Narciclasine Targets STAT3 via Distinct Mechanisms in Tamoxifen-Resistant Breast Cancer Cells

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Abstract

Background: Signal transducer and activator of transcription 3 (STAT3) is constitutively activated in multiple malignant tumors. Compared with regular estrogen receptor (ER)-positive breast cancers, the patients with tamoxifen-resistant breast cancers often exhibit higher level of STAT3 phosphorylation. Narciclasine (Nar) possesses strong inhibiting effects against a variety of cancer cells, however, the underlying antitumor target(s)/mechanism(s) remains barely understood.

Methods: Targets prediction of narciclasine was performed by combining connectivity map (CMAP) and drug affinity responsive target stability (DARTS) strategy. Molecular and biochemical methods were used to elucidate the distinct mechanisms of narciclasine targeting STAT3. The narciclasine nano-delivery system was synthesized by thin film hydration method. Xenograft models were established to determine antitumor activity of narciclasine and its liposome in vivo.

Results: In this study, we successfully identified the STAT3 was the direct target of Nar through the combination strategies of CMAP and DARTS. In ER-positive breast cancer cells, Nar could suppress phosphorylation, activation, dimerization, and nuclear translocation of STAT3 by directly binding with the STAT3 SH2 domain. Additionally, Nar could also specifically promote total STAT3 degradation via proteasome pathway and reduce the STAT3 protein stability in tamoxifen-resistant breast cancer cells (MCF-7/TR). This distinct mechanism of Nar targeting STAT3 was mainly attributed to the various levels of reactive oxygen species (ROS) in regular and tamoxifen-resistant ER-positive breast cancer cells. Meanwhile, Nar loaded nanoparticles could markedly decrease the protein levels of STAT3 in tumor sites, resulting in significant MCF-7/TR xenograft tumor regression without obvious toxicity.

Conclusions: Our findings successfully highlight the STAT3 as the direct therapeutic target of Nar in ER-positive breast cancer cells, especially Nar leaded STAT3 degradation as a promising strategy for the tamoxifen-resistant breast cancer treatment.

Background

Narciclasine (Nar), a plant growth inhibitor, was first isolated from bulbs of several *Narcissus* species in 1967, which has been shown to exhibit anti-tumor, anti-inflammatory, anti-Alzheimer's disease, and obesity suppressing activities[1–5]. In the US National Cancer Institute NCI-60 human tumor cell lines screen, Nar displayed broad cytotoxicity against various cancer cell lines[2]. Although Nar possesses a marked anti-tumor effect, the underlying anti-tumor target(s)/mechanism(s) remains poorly understood. Only a few previous studies showed that Nar inhibited protein synthesis by targeting eEF1A in melanoma cells[6], activated Rho and induced stress fibers in glioblastoma cells[7], regulated the AMPK-ULK1 axis in triple-negative breast cancer cells [8]. In this study, a novel combination strategy was applied to reveal the new underlying anti-tumor target for Nar. The connectivity map (CMAP) database, a pattern-matching software that analyzes gene expression profiles, is one of the most effective tools for predicting pharmacological function and mechanism of action in drug discovery and development[9, 10]. The drug

affinity responsive target stability (DARTS) technique, based on drug binding-incurred changes in protease susceptibility of the target protein, can be used to identify the protein targets of bioactive small molecule-based agents[11]. Thus, we combined this CMAP analysis with DARTS/MS strategy to resolve the molecular targets of Nar.

In this study, we demonstrated that Nar could directly target signal transducer and activator of transcription 3 (STAT3) and significantly inhibit its phosphorylation, which has not yet been reported. The oncogenic transcription factor STAT3 plays pivotal role in cancer progressions, such as the cell proliferation, apoptosis, angiogenesis, metastasis, invasion, immune evasion and drug resistance[12–14]. Constitutive activation of STAT3 is observed in a broad range of human malignancies including colorectal, lung, breast, prostate, gastric, glioma, melanoma, ovary, liver, and pancreas cancers[12, 15]. Therefore, STAT3 is deemed as an attractive therapeutic target for antitumor drug development. In the past two decades, some natural and synthetic compounds have been demonstrated to possess the STAT3 phosphorylation inhibitory effects by directly binding STAT3 domains, such as the DNA and SH2 binding domain [15]. In recent years, a few natural products and proteolysis targeting chimera (PROTAC) molecules can also exert antitumor effects by targeting total STAT3 degradation[16–18]. In addition, STAT3 remains to be a promising clinical target in breast cancer prevention and therapy[15]. STAT3 phosphorylation was also abnormal high expression in tamoxifen-resistant breast cancer, which resulted in ER positive breast cancer resistance to tamoxifen[19–21]. In this study, Nar could target STAT3 and inhibit its phosphorylation without affecting the total protein of STAT3 in regular ER-positive breast cancers. However, distinct mechanism in tamoxifen-resistant MCF-7 (MCF-7/TR) cells, Nar not only inhibited STAT3 phosphorylation, but also significant degraded STAT3 total protein via proteasome pathway, and showed better inhibiting effects.

