Acetyl-cinobufagin Suppresses Triple Negative Breast Cancer Progression by Inhibiting the STAT3 Pathway

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Abstract

Background: The incidence of breast cancer (BC) worldwide has increased substantially in recent years. Epithelial-mesenchymal transition (EMT) refers to a crucial event impacting tumor heterogeneity. Although cinobufagin acts as an effective anticancer agent, the clinical use of cinobufagin is limited due to its strong toxicity. Acetyl-cinobufagin, a pre-drug of cinobufagin, was developed and prepared with greater efficacy and lower toxicity.

Methods: A heterograft mouse model using triple negative breast cancer (TNBC) cell lines, was used to evaluate the potency of acetyl-cinobufagin. Signal transducer and stimulator of transcription 3 (STAT3)/EMT involvement was investigated by gene knockout experiments using siRNA and Western blot analysis.

Results: Acetyl-cinobufagin inhibited proliferation, migration, and cell cycle S/G2 transition and promoted apoptosis in TNBC cells *in vitro*. In general, IL6 triggered the phosphorylation of the transcription factor STAT3 thereby activating the STAT3 pathway and inducing EMT. Mechanistically, acetyl-cinobufagin suppressed the phosphorylation of the transcription factor STAT3 and blocked the interleukin (IL6)-triggered translocation of STAT3 to the cell nucleus. In addition, acetyl-cinobufagin suppressed EMT in TNBC by inhibiting the STAT3 pathway. Experiments in an animal model of breast cancer clearly showed that acetyl-cinobufagin was able to reduce tumor growth.

Conclusions: The findings of this study support the potential clinical use of acetyl-cinobufagin as a STAT3 inhibitor in TNBC adjuvant therapy.

Introduction

Breast Cancer (BC) is the most common tumor among women worldwide. According to statistics from IARC /WHO, there were 2,261,419 new patients in 2020, representing 11.7% of all cancers and 24.5% of female tumors. In addition, the number of deaths from BC was estimated to be 684,996, accounting for 6.9% of all tumors and 15.5% of female tumors [1]. Compared with the other subtypes of BC, triple negative breast cancer (TNBC) is more likely to relapse and metastasize, and is associated with an inferior survival rate. Due to the lack of these specific targets (HR and HER2), TNBC has fewer treatment options than other types of invasive breast cancer and the current treatment regimens, including surgery, chemotherapy and radiotherapy, are inadequate [2]. Therefore, it is imperative to find potential molecular drugs with high potency and lower toxicity.

Signal transducer and stimulator of transcription 3 (STAT3), a member of the STAT protein family, modulates the expression of a variety of genes [3,4]. STAT3 promotes the proliferative activity of cancer cells, immune suppression, angiogenesis, metastatic progression, and development of drug resistance [5,6,7]. Many studies have revealed that STAT3 also plays crucial roles in BC development, as well as in drug resistance development in BC patients receiving chemotherapy [8,9,10]. The regulation of STAT3 activity could effectively suppress carcinogenesis, cancer development, and

invasive behavior in TNBC [11]. Therefore, STAT3 is currently recognized a potential molecular target for the development of an effective treatment for TNBC.

Epithelial-mesenchymal transition (EMT) is the phenotypic conversion of epithelial cells to mesenchymal cell [12]. Several previous studies have shown that EMT is associated with tumor progression and metastasis in TNBC [13,14]. Increasing evidence indicates that various treatment regimens inhibit the migratory behavior by downregulating EMT [15]. In recent years, research has shown that interleukin 6 (IL6) induces EMT of cancer cells by STAT3 activation [16,17,18]. At the same time, STAT3 inhibitors can suppress cancer development, invasive activity, and migratory behavior through inhibition of EMT [19].

Cinobufagin is a key active constituent of Chansu [20]. Cinobufagin has been suggested to possess significant anticancer activity [21,22]. However, although cinobufagin possess satisfactory cytotoxic activity against a variety of cancer cells *in vitro*, its toxicity and severe side effects following its administration *in vivo* are undesirable, clearly revealing the limitations for the clinical use of cinobufagin. In addition, its rapid metabolism, insolubility, and short half-life, further restrict clinical application [23]. In this study, a cinobufagin pre-drug, namely acetyl-cinobufagin, was developed and prepared as per specifications for aspirin and diacerein to obtain new analogs with higher potency and lower toxicity. Yang Lehe *et al.* have applied this processing method to bufalin and obtained good results [24]. Acetyl-cinobufagin contribute to improve drug administration and might enhance the anticancer effect.

