative quantitative method ($2^{-\Delta\Delta Ct}$).

Immunoblotting, Immunofluorescence, and Immunohistochemistry

Antibodies against p-JAK2 (3776), p-STAT3 (9134), JAK2 (3230), STAT3 (9139), p-ERK1/2 (4695), p-AKT (4691), CyclinD1 (55506), BCL-XL (2764), β -Actin (3700), and GAPDH (5174) used for western blot, immunofluorescence or immunohistochemistry were ordered from Cell Signaling Technology (CST, USA). Human MMP9 primary antibody (A2095) was ordered from ABclonal Technology (China). The Alexa Fluor 488-labeled rabbit secondary antibody for immunofluorescence was from Thermo (US). The secondary antibody for immunohistochemistry was obtained from a two-step immunohistochemistry detection kit (PV-9000, ZSGB-Bio, China).

Zymography assay

The secretion levels of MMP9 were analyzed with an MMP Zymography Assay Kit (P1700, Applygen, China). The cell culture supernatant was collected by pipettes and centrifuged (12,000 rpm, 15 min, 4 °C), then diluted with 2× nonreducing PAGE loading buffer (1:1), and 20 μ l samples were loaded and separated directly. An SDS-PAGE gel containing MMP9 substrate protein (substrate G) was prepared. The electrophoresis and stain of gels were performed according to the standard procedures. Gels were scanned on the GelDoc XR System (Bio-Rad, US).

Wound-Healing

Cells were plated in a monolayer with more than 90% fusion. A pipette tip was utilized to draw straight and evenly scratches in each well. Cells were rinsed with PBS. The culture solution was replaced with DMEM containing low serum (0.5% FBS) for each well, and SMY002 was added. Wound healing was observed and photographed with an Olympus CKX41 microscope system.

Transwell

For migratory ability assay, 2×10^5 cells suspended with 100 μ l DMEM were added to the upper chambers (8 μ m pore sizes, Corning, US) and supplemented with or without SMY002. For invasive ability assay, the upper chamber was coated with 100 μ l Matrigel (1 mg/ml, BD, US) prior to seed cells. 800 μ l of DMEM containing 20% FBS was added into the lower chambers. Cultured for 48 h, cells were fixed with methanol for 30 min and stained with 0.1% crystal violet for 30 min at room temperature. The inserts were immersed twice with PBS. The membranes were photographed using an Olympus CKX41 microscope system and counted by ImageJ software.

Cell Cycle and Apoptosis

Cells were trypsinized and rinsed with PBS. 1×10^6 cells were fixed in cold 70% ethanol (diluted with sterile water) for 2 h at 4 °C. The follow-up operations followed the instructions of the DNA content detection kit (Solarbio, China). The fluorescence of PI-stained cells was read by a flow cytometer (BD, US) at the wavelength 630 nm and analyzed with Cell Quest software. Apoptosis assays were performed using the Annexin V-FITC apoptosis detection kit (Solarbio, China) by flow cytometry.

Animal Studies

All animal experiments were conducted following the regulations of the Laboratory Animal Welfare and Ethics Committee and approved by the local authorities. Five-week-old female BALB/c mice were obtained from Beijing Vital River Laboratory Animal Technology. 4T1 cell suspension (1×10^5 cells/mouse) was subcutaneously injected into the mice. All mice were randomly divided into two groups, with five in each. After seven days of implantation, the mice were administered with PBS or SMY002 (30 mg/kg in PBS as a solvent) by daily gavage. The body weight and tumor sizes of mice were detected every other day. Four weeks after implantation, all mice were euthanized with isoflurane. The transplanted tumors were dissected, weighed, imaged, and fixed in 10% neutral formalin.

Statistical Analysis

All data are presented as the mean $\pm$ s.d. Two groups were compared by Student's t-test. For multiple group comparisons, statistical analyses were performed by one-way or two-way ANOVA using Graph-Pad Prism 6.0 software was considered significantly different.