In addition, Nar possessed strong pan-cytotoxicity and poor water solubility[22], which hampering the clinical development. Thus, nanoscale drug delivery was applied in Nar against MCF-7/TR cells *in vivo*. In this study, we integrated CMAP with DARTS/MS revealed that Nar targeted STAT3 and inhibited its phosphorylation and activation in MCF-7 cell, and facilitated the total STAT3 degradation via a ROS-dependent proteasome pathway in MCF-7/TR cells. Meanwhile, nanoparticulate delivery of Nar significantly suppressed MCF-7/TR cells *in vivo*.

Materials And Methods

Materials

Nar was isolated from bulbs of *Narcissus* species in our laboratory. Protease inhibitor, phosphatase inhibitor and pronase were from Roche (Mannheim, Germany). CNBr-activated Sepharose 4B was obtained from Sigma (St. Louis, MO, USA). Recombinant human IL-6 was purchased from Peprotech (Rocky Hill, USA). Bortezomib (BTZ) and N-acetylcysteine (NAC) were from Medchem Express (Monmouth Junction, NJ, USA). Antibodies specific for cyclin D1 (ab40754), c-Myc (ab32072), STAT1 (ab109320), STAT2 (ab32367), STAT4 (ab68156), STAT5 (ab194898) and STAT6 (ab32520) were from

Abcam (Cambridge, UK), and antibodies for STAT3 (#9139 and #4904), p-STAT3 (#9145), P53 (#2527) and survivin (#2808) were from Cell Signaling Technology (Danvers, MA, USA).

Cell lines and culture

Hepatocellular carcinoma (Hep-3B and HepG2), colorectal carcinoma (HCT116 and SW480), and lung carcinoma (A549) obtained from Cell Bank of Shanghai institute of Cell biology, Chinese Academy of Sciences (SIBS, CAS). Prostate cancer (DU145), malignant melanoma (SK-Mel-28), breast cancer (MCF-7), and tamoxifen-resistant breast cancer (MCF-7/TR) were obtained from ATCC (Manassas, VA, USA). The HCT116 was maintained in McCoy's 5A medium (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (Biological Industries, Cromwell, CT, USA). The A549 was maintained in F-12K medium (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS. The SW480 were maintained in L-15 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS. The SK-Mel-28, Hep-3B, HepG2, DU145 and MCF-7 cells were maintained in MEM/EBSS (Hyclone, Logan, UT, USA) supplemented with 10% FBS. The MCF-7/TR cells were maintained in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS, 1 μ M 4-Hydroxytamoxifen, 10 μ g/mL insulin.

CMAp analysis

The gene expression profile of Nar treated MCF-7 cells was obtained from our previous data dataset GSE85871 (<https://www.ncbi.nlm.nih.gov/geo/>)[23]. The differential expression probes of Nar were selected according to fold-change ($FC \geq 3$). The 940 gene expression signatures of Nar were represented by two sets ('up-' and 'down-' probe sets, saved as .grp files and required as the inputs for CMAp), which was made up by the significant up/down regulation probes respectively. The query in the CMAp was performed as a "quick query" in the query section of <http://portals.broadinstitute.org/cmap/>.

DARTS/MS proteomics analysis

The DARTS assay was performed according to the protocol previously described[11]. Briefly, aliquots of MCF-7 cell lysates were treated with Nar (100 μ M) or solvent control (DMSO) for 1 h at room temperature. The samples were digested with pronase at the specified ratios under room temperature for 30 min. Digestion was stopped and samples were boiled for Western blot analysis. For proteomic analysis, the samples were separated by SDS-PAGE and stained with Coomassie Blue. Each lane was excised and divided into three sections, based on molecular weight (<70 kD, 70-100 kD and >100 kD). Each sample was subjected to an in-gel digestion procedure, followed by LC-MS/MS.

Microscale thermophoresis (MST)

The MST measurement for binding of Nar to STAT3 was performed as described previously[24]. Briefly, 5 μ L recombinant human STAT3 (Novoprotein, China) labelling the protein NHS-RED dye was mixed with 5 μ L Nar in different concentrations. Then, samples were incubated 10 min at room temperature and determined by a MonolithTM NT.115 MST device (NanoTemper, Germany).

Cellular thermal shift assay (CETSA)

The CETSA experiment was carried out as described previously[25]. Briefly, MCF-7 cells lysed using liquid nitrogen, and cell lysate samples were subjected to centrifugation and the supernatant collected. Aliquots were incubated with Nar (100 μ M) or solvent control (DMSO) for 30 min at room temperature. Samples were divided in a volume of 50 μ L/tube and heated at a range of temperatures for 3 min, cooled for 3 min at room temperature, and kept on ice. For concentration-response studies, cell lysate samples were incubated with Nar at specified concentrations, heated at 62 °C for 3 min, cooled for 3 min at room temperature, and kept on ice. All samples were subjected to centrifugation and the supernatant (soluble fractions) analyzed by Western blot.