Materials And Methods

Cell culture

The experiments in this study were performed in the commonly used normal (non-tumorigenic) human mammary epithelial cells (MCF-10A) and various human BC cell lines (Hs578T, BT-549, MDA-MB-468 and MDA-MB-231) obtained from the Cell Bank of the Shanghai Chinese Academy of Sciences (Shanghai, China). The Hs578T and MDA-MB-231 cell lines were cultured , in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% Gibco fetal bovine serum (FBS; Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1% penicillin/streptomycin (Solarbio, Beijing, China). The BT-549 cell line was cultured in Gibco Roswell Park Memorial Institute (RPMI)-1640 medium (Thermo Fisher Scientific Inc.) supplemented with 10% Gibco FBS. The MDA-MB-468 cell line was cultured in Gibco Leibovitz's L-15 medium (Thermo Fisher Scientific Inc.) supplemented with 10% Gibco FBS. The MCF-10A cell line was cultured in Gibco DMEM/F12 medium (Thermo Fisher Scientific Inc.) supplemented with 10% Gibco FBS. All cell lines were maintained in an incubator at 37°C, with a humidified atmosphere and 5% CO₂, except for the MDA-MB-468 cell line, which was cultured without CO₂.

Methyl thiazolyl tetrazolium (MTT) cytotoxicity analysis

The MTT assay was used to measure cell viability or cytotoxicity (loss of viable cells) of the test compounds cinobufagin and acetyl-cinobufagin in human BC cell lines and normal breast cells. MTT analysis was performed as follows. Cells were seeded into 96-well plates at a density of 8×10^3 cells per well and incubated overnight. After treatment with the appropriate test compound, 25 μ L of the MTT reagent was added to each well and plates were incubated at 37 °C for an additional 4 h. Afterwards, following removal of the culture medium, the formed formazan crystals were dissolved in 150 μ L of dimethyl sulfoxide (DMSO), and then the optical density (OD) of each well was measured at 490 nm on a Microplate Reader. The cytotoxicity was determined using the following equation: cell activity = (mean OD value of treated wells/mean OD value of vehicle control wells) $\times$ 100%. The half maximal inhibitory concentration (IC₅₀) values were determined using the GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA).

Acute toxicity test

For the acute toxicity assay, thirty 8-week-old female NIH mice were randomly divided into 3 groups of 10 mice each (n = 10), namely the vehicle group, cinobufagin group (30 mg/kg) and acetyl-cinobufagin group (30 mg/kg). The mice in each group were injected intraperitoneally with the corresponding compound on the 1st day. All animals were kept at 25 °C, under a 12 h light/dark cycle and given *ad libitum* access to food and water. As endpoints, the mortality rate and body weight of the animals were recorded for each group daily for 14 d. All animals were sacrificed by cervical dislocation at the end of the experiment.

Colony assay

Cells were seeded into each well of 6-well plates, at a density of 1,500 cells/well. After treatment with drugs or DMSO for 12 h, the cells were cultured in an incubator at 37 °C with a humidified atmosphere and 5% CO₂, for 6-8 days. Afterwards, the cells were fixed with 5% paraformaldehyde (PFA) and stained with 0.1% crystal violet for 15 minutes at 24 °C. All images of stained cells were captured with a camera. Each experiment was performed in triplicate.

Wound-healing migration assay

The cell migration ability was assessed by wound healing assay. The cells were seeded into 6-well plates, allowed to grow to 80–90% confluence and then a linear scratch wound was made on the cell monolayer using a sterile 10- μ L pipet tip. After rinsing the wells with phosphate-buffered saline (PBS) to remove the unbound cells, cells were continued to culture in serum-free medium with vehicle control or acetyl-cinobufagin (0.25, 0.5, 1.0 μ M) for 48 h. The healing of the wound was monitored by capturing images, using an inverted microscope connected to an imaging system (Leica Biosystems GmbH, Wetzlar, Germany), at 0, 24 and 48 h to measure the migration of cells to the wound area.

Transwell invasion assay

Corning® Transwell® polycarbonate membrane cell culture inserts (#3422; Corning Inc., Corning, NY, USA) were used for invasion assay. Cells (MDA-MB-468, BT-549 and Hs578T) were harvested, washed with PBS, resuspended in corresponding serum-free medium and seeded onto the upper side of the Transwell inserts in the upper chamber at a density of 3×10^4 cells per well. A 600- μ L volume of the corresponding medium supplemented with 10% FBS was added to lower chamber. After 24 h, the cells on the upper surface of the Transwell inserts were removed by wiping the insert upper side with cotton swabs. Then, the cells on the lower surface of the inserts were fixed with PFA and stained with 0.4% crystal violet. Finally, invading cells were quantified using a microscope at 20 $\times$ magnification to count positively stained cells in three random fields for each cell line evaluated.

