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To cite this article: Yue Chen, Ming Ji, Shen Zhang, Nina Xue, Heng Xu, Songwen Lin & Xiaoguang Chen (2018): Bt354 as a New STAT3 Signaling Pathway Inhibitor against Triple Negative Breast Cancer, Journal of Drug Targeting, DOI: 10.1080/1061186X.2018.1452244

To link to this article: https://doi.org/10.1080/1061186X.2018.1452244
Bt354 as a New STAT3 Signaling Pathway Inhibitor against Triple Negative Breast Cancer

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Bt354 as a New STAT3 Signaling Pathway Inhibitor against Triple Negative Breast Cancer

Abstract

Inhibition of the signal transducer and activator of transcription 3 (STAT3) signaling pathway has been considered as a novel therapeutic strategy to treat human cancers with constitutively active STAT3. In this study, we screened 1563 compounds and identified Bt354 as a new small-molecule inhibitor of the STAT3 signaling pathway. The effect of Bt354 on STAT3 activity was initially screened and Bt354 significantly inhibited STAT3 activity in a dual luciferase assay. Bt354 inhibited the proliferation of cancer cells in a dose- and time-dependent manner. The phosphorylation of STAT3 at Y705 was suppressed without affecting the phosphorylation of STAT3 at S727 in breast cancer cells. Furthermore, Bt354 inhibited the nuclear translocation of STAT3 and consequently induced cell growth inhibition, apoptosis, and cell cycle G2/M arrest in triple negative breast cancer cells. In vivo, Bt354 also inhibited tumor growth in MDA-MB-435 and MDA-MB-231 xenograft mice without affecting body weight. Computational modeling showed that Bt354 could bind to the SH2 domain of STAT3. These findings suggest that Bt354 may be a potent anticancer agent for STAT3-activated triple negative breast cancer cells.

Keywords: STAT3; breast cancer; apoptosis; cell cycle arrest; inhibitor
Introduction

Signal transducers and activators of transcription (STAT) are a family of seven proteins (STATs 1, 2, 3, 4, 5a, 5b, and 6) that transduce extracellular signals, regulate transcription of target genes [1], and regulate important cellular processes such as cell survival, immune response, angiogenesis, and cell proliferation [2]. STAT3 is constitutively activated and overexpressed in various tumor types, including breast carcinoma, prostate cancer, melanoma, multiple myeloma, and leukemia [3]. Aberrant STAT3 activity contributes to carcinogenesis and tumor progression. STAT3 is a dimer and has a leucine zipper at the N-terminus, a DNA-binding domain, and a Src homology 2 (SH2) transactivation domain at the C-terminus [4].

The STAT3 signaling cascade is initiated by binding of extracellular ligands, such as cytokines and growth factors, to their respective cell surface receptors [5]. Next, Tyr705 in the STAT3 SH2 domain is phosphorylated [6,7], and STAT3 interacts specifically via its SH2 domain with the phosphor-tyrosine docking sites displayed by cell membrane receptors [8,9]. Tyrosine-phosphorylated STAT3 dimerizes and translocates into the nucleus, where it binds to specific promoter sequences on its target genes. Inhibition of STAT3 by antisense oligonucleotide siRNAs, upstream Janus kinase (JAK), Src kinase inhibitors, or direct STAT3 inhibitors suppresses tumor growth and induces apoptosis in cancer cells [10,11]. Thus, the STAT3 pathway is considered to be an attractive therapeutic target for human cancers [12].

Rational design, high-throughput screening, and structure-based virtual screening have identified several classes of small molecules as selective STAT3 inhibitors, such as peptidic inhibitors [13], stattic [14], STA-21 [15], and natural products including dirubin, resveratrol, and cucurbitacin analogues. However, most of these small-
molecule candidates are unsuitable as therapeutic compounds because their off-target effects cause undesirable side effects. It is still important to find and optimize new small-molecule candidates for STAT3 inhibition [16]. In this paper, we screened a small chemical library containing 1563 compounds and identified Bt354 as a potent small molecule that inhibits STAT3 phosphorylation and elicits apoptosis in human breast carcinoma cells.

**Materials and Methods**

**Reagents**

RPMI 1640, DMEM, McCoy’s 5A, fetal bovine serum (FBS), and antibiotic-antimycotic solution were purchased from Gibco (ThermoFisher Scientific). Antibodies against STAT3, p-STAT3 (Y705), p-STAT3 (S727), epidermal growth factor receptor (EGFR), JAK2, Bcl-2, Bcl-xL, Myc-1, and survivin were purchased from Cell Signaling Technology (Beverly, MA). β-Actin antibody was obtained from Santa Cruz Biotechnology. Goat-anti-rabbit and anti-mouse horseradish peroxidase (HRP) conjugates was purchased from ZSBiO. DMSO and chemicals used in buffer solutions were purchased from Sigma-Aldrich Chemical Co.