Pull-down assay

Pull-down assay using immobilized Nar was performed as previously described[26]. Briefly, the CNBr-activated Sepharose 4B was washed and swelled in 1 mM HCl for 30 min, then washed with the coupling buffer (0.1 M NaHCO₃ and 0.5 M NaCl, pH 7.0). CNBr-activated Sepharose 4B beads were mixed with the Nar in coupling buffer at 4°C for 24 h. Nar-conjugated and control beads washed with three cycles of high and low pH buffer solutions (buffer 1, 0.1 M acetate and 0.5 M NaCl, pH 4.0; buffer 2, 0.1 M Tris-HCl and 0.5 M NaCl, pH 8.0). Coupled and uncoupled beads were separately incubated with the cell lysate on a rotator for 12 h at 4°C. The beads were then washed 3 times with the lysis buffer, and the bound proteins were analyzed by Western blot.

Molecular docking

The STAT3 X-ray structure (PDB code: 1BG1) was obtained from the RCSB Protein Data Bank (<http://www.rcsb.org/>) and prepared using the protein preparation wizard Maestro software package (Schrödinger Inc., version 9.0). The SH2 domain of STAT3 protein was defined as the binding pocket[27]. The 3D conformer of Nar was downloaded from PubChem (<https://pubchem.ncbi.nlm.nih.gov>). Nar was docked into grid box using extra precision (XP) mode of Glide program in Schrodinger suite. The OPLS_2005 force field and all default parameters were applied during docking calculation. The docking pose with the lowest Glide score was generated using PyMOL (<http://www.pymol.org/>).

Native PAGE

The native PAGE analysis was performed as previously described[28]. Briefly, native protein samples of MCF-7 cells lysate were mixed with non-denatured gel sample loading buffer (Beyotime, Haimen, China), and loaded onto SDS-free PAGE gels. Following electrophoresis, proteins were transferred to PVDF membranes and immunoblotted with specific antibody as described for Western blot analysis.

Luciferase assay

293T cells were co-transfected with STAT3-Luc and renilla luciferase using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) for 24 h. Following treatment with 50 ng/mL IL-6 and the indicated

concentrations of Nar and stattic. Luciferase activity was measured by the Dual-Glo Luciferase Assay Kit (Promega) using a Luminescence Microplate Reader (BioTek, Winooski, VT, USA).

Cell viability assay

Cell viability was tested by Cell Counting Kit-8 (CCK-8, Dojindo). Cells (5000/well) were seeded in 96-well plates. Overnight, cells were treated with different concentrations of Nar for 24 h. The CCK-8 assay was then carried out to assess the effect of Nar on cell viability, and IC₅₀ values were calculated.

Colony formation assay

Cells were seeded in 12-well plates at 1×10^3 /well, followed by treatment with Nar (5, 10 and 50 nM) for 5 days. Colonies were then subsequently fixed with 4% paraformaldehyde (PFA) and stained with 0.5% crystal violet. Finally, the numbers of cell colonies were imaged and counted.

Apoptosis and cell cycle analysis

MCF-7/TR cells were seeded in 6-well plates were treated with Nar for 24 h. Both floating and adherent cells were harvested for further analysis. The Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, San Jose, CA, USA) was used to detect apoptosis and the cell cycle and apoptosis analysis kit (Beyotime, Haimen, China) for cell cycle analysis, following the manufacturer's instructions.

Wound-healing and transwell assays

For the wound-healing assay, MCF-7/TR cells were cultured in 12-well plates and cells at 90% were scratched with a pipette tip. After scratch, cells were treated with 50 and 100 nM Nar, and the healing process of the wound was measured over a 24 h and 48 h period. For the transwell assay, MCF-7/TR cells were seeded in a cell invasion chamber of 24-well transwell plate. Cells were cultured in the absence or presence of Nar for 24 h, then fixed and stained with crystal violet.

Determination of reactive oxygen species (ROS) levels

The levels of intracellular reactive oxygen species were monitored by using Reactive oxygen species assay kit (Beyotime, Haimen, China). MCF-7 and MCF-7/TR cells were seeded in 96-well plates at 5×10^3 /well and cultured overnight, then followed by treatment with Nar (200 nM) for 1 h and 3 h. Cells were stained with 10 μ M DCFH-DA at 37 °C for 30 min, then detected by a Cytation 5 Cell Imaging Multi-Mode Reader (BioTek, Winooski, VT, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. MCF-7/TR cells were treated with the ROS scavenger, 10 mM NAC for 1 h, followed treatment with or without Nar (200 nM) for 24 hours, the cells were gently washed with PBS and followed by the incubation with 10 μ M DCFH-DA at 37 °C for 30 min. The images were captured with Operetta CLS.

RNA extraction and real-time PCR analysis

MCF-7/TR cells treated with Nar (100 nM) for 3 h, 6 h and 12 h, and total mRNA extracted by using the Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's Protocol. RT-PCR amplifications were performed using the FastStart Essential DNA Green Master and LightCycler® 96 Instrument (Roche, Basel, Switzerland). Total mRNA was reverse transcribed into cDNA using PrimeScript RT reagent Kit (Takara, Shiga, Japan) following the manufacturer's protocol. β -Actin was used as the reference gene. The forward and reverse primers used were listed in Supplementary Table 1.