Immunofluorescence staining

BT-549 cells were seeded on fluorescence glass slides and cultured under treatment with the corresponding compound. Subsequently, cells were fixed with 4% PFA, permeabilized in PBS containing 0.5% Triton X-100, washed four times with PBS, and blocked in 1% bovine serum albumin (BSA) in PBS for 60 min. Then, cell slides were incubated overnight at 4 °C with the corresponding primary antibody (P-STAT3, E-Cadherin, N-Cadherin and Ki67) at the appropriate dilution. Afterwards, the cell slides were incubated with an anti-rabbit secondary antibody or anti-mouse secondary antibody conjugated with Alexa Fluor® 488 (Thermo Fisher Scientific Inc. Waltham, MA, USA) and nuclei were counterstained with the 4',6-diamidino-2-phenyl-indole dihydrochloride (DAPI) (Beyotime Biotechnology, Shanghai, China) in the dark. Then, the cell slides were washed once with PBS and sealed with anti-fluorescence quenching sealing solution and stained cells were visualized by confocal laser scanning microscopy using the Zeiss LSM 710 Confocal Laser Scanning Microscope (Carl Zeiss AG, Oberkochen, Germany) to capture the images.

Hoechst 33342 staining

A Hoechst 33342 staining kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to assess the characteristic apoptotic morphological changes in MDA-MB-468, BT-549 and Hs578T cells. Cells were seeded into 6-well plates (3×10^5 cells/mL) and treated with DMSO (Control) or acetyl-cinobufagin (0.5, 1.0, 2.0 μ M). After incubation for 24 h, cells were washed with PBS, fixed with 4% PFA for 15 min, washed again with PBS, and apoptotic nuclei were stained by incubation at 37 °C with Hoechst 33342 ($v_{\text{Hoechst 33342}}/v_{\text{PBS}}$ at 1:1000) solutions for 20 min in the dark. Finally, cells were washed with PBS, mounted with antifade mounting solution and apoptotic cells were identified after acquiring images by fluorescence microscopy on a Leica fluorescence microscope (Leica Biosystems GmbH).

Apoptosis assay

Cell apoptosis assay was conducted by flow cytometry using the BD Pharmingen™ Annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis detection kit (#556,547; BD Biosciences, Franklin Lakes, NJ, USA). Cells were seeded into 6-well plates and cultured in the absence or presence of acetyl-cinobufagin at the indicated concentrations. After the treatments, cells were harvested, resuspended and subjected to the

Annexin V-FITC/PI apoptosis assay according to the manufacturer's instruction. Briefly, cells were resuspended in 500 μ L of binding buffer, stained by incubation on ice in the dark with 5 μ L of PI for 5 min and 5 μ L FITC-conjugated Annexin V for 15 min. Data from a total of 20,000 fluorescently stained cells/sample were collected by flow cytometry on a BD Accuri C6 Flow Cytometer (BD Biosciences). The Flowjo software (FlowJo, Ashland, OR, USA) was used to analyze the data collected by flow cytometry, so as to calculate the percentage of early (Q3, Annexin V+/PI-) and late (Q2, Annexin V+/PI+) apoptotic cells, in order to determine the total number of apoptotic cells.

Cell cycle assay

The MDA-MB-468 and BT-549 cells were seeded on 6-well plates, incubated for 12 h, and treated with DMSO or acetyl-cinobufagin (0.5, 1.0, 2.0 μ M) for 24 h. After incubation, cells were harvested and fixed with ice cold 70% ethanol at -20°C, digested with 0.1 mg/mL RNase for 10 min at 37°C, then stained with 10 μ g/mL PI, and the DNA content data for cell cycle analysis of each sample were acquired on a FACSCalibur flow cytometer (BD Biosciences).

Western blot analysis

Total protein was extracted from cells treated with either DMSO or acetyl-cinobufagin by lysing the cells on ice for 30 min in radioimmunoprecipitation assay (RIPA) lysis buffer (Solarbio) with 1 mM phenylmethylsulfonyl fluoride (PMSF; Solarbio). After ultrasonic dispersion for 60 s samples were centrifuged at 4°C for 20 min at 10,000 rpm and the protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). After resuspending equal amounts of protein samples in loading buffer solution and denaturing at 95 °C for 10 min, proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) precast gel (BioRad Laboratories, Hercules, CA, USA). Subsequently, the separated proteins were transferred to 0.45- μ m polyvinylidene fluoride (PVDF) membranes (EMD MilliporeSigma, Burlington, MA, USA). The membranes were blocked in 5% skim milk in Tris buffer saline with 0.1% tween-20 (TBST) for 1 h at room temperature, and then the blots were incubated overnight at 4°C with the appropriate primary antibodies. Afterwards, following washes with PBS, the blots were incubated for 1h at room temperature with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000, Abcam, Cambridge, UK). Subsequently, after washing the blots, protein bands were visualized by using the electrochemiluminescence (ECL) detection system (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. The GAPDH protein was developed and used as a loading control using the ImageJ software (National Institute of Health (NIH), Bethesda, MD, USA) to measure the relative intensity of the labeled band. All experiments were performed at least 3 times. The primary antibodies included: E-cadherin (1:5,000, 20874-1-AP, Proteintech, Wuhan, China), N-cadherin (1:2,000, 22018-1-AP, Proteintech), Vimentin (1:2,000, 10336-1-AP, Proteintech), P-STAT3 (1:2,000, ab76315, Abcam), STAT3 (1:1,000, 12640S, Cell Signaling Technology (CST) Inc., Danvers, MA, USA), Cyclin A1 (1:500, AF0143, Affinity Biosciences, Cincinnati, OH, USA), CDK2 (1:1,000, 10122-1-AP, Proteintech), Bax (1:5,000, 50599-2-IG,

Proteintech), Bcl-2 (1:2,000, ab182858, Abcam), lamin B1 (1:1,000, ab133741, Abcam), GAPDH (1:5,000, 10494-1-AP, Proteintech).