Results

Structure design, screening, and synthesis of SMY002

According to the MTT assays (SI. Fig. S1), compounds with 4-nitronaphthol and SERMs (such as Badoxifen and Raloxifene) have moderate anti-tumor activity in TNBC cells. The general structure of 4-nitronaphthol and SERMs were used as the reference scaffold of potent STAT3 inhibitors (Fig.1A). Amino group replacing hydroxyl designation was introducing more polar interaction. The amide group substituted by the ethoxy group aimed to reduce steric hindrance between the phosphorylation binding site of the STAT3 SH2 domain and compounds. Compounds were grouped and ranked in decreasing pharmacophore similarity in MTT assays against 4T1 cells (Fig. 1B and Si. Fig. S2). The compounds in the second group showed potent inhibitory activity, while group compounds had weak activity. The compounds in group showed minor potency in inhibiting cell viability.

Since SMY006 in group showed the most potent activity (Fig. 1B), it was chosen for further study. By preparing and screening various of its salt compounds, SMY002 (a kind of hydrochloride of SMY006) was produced (Fig. 1C) and showed a more selective cytotoxic effect in TNBC cells than MCF-10A (a normal mammary epithelial cell line) cells (Fig. 1D and SI. Fig. S3A-3D).

The optimized synthesis steps of SMY002 are shown in Scheme 1. Compound 2 was synthesized by prepared compound Methylparaben and 2-Piperidinoethanol in the presence of PPh₃ and DIAD through a Mitsunobu reaction. Compound 2 was reduced by LiAlH₄ in THF under 0 °C to give compound 3. Compound 4 was obtained by Compound 3 and 1-Fluoro-4-nitronaphthalene in the presence of NaH in THF through a nucleophilic reaction. Finally, compound 4 was reduced by Fe in 1N. HCl and EtOH mixture solvent to give SMY002. The synthetic details and spectrographic data of the intermediates and compound SMY002 were shown in SI. Materials and methods and SI. Fig. S4-S6.

SMY002 directly interacts and binds to STAT3 with a high affinity

In predicting whether SMY002 interferes with the STAT3 protein, we obtained the crystal structure of the STAT3-SH2 domain from the RCSB protein data bank (PDB code: 1BG1) and prepared its design with Auto Dock Vina. The DFT calculations were carried out using the Gaussian 16 program package^[22]. The geometry optimization of minima was carried out at the B3LYP-D3 level^[23] with the 6-31G(d) basis set. According to the computational docking, SMY002 (cyan) docked in the phospho-Tyrosine-binding pocket of the STAT3-SH2 motif (light blue) (Fig. 2A). Nine residues of Lys591, Glu594, Arg609, Ser613, Ile634, Ser636, Val637, Glu638, and Pro639 were found close to SMY002 (Fig. 2B). The model also indicates that SMY002 interacts stably with the Arg609 and Arg636 residues via two hydrogen bonds (black dash lines) (Fig. 2C). The ether bond oxygen atom in the ligand forms one hydrogen bond with the amine hydrogen atom on the branch chain of Arg609 (Fig. 2C). Meanwhile, the oxygen atom of the ether bond in the ligand forms the other hydrogen bond with the hydroxyl hydrogen atom of the Ser636 branched-chain (Fig. 2C). Thus, SMY002 might interact directly and stably with the STAT3 protein. The Surface Plasmon Resonance (SPR) experiments were carried out as described previously^[20, 21]. The results showed that SMY002 could compete by blocking EGFR pY1068-peptide, the ligand of STAT3, for binding to Stat3 with IC₅₀ values of 2 μM (Fig. 2D, 2E). These data manifested that SMY002 has a high affinity to STAT3.

The inhibitory effects of SMY002 on STAT3 activation

Then, the effects of SMY002 on the transcriptional regulatory function of STAT3 were detected. Fig. 3A-3C shows that SMY002 treatment decreased the STAT3 reporter gene activity in a dose-dependent manner in TNBC cells. In addition, the downstream target genes of STAT3, such as CCND1, BCL-XL, and MMP9, were also dramatically decreased at the mRNA expression levels (Fig. 3D-3F), which confirms that SMY002 inhibits the transcriptional activity of STAT3.