Western blot analysis

For bortezomib (BTZ) experiment, cells were treated with 20 nM BTZ for 12 h before Nar was added for another 8 h treatment, and cells were collected for Western blot analysis. For cycloheximide (CHX) chase studies, cells were treated with Nar for 3 h, then 50 mg/mL of CHX was added, and cells were collected at indicated time points for Western blot analysis. For N-acetylcysteine (NAC) experiment, cells were treated with the ROS scavenger, 10 mM NAC for 1 h, followed treatment with or without Nar, and cells were collected for Western blot analysis. Total cellular proteins were extracted with NP-40 lysis buffer, nuclear and cytoplasmic proteins extracted with a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Haimen, China). Protein concentrations were determined using a BCA protein assay kit (Beyotime, Haimen, China). For Western blot analysis, the samples were separated by SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% nonfat dry milk, the membranes were incubated with primary antibodies at manufacturers' recommended dilutions at 4 °C overnight. A goat anti-rabbit or donkey anti-mouse secondary antibody (IRDye 800, LI-COR, Lincoln, NE, USA) was added at the recommended dilutions and the incubation continued for another 1 h at room temperature. The immunoreactive bands were scanned using an Odyssey Infrared Imaging System (LI-COR).

Immunofluorescence staining

Breast tumor MCF-7 cells were treated with or without Nar 100 nM for 24 h. Cells were washed and fixed in 4% paraformaldehyde for 20 min at room temperature, and then permeabilized in 0.1% Triton X-100 for 30 min at room temperature. After blocking with 5% nonfat dry milk and incubation with indicated antibodies overnight at 4 °C, cells were incubated with corresponding secondary antibodies for 1 h at room temperature, and then stained with DAPI for 30 min. Images were taken using fluorescence microscopy (Leica Microsystems, Inc., Germany).

Preparation and characterization of liposome

Nar encapsulated liposomal nanoparticles were prepared by thin film hydration method. In brief, 1.25 mg Nar was dissolved in 0.5 mL methanol. The lipids, 19.9 mg DPPC and 2.7 mg Cholesterol and 2.4 mg DSPE-MPEG2000 were dissolved in 4.5 mL chloroform. The mixture solutions were placed in round bottom flask and fully removed via rotary evaporator. The resulting thin film was then hydrated with 2.5 mL PBS for 30 min to form liposome. The liposomes were subjected to extruded using 200 nm polycarbonate membrane filter to control size homogeneity. Finally, Nar loaded liposomes was purified to remove unloaded Nar by centrifugation at 12000 rpm for 5 min. The amount of Nar loaded was

quantified using HPLC. Nar loading efficiency was calculated by the following equation: loading efficiency% = (encapsulated Nar/ initial input of Nar)×100%. Drug release was study carried out in PBS according to previously reported method[29]. The particle size and zeta potential of prepared liposomes were measured by Zetasizer Nano ZS instrument (Malvern, U.K.). The microstructure of Nar encapsulated liposome was characterized by Cryo-TEM (Talos F200C G2).

Xenograft models

All animal studies were approved by Committee on the Ethic of Animal experiment of the Shanghai University of Traditional Chinese Medicine (SHUTCM), which complied with the national and international guidelines. Female BALB/c-nu nude mice (4-week-old) were obtained from Shanghai Slake Experimental Animal Co, Ltd. and housed under specific pathogen-free conditions. For MCF-7 xenograft experiment, the breast pad xenografts were established by subcutaneous injections of 8×10^6 MCF-7 cells. Following a two-week inoculation period, the tumor-bearing mice were randomly divided into Nar treatment and control groups. The treatment group was injected with 1 mg/kg Nar every two days for four weeks and those in the control group were injected with the same volume of saline. Body weights and tumor size were recorded two times per week. For MCF-7/TR xenograft experiment, 2×10^6 MCF-7/TR cells were subcutaneously injected into the breast pad of mice. The mice were then randomized divided into three treatment groups: control, Nar (2 mg/kg) and Nar-LPs (5 mg/kg). Nar was administered daily via intraperitoneal injection, and Nar-LPs was administered daily via tail vein injection. Tumor volume was calculated using the equation: volume (mm^3) = length $\times$ (width²)/2. After 8 days, Mice were sacrificed, tumors and other major organs such as liver, spleen, kidney, and heart were isolated.

Immunohistochemistry (IHC) analysis

The IHC analysis was carried out as described previously[30]. The paraffin embedded tissue sections were deparaffinized in xylene, treated with a graded series of alcohol and distilled water. Antigen recovery was performed using microwave boiling treatment in 10 mM sodium citrate buffer (pH 6.0), and the slices were incubated with 3% H_2O_2 to block endogenous peroxide activity. After blocking, the slides were incubated with primary antibodies against p-STAT3 and STAT3 at 4°C overnight. The slides were incubated with secondary antibody at 37°C for 30 min, followed by incubation with DAB and hematoxylin, and images were captured under a microscope.

Statistical analysis

Statistical analysis was performed with unpaired t-test when comparing two different groups or one-way ANOVA with Tukey's multiple comparison tests. All calculated values are shown as mean $\pm$ standard deviation (SD). All statistical analyses were performed using SPSS software (version 16.0, SPSS, Chicago, IL, USA).