Extraction of cytoplasmic and nuclear proteins

BT-549 cells were grown to 80-90% confluence in a 10-cm dish and treated with Acetyl-cinobufagin (0.5, 1.0 and 2.0 μ M). After IL6 stimulation for 0.5 h, the cells were immediately placed on ice. Nuclear and cytoplasmic proteins were extracted using the NE-PER™ Nuclear and Cytoplasmic extraction Kit (Cat# 78833; Thermo Fisher Scientific Inc.). The immunoblotting analysis was performed to detect the expression of related proteins.

Transient transfection of small interfering RNA (siRNA)

Synthetic si-RNAs, specifically STAT3 small interfering RNAs (STAT3 si-RNAs) and negative-control siRNAs (si-NC), were custom made. The siRNAs were transiently transfected into BT-549. The STAT3 si-RNAs were the following: STAT3 si-RNA1 STAT3-Homo-978 5-GCAACAGAUUGCCUGCAUUTT-3; STAT3 si-RNA2 STAT3-Homo-398 5-CCACUUUGGUGUUUCAUAATT-3; and STAT3 si-RNA3 STAT3-Homo-1070 5-CCCGUCAACAAUUAAGAATT-3.

Molecular docking

The molecular docking of acetyl-cinobufagin to the binding sites of the STAT3 molecular docking was determined using the AutoDock Vina 4.2.6 software (<https://ccsb.scripps.edu/autodock/>). The crystal structure of STAT3 derived from the Protein Data Bank was used for the docking research. The input data of the ligand and acceptor were created using the Graphical User Interface program AutoDock Tools 1.5.62. In the docking, the acceptor was deemed as rigid whereas the ligand was elastic.

Animal model

The female BALB/c athymic nude mice used in this study were kept in the experimental animal department of WMU and their use was approved by the ethical committee of WMU. The nude mice were subcutaneously injected with BT-549 cells (5×10^6) which were diluted by mixing with the appropriate quantity of Matrigel (BD Biosciences) and PBS (orthotopically). After 7-10 days, when the tumor measured $\sim 100 \text{ mm}^3$, the animals were assigned into different groups for the assays (control group, positive control group receiving napabucasin at 10 mg/kg, treatment group A receiving acetyl-cinobufagin at 1.0 mg/kg, and treatment group B receiving acetyl-cinobufagin at 2.0 mg/kg, treatment group C receiving cinobufagin 2.0 mg/kg). There were more than 6 mice in every group, and treatment was administered by intraperitoneal injection. The tumor size and body weight variations were recorded every other day for 12 days. After completion of the experiments, mice were sacrificed, and cancer tissue and organs, such as heart, liver, kidneys, and lungs were harvested and used for subsequent immunoblotting assays and hematoxylin and eosin (H&E) staining.

Statistical analysis

Every experiment was independently repeated at least 3 times to ensure the reproducibility and reliability of the study. The data obtained were statistically analyzed using the GraphPad Prism 8.0 software. The difference between experimental groups was analyzed by Student's t-test. For all analyses, if the *p*-value was less than 0.05, the difference was considered to be significant, and indicated evidence of the good outcome of the therapy with acetyl-cinobufagin.