**Cell culture**

Cancer cell lines were obtained from American Type Culture Collection. Human prostate cancer cell lines (DU145 and LNCaP), and human breast cancer cell lines (MDA-MB-468, MDA-MB-435, MDA-MB-453, and MCF-7) were maintained in RPMI 1640. PC3 human prostate cancer cells and MDA-MB-231 human breast cancer cells were maintained in DMEM [17].
**MTT assay**

Cells were cultured in 96-well plates at a density of $1 \times 10^4$ cells/well and treated with Bt354 for 24, 48, and 72 h. The cells were then co-incubated with MTT (1 mg/mL) for another 4 h, and then DMSO (100 μL/well) was added. Absorbance at 570 nm was measured using a multimode plate reader (EnSpire, Perkin-Elmer, Inc., Waltham, MA).

**Transient transfection and dual-luciferase assay**

DU145 cells were seeded at a density of $1 \times 10^5$ cells in 100 mm$^2$ culture plates. The following day, cells were transfected with 21pSTAT3-TA-Luc (27 μg) and pRL-TK (9 μg), a *Renilla* luciferase control reporter plasmid (Promega) using TransFectin (Invitrogen, ThermoFisher Scientific). After 5 h of transfection, cells were trypsinized and seeded onto sterile, black-bottom 96-well plates at a density of $1 \times 10^4$ cells per well, and then incubated with complete medium for 24 h. Cells were treated with either test compounds or 0.1% DMSO for 24 h. After treatment, cells were harvested in passive lysis buffer (20 μL) and luciferase activity was evaluated by the Dual Luciferase Reporter Assay kit (Promega) on a multimode plate reader (EnSpire, Perkin-Elmer, Inc.). Experiments were performed in triplicate and repeated three times. Relative luciferase activity was calculated by relative luciferase activity (%) = $\left[\frac{\text{(normalized luciferase activity of sample treated with a test compound)}}{\text{(normalized luciferase activity of sample treated with 0.1% DMSO)}}\right] \times 100$.

**Western blotting**

Cell lysates were prepared by cell incubation in radioimmunoprecipitation assay buffer [RIPA; 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1%
Triton X-100, 1% sodium deoxycholic acid, 0.1% SDS, 2 mmol/L phenylmethylsulfonyl fluoride, 30 mmol/L Na$_2$HPO$_4$, 50 mmol/L NaF, and 1 mmol/L Na$_3$VO$_4$] containing protease inhibitor cocktail (Roche Applied Science). Proteins (40 μg) were resolved on 7.5% or 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Co.). Membranes were blocked with 5% nonfat dried milk in TBS-T [50 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, and 0.1% Tween 20] and probed with primary antibodies for 3 h. Blots were washed with TBS-T, exposed to HRP-conjugated goat-anti-rabbit or goat-anti-mouse IgG for 1 h, and examined using chemiluminescence POD reagents (Biotanon, China).

**Propidium iodide and Annexin V staining for cell cycle and apoptosis detection**

Cells were treated with Bt354 for 24 h (for cell cycle detection) or 48 h (for apoptosis detection) in culture. For cell cycle detection, the cells were harvested by trypsin (without EDTA), washed in phosphate-buffered saline (PBS) and fixed in cold ethanol (70%) at -20 °C overnight. To calculate DNA content and the proportions of G0/G1, S, and G2/M, 50 μg/mL propidium iodide (PI) and RNase A were added to samples 1 h before flow cytometry analysis. To detect apoptosis, we followed the standard procedure of the Annexin V cell apoptosis analysis kit (Sungene Biotech Co., Ltd., Beijing, China). After trypsin treatment and harvesting, cells were washed in PBS and resuspended in binding buffer (HEPES-buffered saline supplemented with 25 mM CaCl$_2$). Annexin V-FITC (5 μL) was added, the mixture was incubated for 30 min, and then PI (5 μL) was added and the cells were incubated for 5 min in the dark at room temperature. The cells were analyzed by flow cytometry (BD FACSverse, BD Biosciences, San Jose, CA, USA) [18].
**Immunofluorescence assay**

Cells were cultured on a 15 mm confocal dish (catalog no. 801002, NEST) and treated with various concentrations of Bt354 for 12 h. Cells were washed three times with PBS, fixed in 4% formaldehyde for 15 min at room temperature, and blocked with 3% bovine serum albumin/0.3% Triton X-100 in PBS. The cells were subsequently incubated with anti-STAT3 antibodies overnight at 4 °C. Finally, the cells were incubated with Alexa Fluor® 488 donkey anti-rabbit IgG (A11008, Life Technologies) for 2 h and contained with DAPI (4′,6′-diamidino-2-phenylindole). The images of stained cells were obtained using a multiphoton laser scanning microscope (FV1000 MPE, Olympus, Japan).