Results

Potential target of Nar predicted by CMAP-DARTS/MS strategy

Based on strong cytotoxic effects of Nar on cancer cells, the antitumor targets were needed to discover in this study. To investigate the pharmacological functions of Nar, a similarity search against the CMAP was performed. The 940 query genes (>3-fold change; 616 up-regulated and 324 down-regulated; Supplementary Data 1) were submitted to the CMAP database for analysis (Fig. 1A). The top 20 correlated drugs with lower *p*-values and a positive enrichment score are summarized in Fig. 1B (detailed results in Supplementary Data 2). The most similar of these drugs that positively correlated with Nar were protein synthesis inhibitors (anisomycin, emetine, cycloheximide and puromycin). There are also three molecular-targeted drugs niclosamide (STAT3 inhibitor), 15-delta prostaglandin J2 (PPAR γ agonist), and trichostatin A (HDAC inhibitor). Based on the known pharmacological functions of these positively correlated agents, it appeared likely that Nar exerts its antitumor activity by inhibiting protein synthesis, STAT3, HDAC, and/or activating PPAR γ . The CMAP results strongly suggested that one pharmacological function of Nar is inhibiting protein synthesis, which corroborated previous studies [22, 31]. Thus, these previous studies served to further validate both the efficacy and accuracy of CMAP analysis for mechanism of action determination.

To further explore the CMAP predictions, DARTS/MS proteomic characterization was applied. Following DARTS assay, the samples were subjected to proteomics analysis (Fig. 1C). In order to narrow the scope and improve the accuracy, the top 10 potential target proteins (unique peptides ≥ 3 and the intensity ratio ≥ 2) of each molecular weight region were selected (detailed results in Supplementary Data 3). Then, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was performed to analysis these potential target proteins[32]. In these pathways, cell proliferation was the most relevant to an anti-tumor mechanism (Fig. 1D). One of the potential target proteins in cell proliferation pathway was STAT3, which is consistent with CMAP predicting Nar as a STAT3 inhibitor. Thus, combining the direct binding proteins predicted by DARTS/MS proteomics and the CMAP pharmacological function predictions, the antitumor target of Nar was postulated to involve its directly binding STAT3 and inhibiting its activation.

Nar directly binds to STAT3 protein and suppresses its phosphorylation and activation in vitro and in vivo

To determine whether Nar is a potential STAT3 inhibitor, its ability to suppress STAT3 expression was first examined in tumor cells. Among the eight human cancer cell lines examined, SW480, DU145 and MCF-7 expressed STAT3 protein at higher levels (Fig. S1). Each of these cell lines was treated with Nar at a range of concentrations for 24 h. As shown in Fig. 2A-C, Nar significantly inhibited STAT3 phosphorylation in a concentration-dependent manner without affecting the total level of STAT3 in all three lines. Moreover, Nar was the most effective inhibiting STAT3 phosphorylation in MCF-7 cells. The results from a MCF-7 cell-based time course study demonstrated that Nar significantly inhibited the phosphorylation of STAT3 in a time-dependent manner (Fig. 2D). Further, Nar treatment also reduced IL-6-induced STAT3 phosphorylation and activation in MCF-7 cells (Fig. 2E). Thus, Nar could inhibit STAT3 activity by preventing its phosphorylation and activation in MCF-7 cells. In addition, murine MCF-7 xenografts were established to investigate the effect of Nar on STAT3 signaling *in vivo*. Nar treatment effectively reduced

the size and weight of the MCF-7 xenograft tumors in comparison to the vehicle control (Fig. 2F and G). No significant change in body weight or pronounced side effect was observed between the vehicle control and treatment groups (Fig. S2). The levels of phosphorylated and total STAT3 proteins were examined in the excised tumor tissues by IHC staining. As shown in Fig. 2H and I, Nar treatment significantly decreased phosphorylated STAT3 levels. Moreover, the levels of phosphorylated and total STAT3 proteins were examined in the excised tumor tissues by Western blot. As shown in Fig. 2J and K, Nar treatment decreased phosphorylated STAT3 levels. These observations are consistent with Nar as a STAT3 inhibitor that blocks STAT3 phosphorylation and activation *in vitro* and *in vivo*.

To determine whether Nar exerted effect by directly binding STAT3 protein, several binding experiments were carried out. First, microscale thermophoresis (MST) assay was performed to determine the direct binding between Nar and recombinant human STAT3. Expectedly, Nar could readily bind to STAT3 with a K_d estimated at 15 μ M (Fig. 2L). The DARTS assay, which relying on the reduction in the protease susceptibility of the target protein upon drug binding, was conducted[11]. After incubating cell lysate samples with Nar, the STAT3 protein was less susceptible to exogenous pronase at the protein to pronase ratio of 1:300 and 1:200, respectively (Fig. S3A). With increasing Nar concentration, the protease susceptibility of STAT3 markedly decreased (Fig. S3B). Meanwhile, the cellular thermal shift assay (CETSA), which basing on the biophysical principle of ligand-induced thermal stabilization of target proteins, was used to investigate the binding between Nar and STAT3[25]. As shown in Fig. 2M, Nar markedly increased STAT3 accumulation at temperatures ranging from 60 to 66 °C (relative to the DMSO solvent control). The stability of STAT3 protein during heating depended on the concentration of Nar. As shown in Fig. 2N, STAT3 protein accumulation markedly increased as Nar concentration increased. To further evaluate the direct binding of Nar and STAT3, the pull-down assay was performed using Nar-conjugated Sepharose 4B beads with cell lysates of MCF-7 cells. As shown in Fig. 2O, Nar-conjugated beads apparently pulled down the STAT3 from cell lysate compared to control beads. All these data together demonstrated that Nar directly bound to STAT3 and thus inhibited its activity.