We further evaluated whether SMY002 suppresses the phosphorylation of STAT3 (Tyr705), which is crucial for its activation. Fig. 3G-3I shows that SMY002 treatment suppressed STAT3 activation in a dose-dependent manner. Cyclin D1 expression was gradually down in corresponding with the change of p-STAT3. BCL-XL expression was also decreased following SMY002 treatment. However, SMY002 did not impair the p-JAK2, p-ERK1/2, or p-AKT expression. These results displayed that SMY002 can specifically suppress STAT3 activation, not by inhibiting its upstream kinase JAK2 or other off-target effects but by directly interfering with STAT3 phosphorylation.

The dimerization and subcellular localization of p-STAT3 (Tyr705) is crucial for STAT3 binding to specific DNA elements. After SMY002 treatment, the inactive monomers of STAT3 were increased in a dose-dependent manner, which implies STAT3 dimerization was impeded (Fig. 3J-3L). The nucleoprotein separation experiments show that SMY002 considerably diminished the expression of p-STAT3 (Tyr705) in both cytoplasm and nucleus (Fig. 3M-3O). To further support this finding, indirect immunofluorescence experiments were performed. As shown in Fig. 8, the nuclear localization of p-STAT3 (Tyr705) in 4T1 cells without SMY002 treatment was robust regardless of IL-6 stimulation. SMY002 treatment markedly reduced the nuclear staining of p-STAT3 (Tyr705). Therefore, SMY002 can impede the activation, dimerization, and nuclear localization of STAT3 by preventing the phosphorylation of Tyr705.

SMY002 inhibits the survival and cell cycle progression of TNBC

We next assessed the effect of SMY002 on survival and cycle progression of TNBC. The apoptosis ratio of MDA-MB-468 cells was increased from 1.75 % to 17.54 % and 54.47 %, respectively, after treatment with SMY002 10 or 20 μ M (Fig. 4A,4B). Similar data were obtained in MDA-MB-231 cells (Fig. 4C). In addition, SMY002 induced significant G1 phase arrest and visibly shortened S phase in TNBC cells (Fig. 4D-4F). These results reveal that SMY002 can induce cell apoptosis and prolongs the cell cycle.

SMY002 attenuates the motility of TNBC in vitro

One of the critical malignant phenotypes of TNBC cells is their potential for metastasis. To investigate whether SMY002 suppresses STAT3-promoted tumor cell migration, we evaluated the effect of SMY002 on TNBC cell migration by scratch assays (Fig. 5A-5F). Compared with the control, SMY002 treated cells showed significantly lower migration activities, as evidenced by the delay of wound closure observed at different time points. We utilized another approach, transit cell culture, to assess the cell migration and invasion abilities. The results of Fig. 5G-5L reveal that the control treatment cells displayed a remarkable capacity to pass through the membrane or invade through a reconstituted basement membrane (Matrigel) of a branchial chamber.

To explore why SMY002 inhibits cell migration and invasion, we detected the protein expression levels of MMP9, a target gene of STAT3, after treatment with SMY002 for 48 h in TNBC cells. Western blot results displayed that SMY002 repressed MMP9 expression in a dose-dependently manner (Fig. 5M-5O Topline). We performed a zymography assay to disclose the effect of SMY002 on MMP9 secretion. As shown in the bottom line of Fig. 5M-5O, the transparent protein bands on the blue background of the stained gels, which indicate the location of MMP9, were weaker with increasing treatment with SMY002, demonstrating that SMY002 reduced the secretion of MMP9. The above findings suggest that SMY002 effectively suppressed the survival, cell cycle progression, mobility, and invasion of TNBC cells in vitro.

SMY002 reduced the growth and metastasis of TNBC cells

We finally evaluated the therapeutic effect of SMY002 in 4T1 cells transplanted in mice. Compared with the vehicle (PBS) treatment group, SMY002 significantly reduced the volume and weight of tumors (Fig.