**Cell migration assay**

A migration assay was performed by suspending cells in serum-free RMPI-1640 or DMEM medium and seeding them in the upper chambers of permeable supports (Transwell, Corning) in a 96-well plate. The lower chamber of each well contained RPMI1640 or DMEM (500 μL) with 40% FBS. After incubation at 37 °C for 18 h, the non-migratory cells on the upper chamber were removed, and the remaining cells were fixed and stained. The stained cells were visualized by light microscopy and counted in 10 random high-power fields.

**Efficacy study in BalB/c-nu/nu mice bearing MDA-MB-231 and MDA-MB-435 xenograft tumors**

Female athymic nu/nu mice (8–10 week old) were purchased from Vital River Laboratories (Beijing, China). The right flanks of the nude mice were implanted subcutaneously with $5 \times 10^6$ MDA-MB-231 or MDA-MB-435 cells in matrigel solution (0.1 mL). After 2 weeks, the tumors were harvested and transplanted to 40
mice through tissue homogenate. The mice were grouped (six mice per group) with roughly equal distribution of tumor volumes and the treatment started on day 7 after implantation, when tumors reached an average volume of approximately 300 mm$^3$. Tumor volumes (V) were calculated by volume = (width)$^2 \times$ length/2. Bt354 dissolved in the vehicle (5% CMC-Na) was administered at 10, 20, and 40 mg/kg by oral gavage once every 3 days for 21 days. For 21 days, 10 mg/kg docetaxel and 20 mg/kg napabucasin were administered once every 3 days by intraperitoneal injection. The mice were euthanized on day 21, and tumors were weighed and frozen in liquid nitrogen for preparing protein lysates or fixed in 10% neutral buffered formalin for immunohistochemistry.

**TUNEL assay**

A terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed using an In Situ Cell Death Detection Kit, Fluorescein (catalog number 11684795910, Roche Applied Science, Mannheim, Germany), following the manufacturer’s instructions. Cryopreserved tissue sections were fixed in 4% paraformaldehyde for 20 min at room temperature, washed with PBS, and permeabilized with freshly prepared 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. After washing with PBS, the slides were overlaid with TUNEL reaction mixture (100 μL), according to the manufacturer’s instructions, and incubated for 1 h at 37 °C. Finally, cells were washed in PBS, and coverslips were mounted onto slides with Prolong Gold anti-fade reagent with DAPI (catalog number p36931, Molecular Probes, Inc., Eugene, OR). Cells were imaged using a fluorescence microscope (AX70, Olympus). The magnification of the histology photographs was ×20.
**Immunohistochemical analysis**

Tumor tissues were embedded in paraffin and sliced. For Ki-67 staining, tumor sections were de-paraffinized and rehydrated followed by antigen retrieval. Sections were blocked in normal goat serum for 1 h, and incubated with rabbit monoclonal Ki-67 antibody (1:400; CST) overnight at 4 °C. Ki-67-stained cells were visualized with a DAB kit after incubating with HRP-labeled secondary antibodies (ZSBiO). The section was scanned at a magnification of ×200. The Ki-67-positive rate was calculated by Image-Pro Plus 5.1 (Media Cybernetics).

**Molecular modeling and docking**

To understand why Bt354 performs distinct functions in the inhibition of STAT3 activity, we created a molecular model. The crystal structure of STAT3 at 2.25 Å resolution was obtained from the Protein Data Bank (PDB ID: 1BG1) and used for modeling. Docking of Bt354 to the binding site of the STAT3 SH2 domain was performed in silico by using the LibDock module in the Discovery Studio 2.5 package (Accelrys). Prior to docking, water molecules and binding DNA were removed, and the polar hydrogen and incomplete residues were added to the STAT3 model. The three-dimensional structures of Bt354 were constructed by using the Prepare Ligands module. The binding pocket for ligand molecules was defined as the STAT3 SH2 domain with a 14 Å radius from the center of the binding pocket.

**Statistical analyses**

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Prism Software, Inc., La Jolla, CA) and the significance levels were evaluated by using the unpaired t test. *p* values were two-sided. Three asterisks (***)**, two asterisks (**), one asterisk (*), and no asterisk in figures and tables indicate *P* values of <0.001, <0.01,
0.01 < P < 0.05, and >0.05, respectively.

**Results**

*Bt354 inhibited STAT3-dependent luciferase activity in prostate and breast cancer cells*

To identify specific inhibitors of STAT3, we screened a small compound library of 1563 compounds using a dual-luciferase assay system reflecting STAT3 activity and found that Bt354 exhibited good inhibition activity. The structure of Bt354 is shown in Fig. 1A. To confirm whether Bt354 is an inhibitor for the STAT3 signaling pathway, we examined the expression level and phosphorylation status of STAT3 in various cancer cell lines. Cell lines DU145 (prostate cancer), MDA-MB-435 (breast carcinoma), and MDA-MB-231 (breast carcinoma) displayed high levels of activated STAT3, as indicated by STAT3 Tyr705 and Ser727 phosphorylation (Fig. 1B). Based on these results, we investigated the inhibition effect of Bt354 in these three cell lines via dual-luciferase assay. The results showed that Bt354 inhibited STAT3 activity in a dose-dependent manner, with IC50 values of 4.6 μM (DU145), 6.5 μM (MDA-MB-435), and 7.2 μM (MDA-MB-231) (Fig. 1C). These data provide strong evidence that Bt354 significantly inhibits STAT3 activity in prostate and breast cancer cells.