Nar binds to SH2 domain, decreases STAT3 dimerization, and inhibits STAT3 nuclear translocation

To further characterize the Nar-STAT3 interaction, the binding mode of Nar and STAT3 (PDB ID: 1BG1) was modeled by docking simulation using Maestro software (Schrödinger, version 9.0). As shown in Fig. 3A, Nar docked into the SH2 domain of STAT3 and potentially interacted with the SH2 domain via four pronounced hydrogen bonds (red lines). In general, STAT3 undergoes SH2 domain-mediated dimerization upon phosphorylation at the tyrosine 705 residue and subsequently translocate to the nucleus[33]. To determine if Nar binding to SH2 domain would consequently reduce STAT3 dimerization, a native PAGE analysis of dimeric STAT3 following Nar treatment was performed. As shown in Fig. 3B, Nar inhibited STAT3 dimerization by decreasing the level of dimeric STAT3 while increasing the level of monomeric STAT3. The luciferase reporter gene assay was performed to assess the effect of Nar on STAT3 transcriptional activity. As shown in Fig. 3C, Nar significantly inhibited IL6-induced STAT3-driven luciferase activities dose-dependently. The subcellular localization of STAT3 was examined using immunofluorescence staining. Nuclear STAT3 protein levels were drastically reduced following Nar

treatment (Fig. 3D). Western blot analysis of nuclear and cytoplasmic extract samples further revealed that Nar reduced nuclear STAT3 level and phosphorylated STAT3 (p-STAT3) protein in both the nuclei and cytoplasm (Fig. 3E). Following nuclear translocation, STAT3 controls the transcription of downstream target genes that are critical to tumor growth, angiogenesis, and invasion[34]. In MCF-7 cells, Nar treatment suppressed the expression of representative STAT3 target genes (cyclin D1, c-Myc, and survivin) while increased p53 protein levels (Fig. 3F).

Nar specifically targets degradation of STAT3 via a ROS-dependent proteasome pathway in MCF-7/TR cells

In clinical practice, selective estrogen receptor modulator tamoxifen has been used widely to treat ER-positive breast cancer. However, elevated levels of STAT3 phosphorylation have been observed in tamoxifen-resistant breast cancer[19, 20]. Here, we observed that basal expression levels of ER α and ER β were significantly suppressed in MCF-7/TR cells, and also found abnormal high expression of phosphorylated STAT3 in MCF-7/TR cells compared with MCF-7 cells (Fig. 4A). The above data suggested that Nar directly bound to STAT3 and inhibited its phosphorylation without affecting the total level of STAT3 in MCF-7 cells. Strikingly and unexpectedly, MCF-7/TR cells treated with Nar, not only the phosphorylation level of STAT3 was reduced, but also the total protein level of STAT3 was significantly reduced (Fig. 4B). Nar also significantly inhibited the total protein level and phosphorylation level of STAT3 in a time-dependent manner (Fig. 4C). To determine the selectivity of Nar to STAT3, other members of STAT family were also detected. Except for STAT2 and STAT4 that were not detected, Nar treatment had a slight inhibitory effect on the protein levels of STAT1, STAT5A/B and STAT6 (Supplementary Fig. S4). Therefore, Nar possessed a certain selective and specific suppression effect on STAT3 protein. Meanwhile, the expression of STAT3 target genes cyclin D1 and c-Myc, which are related to cell cycle and proliferation, were significantly inhibited by Nar treatment (Fig. 4D), and the mRNA levels of cyclin D1 also significantly decreased by Nar (Fig. 4E).

To investigate how Nar downregulated the protein level of total STAT3 in MCF-7/TR cells, the levels of STAT3 mRNA transcript were examined. The results showed that no significant changes in STAT3 mRNA levels between control and Nar treatment (Fig. 4E), which suggesting that downregulation of STAT3 occurred post-transcriptionally. Thus, we hypothesized that Nar-mediated STAT3 degradation might probably be attributed to the ubiquitin proteasome pathway. As shown in Fig. 4F, the reduction in STAT3 protein caused by Nar treatment can be efficiently rescued by proteasome inhibitor BTZ, which indicated that Nar targeted STAT3 to proteasome degradation. Then, MCF-7/TR cells treated CHX blocking protein synthesis to determine the effect of Nar on the protein stability of STAT3. The results showed that Nar treatment reduced STAT3 protein half-life from 123 to 38.8 min in MCF-7/TR cells (Fig. 4G and H).