Results

Acetyl-cinobufagin inhibits the viability and proliferation of TNBC cells

To fully understand the metabolic effect of cinobufagin and identify novel analogs with higher potency and lower toxicity, the pre-drug of cinobufagin, namely acetyl-cinobufagin was developed and prepared (Figure 1a). To evaluate the potency of cinobufagin and acetyl-cinobufagin on human BC cell lines, the cell viability was determined using the MTT assay. The results revealed that the IC₅₀ of acetyl-cinobufagin against MDA-MB-468, BT-549 and Hs578T cells were 1.120, 1.267 and 1.195 μM, respectively (Figure 1b); while IC₅₀ of cinobufagin against MDA-MB-468, BT-549 and Hs578T were 4.708, 21.550 and 8.081 μM, respectively (Figure 1c). In addition, the IC₅₀ of acetyl-cinobufagin against MDA-MB-231 were 13.86 μM, respectively (Supplementary Figure S1a). Meanwhile, the IC₅₀ of acetyl-cinobufagin against normal breast cell line (MCF-10A) was 35.90 μM (Supplementary Figure S1b). Acute toxicity experiments were conducted to determine the safety of the compounds. The majority of the animals in the cinobufagin group (8/10) died after receiving the injection of 30 mg/kg cinobufagin, whereas only a few (2/10) animals died after the injection of acetyl-cinobufagin (Figure 1d). In contrast to the negative control (vehicle) group, the weight of the animals in the acetyl-cinobufagin group remained steady (Figure 1e). Moreover, acetyl-cinobufagin significantly reduced the colony formation ability of the TNBC cell lines (Figure 1f). The evaluation of the effect of acetyl-cinobufagin on the proliferative activity of TNBC cells through immunofluorescence analysis of the expression of Ki67 in BT-549 cells revealed that the fluorescence intensity of Ki67 was markedly dose-dependently attenuated (Figure 1g), indicating that acetyl-cinobufagin suppresses cell growth and proliferation in a dosage-dependent manner.

Acetyl-cinobufagin inhibits the migration of TNBC cells

The analysis of the inhibitory effect of acetyl-cinobufagin on the migration of the TNBC cell lines MDA-MB-468, BT-549 and Hs578T by the wound healing assay revealed that acetyl-cinobufagin dose-dependently markedly inhibited their migration (Figure 2a, Supplementary Figure S1c and d). In addition, our evaluation of the inhibitory effect of acetyl-cinobufagin on the migration of the TNBC cell lines MDA-MB-468, BT-549 and Hs578T by the Transwell assay also showed that acetyl-cinobufagin dose-dependently markedly blocked their migration (Figure 2b). since the hallmark of EMT is the upregulation of the expression of N-cadherin followed by the downregulation of the expression of E-cadherin, we analyzed their expression in the TNBC cells by immunofluorescence staining. The immunofluorescence analysis indicated that treatment with acetyl-cinobufagin led to increased expression of E-cadherin, and

reduced expression of N-cadherin (Figure 2c and d), suggesting an inhibitory effect on EMT. A similar effect of acetyl-cinobufagin on the expression of EMT marker proteins was indicated by Western blot analysis, which revealed increased expression of E-cadherin and decreased expression of vimentin in the presence of acetyl-cinobufagin compared with the control group (Figure 2e). Together, these findings demonstrated that acetyl-cinobufagin not only inhibited migration and proliferation of TNBC cells, but also regulated the expression of t protein involved in migration.

Acetyl-cinobufagin promotes apoptosis of TNBC cells

The effect of various concentrations of acetyl-cinobufagin (0, 0.5, 1.0, and 2.0 μ M) on apoptosis in the TNBC cell lines MDA-MB-468, BT-549 and Hs578T was analyzed by Hoechst 33342 staining of apoptotic cell nuclei. The results showed that cells treated with acetyl-cinobufagin exhibited higher nuclear staining with Hoechst 33342 (blue fluorescence) indicating the induction of substantial apoptosis (Figure 3a). A similar apoptotic effect of acetyl-cinobufagin on these 3 TNBC cell lines was observed by flow cytometry analysis, after treating these cells with acetyl-cinobufagin for 24 h. Apoptotic cells were positively stained for Annexin V alone or both Annexin V and PI. However, the dead cells were merely PI-positive. Specifically, the results showed that acetyl-cinobufagin significantly increased the proportion of apoptotic cells (Figure 3b). In addition, the results also revealed that the expression of apoptosis-associated proteins was also dose-dependently regulated by acetyl-cinobufagin, in particular, the expression of BAX was increased, while that of BCL2 was reduced (Figure 3c and d). In general, acetyl-cinobufagin significantly induced apoptosis in TNBC cells.

Acetyl-cinobufagin inhibits S/G2 transition in TNBC cells

The acetyl-cinobufagin-induced retardation of cell cycle progression was evaluated in MDA-MB-468 and BT-549 cells treated with acetyl-cinobufagin for 12 h by calculating the proportion of cells in the S to G2 phase transition by flow cytometric analysis. In contrast to the untreated control (vehicle) group, acetyl-cinobufagin markedly induced the retardation of cell cycle progression in the S/G2 phase transition (Figure 4a). Since CDK2 and cyclin A1 are well-known key proteins for S-phase arrest, we analyzed their expression by immunoblotting analysis. The results revealed that treatment with acetyl-cinobufagin dosage-dependently suppressed the expression of cyclin A1 and CDK2 in human TNBC cells (Figure 4b and c). Thus, these finding indicate that the inhibitory effect of acetyl-cinobufagin on the proliferative activity of human TNBC cells may be partially related to the induction of retardation of cell cycle progression in the S to G2 phase transition.