*Bt354 inhibited the constitutive phosphorylation and nuclear localization of STAT3*

Because Bt354 inhibits STAT3 transcriptional activity, we explored the effect of Bt354 on the STAT3 signaling pathway. The protein and phosphorylation levels of STAT3, JAK2, and Src were evaluated by immunoblotting in both MDA-MB-435 and MDA-MB-231 cells, which harbor constitutively activated STAT3. Bt354
decreased the phosphorylation level of STAT3 at Tyr705 in a dose- and time-dependent manner, whereas the phosphorylation level of STAT3 at Ser727 was not significantly changed (Figs. 2A and B). The protein and phosphorylation levels of JAK2 and Src were not changed after cells were exposed to Bt354.

Immunofluorescence assays were carried out in MDA-MB-435 and MDA-MB-231 cells using confocal microscopy. Without compound exposure, STAT3 was mainly located in the nucleus (Figs. 2C and D). After treatment with 1 and 5 μM Bt354 for 12 h, nuclear STAT3 levels decreased substantially in a dose-dependent manner, implying that Bt354 inhibits the translocation of STAT3 into the nucleus. These results suggest that Bt354 may directly and specifically inhibit STAT3.

**Bt354 inhibited the proliferation of DU145, MDA-MB-435, and MDA-MB-231 cells with constitutively activated STAT3**

The STAT3 signaling pathway plays an important role in cellular processes such as cell proliferation, survival, migration, and angiogenesis. Because Bt354 directly inhibited STAT3, we assessed the proliferation inhibition of Bt354 in cells with constitutively activated STAT3. Bt354 showed significant inhibition of proliferation in the DU145 prostate cancer line with an IC\(_{50}\) value of 0.15 μM. Bt354 also inhibited growth in MDA-MB-435 and MDA-MB-231 cells with IC\(_{50}\) values of 0.07 and 1.25 μM (Fig. 3A). Additionally, to test the long-term effects of Bt354 on cancer cell growth, DU145, MDA-MB-435, and MDA-MB-231 cells were treated with different concentrations of Bt345 for up to 72 h. The cell proliferation was significantly inhibited in a time-dependent manner. The IC\(_{50}\) values for 24, 48, and 72 h incubation for DU145 cells were 20, 0.3, and 0.15 μM; for MDA-MB-435 cells they were 25, 2.5, and 0.07 μM; and for MDA-MB-231 they were 35, 15, and 1.25 μM, respectively
The immunofluorescence staining using EdU confirmed that Bt354 exerted potent anti-proliferative effects against MDA-MB-435 and MDA-MB-231 cells in a dose-dependent manner (Fig. 3C).

**Bt354 caused G2/M arrest in breast tumor cells and induced late apoptosis**

After we confirmed that Bt354 inhibited the proliferation of cancer cells, we evaluated the effect of Bt354 on cell survival. The MDA-MB-435 and MDA-MB-231 cell lines were detected via flow cytometric analysis after exposure to Bt354. We found that Bt354 caused G2/M accumulation in both cell lines. When treated with 0.5 μM Bt354, the G2/M phase accumulated to 28.68% and 27.50% in MDA-MB-435 and MDA-MB-231 cell lines, respectively (Figs. 4A and B). We also detected the expression of CDC25c and CDC2, which are the key regulators of the G2/M checkpoint (Fig. 4E). Both were significantly decreased in a dose-dependent manner by Bt354 in both the MDA-MB-435 and MDA-MB-231 cell lines while the phosphorylation of CDC25c increased considerably.

Late apoptosis and cell death was also investigated in these cell lines by Annexin V and PI staining (Figs. 4C and D). The percentage of late apoptosis cells increased from 4.06% to 26.0% in MDA-MB-435 and 1.47% to 29.5% in MDA-MB-231 cell lines. The percentage increased in a concentration-dependent manner. The expression of proteins downstream of STAT3 related to the cell cycle, including cyclin D1, Bcl-2, Bcl-xL, Mcl-2, survivin, and C-myc, were detected, and they were dramatically attenuated by Bt354 treatment in a dose-dependent manner (Fig. 4E). These results suggested that Bt354 induced cell cycle arrest at the G2/M phase and cell apoptosis.
**Bt354 blocked the migration of MDA-MB-435 and MDA-MB-231**

To assess the ability of Bt354 to block STAT3-dependent tumor progression processes further, wound healing and transwell assays were performed to measure the migration of malignant cells. Migration was significantly reduced in MDA-MB-435 and MDA-MB-231 cells by Bt354 in a dose-dependent manner (Figs. 5A and B). A wound healing assay was used to confirm that Bt354 suppressed cell migration (Fig. 5C). MDA-MB-435 cells were treated with Bt354 at different concentrations, and the migration was clearly inhibited at 1 μM. The inhibition effect increased in a dose-dependent manner. These findings demonstrate that Bt354 inhibited the migration of malignant cells.