6A-6C). Xenografts in the SMY002 treatment group developed less lung metastasis than those in the vehicle group (white nodule in the metastatic loci decreased from 35 to 9.8) (Fig. 6D, 6E). Immunohistochemical staining results meant that p-STAT3 (Tyr705) and MMP9 expression in xenograft tumors treated with SMY002 was weaker than that of the vehicle group (Fig. 6F, 6G). These data are compatible with the results in vitro that SMY002 blocks the growth and invasion of TNBC cells via suppressing STAT3 activation. Compared with the vehicle group, the body weight of mice in the SMY002 administration group was not significantly reduced (Fig. 6H). Additional adverse reactions, such as fever, bleeding, or death, did not occur during the experiment, implying that SMY002 has few side effects in mice. These data underlined that SMY002 potently suppressed the growth and dissemination of TNBC by inhibiting STAT3 activity.

Conclusion

At present, many small molecule compounds targeting STAT3 signal have entered the clinical trials, including WP1066 (targeting JAK2)^[24], AZD9150 (the antisense oligonucleotide of STAT3)^[25], C188-9 (targeting the STAT3 SH2 domain)^[26], OPB-31121 (targeting JAK2)^[27], OPB-51602 (targeting the STAT3 SH2 domain)^[28], Ruxolitinib (a JAK1/2 inhibitor)^[29], Ritonavir (a P-glycoprotein inhibitor)^[30], TT-00420 (an aurora kinase A/B inhibitor), Icaritin (a regulator of MAPK/ERK/JNK and JAK2/STAT3/AKT signals)^[31] and Napabucas (targeting the STAT3 SH2 domain)^[32], et al. However, they are limited due to the indirect targeting, weak selectivity, low bioavailability, lack of therapeutic advantage or high toxicity. Compared with these compounds, SMY002 has the advantage of direct targeting STAT3 and can remarkably reduce the growth and dissemination of transplanted TNBC cells in mice. Further studies on pharmacokinetics and toxicology will be carried out to ensure the application of SMY002 in TNBC therapy.

Abbreviations

STAT3, signal transducer and activator of transcription 3

TNBC, triple-negative breast cancer

SERM, selective estrogen receptor modulator

ER, estrogen receptor

PR, progesterone receptor

HER2, human epidermal growth factor receptor 2

GP130, glycoprotein 130 KD

JAKs, Janus kinases

SH2, Src homology domain 2

MMPs, matrix-metalloproteases

Tyr705, tyrosine 705

IC50, half-maximal inhibitory concentration

DMSO, dimethyl sulfoxide

SPR, surface plasmon resonance

MTT, 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide

DAPI, 4,6-diamidino-2-phenylindole

Declarations

Acknowledgments

We acknowledge the National Natural Science Foundation of China for funding the project (No. 81803575) and the Henan Science and Technology Department of China for financing the project (No. G20190126005). We also thank the Department of Science and Technology, Government of Kaifeng, China, for funding the project (No. 21SSF003).

Author contributions

All authors contributed to the study's conception and design. Zhengyan Yang: contributed to conceptualization and writing-original draft; Hongyun Xu: contributed to investigation and validation. Yupo Yang and Chaoqun Duan: contributed to the synthesis and analysis of the compounds. Pai Zhang: contributed to the data curation. Yang Wang: contributed to resource collection. Kai Fu: contributed to methodology and formal analysis. Yonghong Shen: contributed to the writing-reviewing, and editing of the manuscript. Marvin Xuejun Xu: contributed to funding acquisition and project administration. All authors read and approved the final manuscript.

Funding

This work was supported by grants from the High-End Foreign Experts Introduction Plan of Henan Science and Technology Department (No. G20190126005), Kaifeng Science and Technology Development Plan Project (No. 21SSF003), Youth Fund of National Natural Science Foundation of China (No. 81803575)

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

The study was approved by the Institutional Ethical Committee. Informed consent was obtained from all individual participants included in the study.