Acetyl-cinobufagin inhibits EMT *via* STAT3 signaling

The molecular docking results revealed that acetyl-cinobufagin can occupy the STAT3 binding sites and form hydrogen bonding with the GLN-326 residue (Figure 5a and b). Given that a derivative of cinobufagin has been previously found to exhibit anticancer activity by suppressing the STAT3 signaling pathway, we hypothesized that acetyl-cinobufagin might impact STAT3 phosphorylation (P-STAT3). We performed Western blot analysis to investigate whether acetyl-cinobufagin impacts the expression of P-

STAT3 in MDA-MB-468, BT-549, and Hs578 cells and found that acetyl-cinobufagin time- and dose-dependently regulates the expression of P-STAT3 (Figure 5c, Supplementary Figure S1e). In addition, immunofluorescence staining revealed that P-STAT3 was localized to the cytoplasm and subsequently, in response to IL6 activation, translocated to the nucleus, as indicated by the increased accumulation of the green fluorescence protein in the cell nuclei. We also found that such IL6-induced translocation to the nucleus can be reversed by treatment with acetyl-cinobufagin (Figure 5d). These results strongly suggested that IL6-induced STAT3 nuclear translocation was suppressed by acetyl-cinobufagin. Additionally, we also analyzed the levels of nuclear and cytoplasmic STAT3 proteins after treatment with IL6. Compared with non-stimulated cells, after stimulation with IL6 for 30 min, the level of nuclear STAT3 protein was increased, whereas the levels of cytoplasmic STAT3 protein was decreased. We also found that acetyl-cinobufagin suppressed IL6-induced STAT3 nuclear translocation in a dose-dependent manner (Figure 5e). These findings indicate that acetyl-cinobufagin suppresses STAT3 signaling and interferes with the STAT3 nuclear translocation.

Past research has revealed that EMT occurs downstream of the STAT3 signaling pathway. Thus, we further investigated whether acetyl-cinobufagin suppresses the role of EMT in tumorigenesis and migration by inhibiting the STAT3 signaling pathway. We found that the treatment of TNBC cells with IL6 (20 ng/mL) for 30 min led to a remarkable increase of the levels of P-STAT3 compared with the control group. In addition, the expression of E-cadherin was decreased, while the expression of N-cadherin was increased. Such effects were suppressed by treatment with acetyl-cinobufagin, which reduced P-STAT3 and inhibited EMT (Figure 5f). Moreover, we designed and selected si-STAT3, a siRNA with maximal inhibitory effect against STAT3, through Western blot analysis, which showed that STAT3 si-RNA3 had the maximal inhibitory effect on STAT3 (Supplementary Figure S1f). However, as shown in Figure 5g, the results revealed that knockdown of STAT3 expression by STAT3 siRNA3 had no significant effect on EMT. These results were further verified by knockdown of STAT3 in TNBC cells before the treatment with 1.0 μ M acetyl-cinobufagin. As shown in Figure 5h, knockdown of STAT3 had no noticeable effect on invasive and metastatic properties of the TNBC cells. In general, acetyl-cinobufagin exerted a potent antitumor effect by suppressing EMT in TNBC cells through the STAT3 signaling pathway.

Acetyl-cinobufagin inhibits the growth of the TNBC xenograft model

We further investigated whether acetyl-cinobufagin suppresses STAT3 and EMT *in vivo*. To this end, a human xenograft model was established by orthotopically injecting BT-549 cells into the mammary fat pad of nude mice and used it to verify the inhibitory effect of acetyl-cinobufagin on TNBC cells *in vivo*. We treated the mice with napabucasin (10 mg/kg; positive control), or cinobufagin (2.0 mg/kg) or different concentrations of acetyl-cinobufagin (1.0 or 2.0 mg/kg) by intraperitoneal injection every other day. The growth curves of the human xenograft tumors in the nude mice showed a significant decline in tumor growth in the treated animals and the tumor volume also decreased (Figure 6a and b). At the same time, the tumor weight was also reduced (Figure 6c). No significant difference in body weight was detected during the treatment period (Figure 6d). H&E staining of the primary organs displaying morphogenetic processes revealed that no change had occurred (Figure 6e). These results revealed no

evidence of cytotoxicity in acetyl-cinobufagin-treated mice, verifying its safety *in vivo*. We further investigated the effect of acetyl-cinobufagin on cancer development by immunohistochemical analysis of the expression of Ki67. The results showed a decrease in the expression level of Ki67 (Figure 6f). These findings evidenced that acetyl-cinobufagin markedly suppressed the proliferative activity of TNBC cells through the STAT3 signaling pathway *in vivo*. The immunoblotting assay also confirmed that acetyl-cinobufagin suppressed STAT3 signal transmission and EMT (Figure 6g). Furthermore, these results revealed that acetyl-cinobufagin is a potential candidate for the treatment of TNBC. Our study provides inspirations for the development of tumor therapy by suppressing the migratory behavior of TNBC cells through regulation of STAT3 signaling.