**Bt354 exerted antitumor activity in MDA-MB-435 and MDA-MB-231 cell xenograft mice**

Given the potent antitumor activity of Bt354 in vitro, its efficacy in the MDA-MB-435 and MDA-MB-231 xenograft mice model was investigated. Bt354 exerted significant antitumor activity in the MDA-MB-231 model (Fig. 6A, Table 1). The mean tumor volumes in groups given Bt354 given orally (10, 20, 40 mg/kg) were 1303.2 ± 231.9, 976.0 ± 234.0, and 811.0 ± 123.0 mm³ (P < 0.001), respectively, compared with 2604.3 ± 345.0 mm³ in the control group. The positive control group given napabucasin (1432.0 ± 210.5 mm³) also exhibited an antitumor effect. The tumor growth inhibition values (T/C) were 50%, 37.4%, and 31.3% for the 10, 20, and 40 mg/kg Bt354 groups. The tumor weight decreased by 74.6% in the 40 mg/kg group (P < 0.001, Fig. 6B, Table 1). We found similar results in the MDA-MB-435 model; the mean tumor volumes in the 10, 20, and 40 mg/kg Bt354 groups were 393.2 ± 31.9, 351.1 ± 63.9, and 321.4 ± 62.9 mm³ (P < 0.001), respectively, compared with
1365.8 ± 204.1 mm³ in the control group. The T/C values were 28.7%, 25.7%, and 23.5% for the groups given 10, 20, and 40 mg/kg Bt354 orally, respectively. The tumor weights were decreased by 56.2%, 63.8%, and 77.1% in the 10, 20, and 40 mg/kg groups (P < 0.001), respectively (Figs. 6C and D, Table 2).

Because Bt354 exerted potential antitumor activity in breast tumor xenograft models, we confirmed the pharmacological mechanism of Bt354 in these models. Western blot analysis of three tumors from four groups (Control, 10, 20, and 40 mg/kg) showed that p-STAT3(Y705) decreased in a dose-dependent manner (Fig. 7B). The number of Ki-67-positive cells in the tumor sections decreased in the 10, 20, and 40 mg/kg Bt354 groups (P < 0.01), which indicated that Bt354 suppressed tumor proliferation (Fig. 7A). To confirm the effect of Bt354 in inducing apoptosis, we performed a TUNEL assay on the MDA-MB-435 and MDA-MB-231 tumor slices. Bt354 increased the tumor cell apoptosis in the 10, 20, and 40 mg/kg groups in a dose-dependent manner (Fig. 7C).

**Bt354 binds to the SH2 domain of STAT3**
Molecular docking between STAT3 and Bt354 was performed with the LibDock module in the Discovery Studio 2.5 package. The LibDock score, absolute energy, and relative energy are summarized in Table 3. The *in silico* molecular docking study results showed that Bt354 may bind to the SH2 domain of STAT3 (Fig. 8). Bt354 formed several hydrogen bonds and π--π interactions with nearby amino acid residues, including Ser649, Glu652, Asn646, Arg688, and Pro689.
Discussion

STAT3 is a bifunctional protein present in the cytoplasm and is coupled with the tyrosine phosphorylation signal pathway. STAT3 plays an important role in signal transduction and transcription. Many experiments in vivo and in vitro have shown that STAT3 controls numerous tumorigenic signaling pathways. The transcription of the corresponding target genes can be regulated by activating growth factors, such as EGFR, Src, IL6, vascular endothelial growth factor, and cytokines, through the JAK2-STAT3 signaling pathway [19]. The STAT3 pathway is closely related to cell differentiation, proliferation, angiogenesis, apoptosis, immune escape, and metastasis [20]. STAT3 overexpression had been observed in many cancers, such as head and neck, prostate, breast, and gastric cancers [22].

A number of strategies, including anti-sense oligonucleotide targeting of STAT3, synthetic drugs, natural small molecules, and gene therapy techniques, have been used over the last decade to suppress the deregulated STAT3 signaling cascade in cancer. Currently, there is no approved drug that targets STAT3, although a number of small-molecule inhibitors of STAT3 have been discovered [21].