To investigate why Nar degraded STAT3 in MCF-7/TR cells but not in MCF-7 cells, cellular ROS levels were determined. Previous studies showed that the level of ROS in MCF-7/TR cells was higher than that in MCF-7 cells, which prompted us to test whether ROS contribute to STAT3 degradation[35, 36]. As shown in Fig. 4I, we found that the level of ROS in MCF-7/TR cells was significantly higher than that in

MCF-7 cells, and Nar treatment also could significantly increase the ROS level in MCF-7/TR cells. Meanwhile, Nar treatment increased the ROS level (green) in MCF-7/TR cells, and this ROS signal was significantly diminished by pretreating with the NAC (Fig. 4J). Next, NAC experiment was performed to determine whether degradation of STAT3 was related to ROS levels. Consistent with expectation, treatment with the ROS scavenger NAC efficiently rescued STAT3 from proteasome degradation (Fig. 4K). Taken together, these results demonstrated that Nar targeted STAT3 to proteasome degradation through a ROS-dependent mechanism in MCF-7/TR cells.

Nar inhibits cell proliferation, promotes cell cycle arrest in G2/M, and suppresses the migration in MCF-7/TR cells

Nar, as a direct STAT3 inhibitor, possessed a role to degrade STAT3 protein via ROS-dependent proteasome pathway in MCF-7/TR cells. Subsequently, the cell counting kit-8 (CCK-8) assays showed that Nar exerted higher cytotoxicity in MCF-7/TR compared to MCF-7 cells (Fig. 5A). To further assess the growth inhibition of Nar on MCF-7/TR cells, a colony formation assay was performed. The results showed that Nar treatment significantly suppressed the colony-formation efficiency of MCF-7/TR cells (Fig. 5B and C). To confirm whether Nar inhibited cell proliferation by inducing cell cycle arrest, flow cytometry was performed. Nar treatment significantly induced G2/M phase arrest in MCF-7/TR cells in a concentration-dependent manner (Fig. 5D and E). Moreover, the annexin V-FITC/PI double-staining assay showed that only higher concentration of Nar could cause an increase in the proportion of apoptotic cells (Fig. 5F and G). Hence, Nar killed MCF-7/TR cells mainly by inhibiting cell proliferation and inducing cell cycle G2/M phase arrest.

Cell migration and invasion play an important role in tumor development. To investigate whether Nar affects cell migration, wound healing assays were performed. The results showed that Nar significantly inhibited cellular migration of MCF-7/TR cells (Fig. 5H). Then, transwell assay was performed to further confirm the inhibition effect of Nar on cell invasion. The result showed that the invasive ability of MCF-7/TR cells was significantly reduced by Nar compared with that in controls (Fig. 5I and J).

Nar suppresses the growth of MCF-7/TR xenograft tumors

The previously reported toxicity and the poor water solubility of Nar were important obstacle hampering the efficacy *in vivo*. Thus, the nano-drug delivery system was used to reduce the toxicity and increase the therapeutic efficacy. We successfully encapsulated Nar into liposomes using thin-film hydration method. Nar liposomes (Nar-LPs) were spherical in shape with smooth surface in the observation using cryoelectron microscope (Cryo-EM) (Fig. 6A). Nar-LPs exhibited the hydrodynamic size of 176.1 nm (Fig. S5A) and zeta potential of -2.8 mV (Fig. S5B). Drug loading and encapsulation efficiency were 90% and 10%, respectively. Nar could be released from liposomes in a sustained manner (Fig. S5C). The cytotoxicity of Nar-LPs against MCF-7 and MCF-7/TR cells were further evaluated using free Nar as control. As expected, the cytotoxicity of Nar-LPs was comparable to that of free Nar in MCF-7 and MCF-7/TR cells (Fig. 6B and C).

Furthermore, MCF-7/TR xenograft model was performed to evaluate the anti-tumor effect of Nar-LPs *in vivo* (Fig. 6D). As shown in Fig. 6E-G, liposomal nanoparticles enhanced the water solubility and reduced the toxicity of Nar, Nar-LPs (5mg/kg) significantly inhibited tumor growth *in vivo*. In addition, no significant weight loss (Fig. 6H) and no histological differences of the lung, heart, liver, kidney, or spleen were found in Nar-LPs treatment groups (Fig. 6K). These results indicated that Nar-LPs was no side effect at 5 mg/kg *in vivo*. Consistent with the *in vitro* data, Nar-LPs significantly decreased the protein levels of STAT3, p-STAT3 and cyclin D1 in xenograft tumors (Fig. 6I and J).

Discussion

Mounting evidence supports the application of Nar as an anti-tumor agent. Several studies have reported some anti-tumor mechanisms of Nar, but natural products often hit multiple molecular targets. Therefore, we used two omics strategies to identify STAT3 as another important Nar antitumor target. This STAT3-mediated mechanism of action for Nar was first confirmed in this study. The data *in vitro* and *in vivo* suggested that Nar could suppress STAT3 phosphorylation and activation, directly bound to the SH2 domain of STAT3 protein, inhibited STAT3 dimerization and nuclear translocation in MCF-7 cells.