Discussion

Cinobufagin is a natural product extracted from toad venom, one of the major active ingredients in cinobufotalin injection, which is mainly used to treat intermediate and advanced tumors [25]. However, the rapid metabolism of cinobufagin, toxicity, insolubility and short half-life have limited its clinical use [23]. In this study, we used a similar approach as that used for aspirin and acetyl-bufalin to acetylate cinobufagin, which improved its curative effect without its toxic side effects. For example, acetyl-cinobufagin exhibited better tolerance in animals treated with a high-dose compared to treated with cinobufagin (Figure 1d and e). Moreover, acetyl-cinobufagin had a better therapeutic effect than cinobufagin (Figure 6a, b and c). In addition, we used TNBC cells to determine the functional role of acetyl-cinobufagin. It was found the lowest IC₅₀ of acetyl-cinobufagin reached 1.12 μ M against MDA-MB-468 cells. Furthermore, acetyl-cinobufagin was shown to suppress the proliferation of TNBC cells and promote cell apoptosis. Furthermore, like cinobufagin, acetyl-cinobufagin delayed S/G2 phase transition in TNBC cells [26]. Additionally, we also verified the distinct anticancer mechanisms, as well as the long-term stable efficacy and low toxicity of acetyl-cinobufagin. Together, our results suggest that acetyl-cinobufagin had a more effective anticancer activity than cinobufagin.

EMT has been strongly associated with cancer development and metastasis in a variety of tumor types [27]. It involves multiple signaling pathways, (including the JAK/STAT, TGF- β , PI3K/AKT, Ras/MAPK, Wnt, Notch, and Hedgehog signaling pathways) and transcription factors [28,29]. EMT involves various processes, such as loss of epithelial polarity detachment of the basement membrane, and acquisition of mesenchymal features, but not acquisition of migration and invasion features [30]. One of the most prominent hallmarks of TNBC is EMT, a conservative development process often hijacked by cancer cells to enhance their migration and invasion abilities [31]. It is widely established that IL6 promotes TNBC invasion and migration [32]. This study found increased expression of E-cadherin, whereas the expression of vimentin and N-cadherin was decreased. Therefore, our findings indicate that acetyl-cinobufagin decreased migration of TNBC cells by inhibiting EMT.

In the tumor microenvironment (TME), the IL6/JAK/STAT3 signaling pathway promotes the proliferative, survival, invasive, and metastatic activities of cancer cells, and strongly inhibits the anticancer immune response [33]. Signaling through the IL6/JAK/STAT3 pathway after activation by the binding of IL-6

family of cell factors (IL6 and IL-11) to the receptors has been shown in BC progression [34]. After IL6 binds to its receptor, STAT3 is stimulated by phosphorylation at residue Tyr-705, which leads to p-Tyr-705-SH2 dimer formation, nuclear translocation, recognition of STAT3-specific DNA binding elements, and induction of targeted genetic transcription [35]. The IL6R/STAT3/miR-34a feed-back loop was discovered in breast, an activated IL6R/STAT3/miR-34a feedback loop was necessary for EMT, invasive, and metastatic activities [36]. In this study, by suppressing STAT3 phosphorylation and nuclear translocation, acetyl-cinobufagin modulated the STAT3-mediated transcriptional activation. Acetyl-cinobufagin failed to reduce the migration and invasion ability of cells with knocked down STAT3. In summary, our findings indicate that acetyl-cinobufagin is a potential candidate for tumor therapy, which acts through suppressing EMT in BC by targeting the STAT3 signaling pathway.

Holistically, this research reveals that acetyl-cinobufagin induces the suppression of TNBC *in vitro* and *in vivo* by inhibiting the stimulation of STAT3. The findings of this study, as well as the contributions from past studies, indicate the treatment value of acetyl-cinobufagin. However, more research is needed to achieve the optimization of acetyl-cinobufagin for its clinical application.

Abbreviations

Hormone receptor (HR)

Epithelial to mesenchymal transition (EMT)

Optical density (OD)

Tyrosine residue 705 (Tyr-705)

Phenylmethanesulfonyl fluoride (PMSF)

Bicinchoninic acid (BCA)

Polyacrylamide gel electrophoresis (PAGE)

Polyvinylidene difluoride (PVDF)

Chemiluminescence (ECL)

Declarations

Ethical approval and consent to participate

The Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University issued the ethical approval, and all individual participants included here offered informed consent.

Consent for publication

To publicize this study and relevant figures, the researchers distributed written informed consent to patients.

Availability of data and materials

Raw information supporting the concluding remarks here are available on the major electronic information storage system pertaining to The First Affiliated Hospital of Wenzhou Medical University, and access can be offered in line with the authors.

Competing interests

The researchers announce no interest clashes.