In this study, we used a dual-luciferase assay to identify STAT3 inhibitors from 1563 chemicals, and found that Bt354 is a potent inhibitor of STAT3. Our screening results show that DU145 (prostate cancer), SK_OV_3 (ovarian cancer), MDA-MB-435 (breast cancer), and MDA-MB-231 (breast cancer) cells exhibited high STAT3 expression and extremely low levels of STAT3 in PC3 (prostate cancer) cells. DU145, MDA-MB-435, and MDA-MB-231 cells also exhibited high expression of p-STAT3 (Y705) and p-STAT3 (S727). This result was confirmed by the inhibitory effect of Bt354 in a dual luciferase assay, suggesting that Bt354 is a strong STAT3 inhibitor. An MTT assay showed that Bt354 strongly inhibits DU145, MDA-MB-435, and
MDA-MB-231 proliferation in a dose-dependent and time-dependent manner. The EdU assay confirmed the inhibitory effect of Bt354 on cancer cells.

STAT3 is activated by upstream molecules, such as the JAK family of protein tyrosine kinases [23], Src, and growth factor receptors, such as EGFRs, fibroblast growth factor receptors, hepatocyte growth factor receptor, platelet-derived growth factor receptors, and vascular endothelial growth factor receptors [24,25,26]. Our results elucidate the underlying mechanism of the selective toxicity of Bt354: Bt354 specifically inhibited STAT3 phosphorylation at Tyr705 in a dose- and time-dependent manner in MDA-MB-435 and MDA-MB-231 cells. Ser727 phosphorylation may be a secondary event after Tyr705 phosphorylation that is required for the maximal transcriptional activity of STAT3. STAT3 serine phosphorylation (Ser727) by ERK negatively regulates the tyrosine phosphorylation of STAT3 (Y705) [27]. Notably, we only observed a small effect on STAT3 Ser727 at 1 μM Bt354. Moreover, there were no significant effects on Jak2, Src, and their phosphorylation. These data suggest that Bt354 specifically blocks the phosphorylation of STAT3 Tyr705. The inhibition of STAT3 activation was independent of general upstream kinases, which may be a result of other signaling events.

The localization experiments using confocal microscopy indicated that Bt354 interfered with the translocation of STAT3 to the nucleus. After treatment with Bt354, the amount of STAT3 in the nucleus decreased in MDA-MB-435 and MDA-MB-231 cells in a dose-dependent manner, and most STAT3 was localized in the cytoplasm. The decrease in STAT3 in the nucleus decreased the downstream gene expression of Bcl-xL, Bcl-2, Mcl-1, and survivin, which are involved in cell proliferation, survival,
and apoptosis [28,29].

Mechanistic insight into the biological effects of Bt354 reveals the suppression of the constitutive expression of known STAT3-regulated genes, including C-myc, Bcl-xL, survivin, and cyclin D1, which control cell growth and apoptosis, promote tumor angiogenesis, and modulate tumor cell invasion, respectively [30,31,32]. Subsequently, we detected the expression of the corresponding proteins in both MDA-MB-435 and MDA-MB-231 cells, the results show that Cdc25c, Cdc2, and cyclin D1 decreased as p-cdc25c increased at 0.5 μM Bt354, and these proteins are related to the accumulation of cells in the G2/M phase of the cell cycle (Fig. 4A). The suppression of Bcl-xL, Bcl-2, Mcl-1, survivin, and C-myc expression by Bt354 is shown in Fig. 4E. The percentage of late apoptosis cells increased in a dose-dependent manner as expected. The downregulation of Bcl-xL, Bcl-2, Mcl-1, survivin, and C-myc is likely linked to the ability of Bt354 to induce cell death in breast cancer cells.

The decrease of STAT3 in the nucleus also interferes with the migration of malignant tumors [33,34,35]. To assess whether Bt354 blocks STAT3-dependent tumor progression, a transwell study was performed as a measure of the migration of MDA-MB-435 and MDA-MB-231 human breast cancer cells. The numbers of MDA-MB-435 and MDA-MB-231 cells migrating into the denuded area were significantly reduced after 24 h treatment with Bt354, with a statistically significantly reduction in number after treatment with 1 μM Bt354. The results were confirmed by the wound healing assay on MDA-MB-435 cells. These finding demonstrated that Bt354 may suppress the migration of MDA-MB-435 and MDA-MB-231 human breast cancer cells, which harbor aberrant STAT3.
Furthermore, the *in vivo* administration of Bt354 on STAT3 induced a significant antitumor response in human breast cancer tumor xenografts. Bt354 effectively inhibited the increase in tumor mass in nude mice with a slight loss of body weight, indicating that Bt354 may possess low toxicity and high anti-tumor activity *in vivo*. Furthermore, Bt354 increased tumor apoptosis, which is consistent with the suppression of cell proliferation by Bt354 observed by Ki-67 staining.

Computational modeling showed that Bt354 may directly bind to the SH2 domain of STAT3. Bt354 forms a number of hydrogen bonds with nearby amino acid residues, including Ser649, Glu652, Asn646, Arg688, and Pro689. This result suggests that the interaction in the SH2 pocket of STAT3 allows Bt354 to inhibit the phosphorylation and nuclear localization of STAT3.