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Supplementary Figures

Supplementary Figures are not available with this version

Figures

Figure 1

Structure design and synthesis of naphthalene derivatives. A, The initial hit compounds of 4-nitronaphthol and SERMs. B, Structural characteristics and groupings of potential TNBC inhibitors. The compounds can be divided into three structural groups. The compounds of group I (including SMBAa007) contain an amide substitution at the 4-position (the -R group highlighted in yellow) with the highest IC_{50} values. The group II compounds showed the most vital activity (containing SMY006) with ether (the -R group highlighted in blue). Compounds in group III with medium activities have a carboxyl

that combines the 4-nitronaphthol and SERM scaffold, such as a (SMY001) (the -R group highlighted in pink). C, SMY002, a kind of hydrochloride of SMY006. D, Anti-proliferation activity of SMY002 and SMY001 against mammary epithelial cells and TNBC cells was detected by MTT assay.

Figure 2

Docking and SPR assay of SMY002. A, Surface structure of STAT3 and ligand SMY002, the color in light blue is STAT3 SH2 domain. B, The schematic diagram of the interactions between STAT3 SH2 domain

and SMY002. C, Residues R609 and S613 have hydrogen bonding with SMY002, respectively. Interaction mode and affinity assay of SMY002 with STAT3 protein. D, Binding of recombinant STAT3 (500 nM) to Biacore sensor chip-coated with the phosphododecapeptide ligand (surrounding pY1068 of the EGFR) containing SMY002 (1 to 1000 μ M) was measured by SPR. E, The competitive binding curve and IC50 value of SMY002 to STAT3 in the SPR assay.

Figure 3

SMY002 inhibits the phosphorylation and activation of STAT3 in 4T1, MDA-MB-231, and MDA-MB-468 cells. A-C, Luciferase assay of TNBC cells treated with SMY002 for 48 h. D-F, Effects of SMY002 on CCND1, BCL-XL, and MMP9 mRNA expression levels were examined by real-time PCR. G-I, TNBC cells were treated with different doses of SMY002 for 48 h. The protein expression levels of p-JAK2 (Y1007/1008), p-STAT3 (Tyr705), T-STAT3, p-ERK 1/2, p-Akt (Ser473), Cyclin D1, BCL-XL, and β -actin were assayed by Western blot. J-L, TNBC cells were incubated with SMY002 in various concentrations for 48 h and then extracted. The cell lysis mixture was separated using a non-denaturing PAGE. STAT3 dimers and monomers were detected by Western blot with the anti-STAT3 antibody. M-O, Cells were dealt with or without SMY002 for 24 h, followed by nuclear and cytoplasmic fractionation. The expression of p-STAT3 (Tyr705), total STAT3, Histone 3, and GAPDH was analyzed by Western blot. ($**p<.01$, $***p<.001$, $****p<.0001$).

Figure 4

SMY002 induces apoptosis in TNBC cells (A-D) and cycle blockage (E-H) in TNBC cells. (* $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$).

Figure 5

SMY002 attenuates the migration and invasion of TNBC cells in vitro. A-C, Cells were scratched and treated with or without SMY002 was imaged at indicated times. E-F, The healing area was calculated with Image J software. G-L, SMY002 inhibited cell migration and invasive activities were examined by the transit chamber assays. Cells that transited the filter membranes of chambers were photographed (G-I) and counted by ImageJ software (J-L). M-O, TNBC cells were cultured with SMY002 for 48 h. Secreted

MMP9 in the supernatant of cell culture was pipetted, centrifuged, concentrated, and then analyzed by Western blot (Top) and zymography (Bottom). (** $p < .01$, *** $p < .001$, **** $p < .0001$)

Figure 6

SMY002 prevents xenograft TNBC cell growth and dissemination in vivo. A, The volume of transplanted tumors with vehicle (PBS) or SMY002 administration. B, C, Gross anatomy (B) and wet weight (C) of primary tumors were photographed and recorded. D, E, Lungs of mice in the experimental and placebo

groups were dissected and fixed in Bouin's solution. The white nodules representing the tumor metastasis were imaged (D) and counted (E). F, G, Expression of p-STAT3 (Tyr705) and MMP9 in the xenograft tumors was assayed by IHC using specific antibodies (F) and quantified by Image-Pro Plus 6.0 (G), Bars, 200 μ m. H, The body weight of mice before they were executed. (** $p < .01$, **** $p < .0001$).