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Authors' contributions

Yufeng Qi: Investigation and Methodology; Tianru Zhu: Data curation; Zitian Liu: Formal analysis; Conghui Liu: Software; Congzhi Yan: Validation; Zhixuan Wu: Visualization; Yiyi Xu: Methodology; Ying Bai: Writing-original draft; Dezhi Cheng and Xiaohua Zhang: Resources; Haiyang Zhao: Project administration; Chengguang Zhao: Writing-review&editing; Xuanxuan Dai and Lehe Yang: Conceptualization.

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Figures

Figure 1

Acetyl-cinobufagin inhibits the proliferation of TNBC cells. (A) Chemical composition of acetyl-cinobufagin. (B, C) The suppression rate was determined by the MTT assay in MDA-MB-468, BT-549, Hs578T cells. (D) The cinobufagin and acetyl-cinobufagin groups were intraperitoneally injected with 30 mg/kg on the 1st day, and the death rate of the animals were recorded for 14 d. (E) Body weight curve of the animals. (F) Colony forming analysis was completed with the aforementioned three cell lines

with/without acetyl-cinobufagin treatment. (G) Changes in the signals received by proteins (Ki67) in BT-549 cells were analyzed by immunofluorescence staining. Statistical analysis is illustrated as follows: (D) Log-rank (Mantel-Cox) test; (F) t-test. * $p < 0.05$; ** $p < 0.01$.

Figure 2

Acetyl-cinobufagin inhibits the migration of TNBC cells. (A) Wound healing assay was performed using the BT-549 cells with/without acetyl-cinobufagin treatment. (B) Transwell migration assay was performed with the MDA-MB-468, BT-549 and Hs578T cells. (C, D) Changes in the expression of EMT marker proteins (E-cadherin and N-cadherin) in Bt-549 cells were verified by immunofluorescence staining. (E) Expression of proteins related to the EMT signaling pathways as identified by Western blotting. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to the control as per Student's t-test.

Figure 3

Acetyl-cinobufagin induced cell apoptosis in TNBC cells. (A) Hoechst 33342 staining and (B) Flow cytometric analysis were performed to study the cell apoptosis rate after treatment with 0, 0.5, 1.0, and 2.0 μM of acetyl-cinobufagin for 24 h in MDA-MB-468, BT-549 and Hs578T cells by Annexin V and PI. (C, D) The expression levels of BAX and BCL-2 in above-mentioned three TNBC cell lines were determined by immunoblotting after treatment with acetyl-cinobufagin at three concentrations for 24 h. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared with the control using the Student's t-test.

Figure 4

Acetyl-cinobufagin induced S/G2 cell cycle arrest. (A) Cell cycle distribution in MDA-MB-468 and BT-549 cells treated with acetyl-cinobufagin treatment evaluated by flow cytometry. (B, C) The immunoblotting analysis was performed to determine the levels of cyclin A1 and CDK2 after treatment with acetyl-cinobufagin. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with the control based on Student's t-test.

Figure 5

Acetyl-cinobufagin inhibits EMT through the STAT3 signaling pathway. (A, B) Molecular docking of acetyl-cinobufagin to the STAT3 binding sites. (C) Expression of proteins involved in the STAT3 signaling pathway as detected by Western blot analysis. (D) The subcellar location of STAT3 was examined by immunofluorescence staining and confocal laser scanning microscopy (CLSM) in BT-549 cells. (E) The levels of STAT3 protein in the cytoplasm and nuclei was determined by immunoblotting analysis after extraction from BT-549 cells. (F) BT-549 cells treated with IL6 for 30 min after treatment with acetyl-cinobufagin for 10-20 h; expression levels of proteins associated with STAT3 and EMT signaling pathways as detected by Western blot analysis. (G) STAT3 knockdown in BT-549 cells treated with control, IL6 or IL6 + acetyl-cinobufagin (1.0 μ M), expression levels of proteins related to STAT3 and EMT signaling pathways as detected by Western blot analysis. (H) Evaluation of metastatic abilities of BT-549 and Hs578T after knockdown of STAT3.

Figure 6

The role of acetyl-cinobufagin in growth suppression of TNBC in vivo in a human xenograft model established by orthotopically injecting BT-549 cells. (A) BALB/c animals were divided into Control, napabucasin 10 mg/kg, cinobufagin 2.0 mg/kg, acetyl-cinobufagin 1.0 mg/kg, and acetyl-cinobufagin 2.0 mg/kg groups. The cancer volumes were recorded every 2 days and the curve was eventually drawn. (B) Gross view of xenograft tumor tissue. (C) Ultimately, the animals were sacrificed, and the cancer

tissue was harvested and weighed. (D) The weight of the animals was recorded during the assay. (E) No histology abnormality was detected by H&E staining of the main organs in the 5 animal groups. (F) Immunohistochemical staining images of the expression of the cellular proliferative biomarker Ki67 in cancer samples. (G) Expression of STAT3- and EMT-related proteins detected by Western blot analysis. Two-way analysis of variance (ANOVA) was used to study the tumor volume and the Student's t-test was used for others with p-value significant codes: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

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