In this work, we identified a STAT3 SH2 domain binder and inhibitor of STAT3 signaling that is structurally different from previously identified dimerization disrupters, peptides and peptidomimetics, and other STAT3 inhibitors, such as STA-21 [36], cucurbitacin, and peptide apatamers [37]. Moreover, Bt354 did not downregulate STAT3 protein expression, even at the highest concentrations tested. Our results suggest that Bt354 is a lead compound for inhibitors of constitutive STAT3 signaling in human breast cancer cells.

**Disclosure statement**

We declare that none of the authors has any potential conflict of interest related to the present work.
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Table 1. MDA-MB-231 tumor inhibitory activity of Bt354 in vivo.

Table 2. MDA-MB-435 tumor inhibitory activity of Bt354 in vivo.

Table 3. Molecular docking LibDockscore, absolute energy, and relative energy of Bt354.

Figure 1. Bt354 inhibits STAT3-dependent luciferase activity. (A) Chemical structures of Bt354. (B) Constitutive expression of STAT3 and phosphorylated STAT3 in seven cancer cell lines. (C) Inhibitory effect of Bt354 on STAT3-dependent luciferase activity in DU145, MDA-MB-435, and MDA-MB-231 cells.

Figure 2. Bt354 inhibits phosphorylation of STAT3 Tyr705 independent of JAK2 activity, and Bt354 inhibits STAT3 nuclear localization in MDA-MB-435 and MDA-MB-231 cells. MDA-MB-435 and MDA-MB-231 cells were dose- and time-dependently treated with Bt354. Cell lysates were prepared with RIPA lysis buffer. Proteins were analyzed by immunoblotting with specific antibodies. (A) Bt354 inhibition of the phosphorylation of STAT3. Tyr705 was inhibited in a dose-dependent manner, whereas Ser727 was only slightly inhibited. (B) Time-dependent Bt354 suppression of phosphorylated STAT3 levels. MDA-MB-231 and MDA-MB-435 cells were treated with 1 μM Bt354 for the indicated times. (C, D) Inhibition of subcellular localization of STAT3 evaluated by immunofluorescence staining. Following treatment with 1 and 5 μM Bt354 for 12 h, MDA-MB-435 and MDA-MB-231 cells were stained with anti-STAT3 to label STAT3 (green). DAPI was used to label the nucleus (blue). Similar results were obtained in three independent experiments.

Figure 3. Bt354 inhibits the proliferation of prostate and breast cancer cells. (A) Cell viability determined by an MTT assay of DU145, MDA-MB-435, and MDA-MB-231 cells treated with different concentrations of Bt354 for 72 h. (B) Cell viability determined by an MTT assay of DU145, MDA-MB-435, and MDA-MB-231 cells treated with Bt354 for 24, 48, and 72 h. (C) Anti-proliferative effects of Bt354 in MDA-MB-435 and MDA-MB-231 evaluated by using immunofluorescence staining with EdU. (D) Change in the number of EdU-labeled MDA-MB-435 and MDA-MB-
231 cells. The results are representative of three independent experiments. 

Figure 4. Bt354 induces G2/M arrest and apoptosis in MDA-MB-435 and MDA-MB-231 cells. (A) PI staining and flow cytometry analysis of MDA-MB-435 and MDA-MB-231 exposed to different concentrations of Bt354 for 24 h. (B) Cell cycle analysis via DNA content and cell cycle distribution shown as histograms. (C) Fluorescence correlation spectroscopy images of PI and Annexin V double staining of MDA-MB-435 and MDA-MB-231 cells that were exposed to different concentrations of Bt354 for 48 h. (D) Histograms of the percentage of apoptotic cells. (E) Western blot analysis of cell cycle- and cell apoptosis-related protein expression in breast cancer cells treated with different concentrations of Bt354 for 24 h. The results are representative of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the control group.

Figure 5. Bt354 inhibits the migration of MDA-MB-435 and MDA-MB-231 cells. (A) Micrograph images of MDA-MB-435 and MDA-MB-231 cells treated with vehicle (0.1% DMSO) or Bt354 (0.5, 1, 5 μM). Five fields were selected at random. (B) Cell populations in each image. The inhibition of migration occurred in a dose-dependent manner. (C) Wound healing assay of MDA-MB-435 cells treated with Bt354 (0.5, 1, 5 μM) for 24 h and allowed to migrate into the denuded area. The results are representative of three independent experiments. *P < 0.05 and **P < 0.01 compared with the control group.

Figure 6. Bt354 inhibits tumor growth of MDA-MB-435 and MDA-MB-231 cells. (A) Weight of the stripped tumors of MDA-MB-231 xenograft BalB/C nu/nu mice that received the indicated dosage of Bt354, docetaxel, or nabapuca. On day 21, the mice were euthanized and the tumor tissues were exfoliated and photographed. The weights are presented as means with standard errors (**P < 0.01 and ***P < 0.001). (B) Tumor volume as a function of time in MDA-MB-231 xenograft tumor mice that received the indicated concentrations of Bt354 and docetaxel. (C, D) Tumor volume of the stripped tumors of MDA-MB-435 xenograft mice and weight of mice. The measurements are presented as means with standard errors. *P < 0.05, **P < 0.01, and ***P < 0.001.
Figure 7. (A) Sections of individual tumors from six groups immunohistochemically stained with Ki-67 to evaluate cell proliferation (×200). **P < 0.01 and ***P < 0.001. (B) Western blot analysis of three tumors from four groups. The expression of STAT3 and p-STAT3 (Y705) was detected. (C) TUNEL assays of tumor sections from MDA-MB-231 and MDA-MB-435 cells to detect apoptosis in tumor tissue.

Figure 8. Schematic diagrams of Bt354 docking with the STAT3 SH2 domain. (A) Predicted model of Bt354 binding to the STAT3 SH2 domain as shown by computational modeling. Protein structure information was obtained from Protein Data Bank entry 1BG1. (B, C) Model of Bt354 binding to the SH2 domain. (D) Predicted interaction between the amino acid residues of the SH2 domain and Bt354. Hydrogen bonding is shown between Ser649 and Bt354. The π-cation interaction is shown between Arg688 and Bt354.

Supplementary Fig. S1 (A) Body weight of MDA-MB-231 xenograft mice. (B) Body weight of MDA-MB-435 xenograft mice.

Supplementary Fig. 2 Inhibition of MCF 10A.

Supplementary Fig. 3 Inhibition of MCF-7.

Supplementary Fig. 4 Effect of Bt354 on PARP and caspase (3,8,9).

Supplementary Fig. 5 Effect of Bt354 inhibitor (Napabucasin) on PARP and caspase 3.
(A) MDA-MB-231  (B) MDA-MB-231  (C) MDA-MB-435  (D) MDA-MB-435

(A) 
(B) 
(C) 
(D)

(A) MDA-MB-231  (B) MDA-MB-231  (C) MDA-MB-435  (D) MDA-MB-435

(A) 
(B) 
(C) 
(D)

(A) MDA-MB-231  (B) MDA-MB-231  (C) MDA-MB-435  (D) MDA-MB-435

(A) 
(B) 
(C) 
(D)

(A) MDA-MB-231  (B) MDA-MB-231  (C) MDA-MB-435  (D) MDA-MB-435

(A) 
(B) 
(C) 
(D)

(A) MDA-MB-231  (B) MDA-MB-231  (C) MDA-MB-435  (D) MDA-MB-435

(A) 
(B) 
(C) 
(D)

(A) MDA-MB-231  (B) MDA-MB-231  (C) MDA-MB-435  (D) MDA-MB-435

(A) 
(B) 
(C) 
(D)
Table 1. MDA-MB-231 tumor inhibitory activity of Bt354 in vivo.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Number</th>
<th>Body Weight (g)</th>
<th>Tumor volume (mm$^3$)</th>
<th>Tumor Weight (g)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Begin / End</td>
<td>X ±SD</td>
<td>Begin / End</td>
<td>X ±SD</td>
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<tr>
<td>Control</td>
<td>-</td>
<td>6/5</td>
<td>20.0±0.6</td>
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<td>133.7±38.0</td>
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<td>Docetaxel</td>
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<td>19.9±0.3</td>
<td>17.6±0.9</td>
<td>138.1±28.5</td>
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<tr>
<td>Napabucasin</td>
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<td>18.8±0.7</td>
<td>123.4±20.9</td>
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NA: Not Applicable, T/C%: Tumor volume of Treatment group/Control group×100, TGI: Tumor Growth Inhibition (100-Tumor weight of Treatment group/Control group×100) (*P < 0.05, **P < 0.01, ***P < 0.001)
Table 2. MDA-MB-435 tumor inhibitory activity of Bt354 in vivo.

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<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Number Begin / End</th>
<th>Body Weight (g) Begin / End</th>
<th>Tumor volume (mm$^3$) Begin / End</th>
<th>Tumor Weight (g) Begin / End</th>
<th>T/C% Begin / End</th>
<th>TGI X ±SD T/C%</th>
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<td>Docetaxe</td>
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<td>19.8±0.8 20.0±0.6</td>
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<td>23.5***</td>
<td>0.24±0.04</td>
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NA: Not Applicable, T/C%: Tumor volume of Treatment group/Control group×100, TGI: Tumor Growth Inhibition (100-Tumor weight of Treatment group/Control group×100) (*P < 0.05, **P < 0.01, ***P < 0.001)

Table 3. LibDockscore, absolute energy, and relative energy of Bt354 in molecular docking.

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<th>Compound</th>
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<th>Relative energy</th>
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