At present, endocrine therapy is highly effective in treatment with ER-positive breast cancer by suppressing estrogen production and/or targeting the ER directly[37]. Thus, selective estrogen receptor modulators, such as tamoxifen, have been used widely to treat ER-dependent tumors. However, *de novo* resistance or acquired resistance limited clinical efficacy of tamoxifen. In recent studies, the expression and activation of STAT3 phosphorylation could contribute to tamoxifen resistance in breast cancer[21, 38]. In addition, overexpression of STAT3 target gene cyclin D1 in breast tumors correlated with refractoriness to tamoxifen clinical treatment[39, 40], and blocking cyclin D1 expression or function could inhibit development and growth of tamoxifen-resistant breast tumors[41, 42]. Thus, Nar targeting STAT3 may be better for the treatment of tamoxifen-resistant breast cancer. To our surprise, Nar not only inhibited STAT3 phosphorylation, but also specifically degraded the level of total STAT3 protein and reduced STAT3 protein stability in MCF-7/TR cells. The level of ROS was the primary factor for distinct mechanism of Nar targeting STAT3 in MCF-7 cells and MCF-7/TR cells. In previous works, the level of ROS was elevated in tamoxifen treatment of breast cancer, which also could build the resistance to treatment[35, 36]. Moreover, an increasing of ROS can modify the cell-signaling proteins, including degradation of ubiquitination/proteasome system[43]. Indeed, we found that the level of ROS in MCF-7/TR cells was higher than that in MCF-7 cells, and Nar also could significantly increase the level of ROS in MCF-7/TR cells. In addition, inhibition of ROS accumulation by the ROS scavenger NAC also restored the level of STAT3 protein in MCF-7/TR cells. Our results strongly suggested that Nar targets STAT3 to proteasome degradation through accumulation of ROS, but the specific mechanism is still needed to be further explored.

The cytotoxic effect of Nar in MCF-7/TR cells was better than that in MCF-7 cells, suggesting that Nar could be more effective against tamoxifen-resistant breast cancer by targeting degradation of STAT3 *in vitro*. However, the considerable toxicity and poor water solubility of Nar were important obstacles

hampering the *in vivo* efficacy and clinical development[22, 44, 45]. In previous works, the medicinal chemistry approach generated a large number of Nar derivatives, but the antitumor effect of most of these modifications did not improve[44, 46]. Therefore, the nanoscale drug targeted delivery system was used to enhance the water solubility of Nar. In the case of non-toxic side effects, Nar dose of 5 mg/kg had more significant inhibitory effect on MCF-7/TR subcutaneous tumor growth.

Conclusions

In this study, we used two omics targets identification modalities (CMAP and DARTS/MS) revealed STAT3 as an anti-tumor target of Nar (Fig. 7A). Meanwhile, Nar targeting STAT3 had distinct mechanisms in MCF-7 cells and MCF-7/TR cells. Nar directly bound to STAT3 protein and suppress STAT3 phosphorylation and activation in MCF-7 cells, but in MCF-7/TR cells could specifically promote total STAT3 degradation via a ROS-dependent proteasome pathway (Fig. 7B). In addition, Nar encapsulated liposomal nanoparticles exerted a strong antitumor effect *in vivo*, which established Nar as a novel therapeutic against MCF7/TR (Fig. 7C). Therefore, this research provided an efficient strategy for the discovery of natural product targets, and also highlighted targeting STAT3 degradation as a promising therapeutic strategy for the treatment of tamoxifen-resistant breast cancer.

Abbreviations

Nar: Narciclasine; STAT3: Signal transducer and activator of transcription 3; CMAP: connectivity map; DARTS: drug affinity responsive target stability; CETSA: Cellular thermal shift assay; MST: Microscale thermophoresis; MCF-7/TR: Tamoxifen-resistant MCF-7; ROS: Reactive oxygen species.

Declarations

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Not applicable

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Availability of data and materials

All data generated or analysed during the present study are included in this published article.

Authors' contributions

Weidong Zhang, Xin Luan and Sanhong Liu designed the research. Chao Lv and Yun Huang carried out the experiments and performed data analysis. Qun Wang, Hongwei Zhang and Jinmei Jin participated part of the experiments. Dong Lu, Yudong Zhou and Yunheng Shen participated part of data analysis. All authors have approved the final article.

Ethics approval and consent to participate

The experimental protocol was established, according to the Guide for the Committee on the Ethic of Animal experiment of the Shanghai University of Traditional Chinese Medicine (SHUTCM).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

Figure 1

The prediction of antitumor targets of Nar by CMAP-DARTS/MS strategy. (A) The query genes of Nar were submitted to the CMAP database for analysis. (B) The top 20 drugs/agents that were positively correlated with Nar by the CMAP analysis. (C) Following DARTS assay, the samples were subjected to LC-MS/MS for proteomic analysis, and the possible Nar binding proteins were obtained. (D) The KEGG pathway was performed to analysis these potential binding proteins of Nar.

Figure 2

Nar directly binds to STAT3 protein and suppresses its phosphorylation and activation in vitro and in vivo. (A-C) MCF-7, SW480 and DU145 cells were treated with different concentrations of Nar for 24 h followed by Western blot analysis of total and phosphorylated STAT3. (D) MCF-7 cells were treated with Nar at 100 nM for various time points. (E) Nar inhibition of IL-6-induced STAT3 phosphorylation and activation in MCF-7 cells. (F) Images of representative MCF-7 xenograft tumors from the vehicle and Nar-treated groups. (G) The weight of dissected xenograft tumor masses. (H, I) The IHC staining analyzed STAT3 and p-STAT3 in MCF-7 xenograft tumors. (J, K) Total proteins were extracted from excised tumors, and the expression levels of STAT3 and p-STAT3 were determined by western blotting. (L) MST analysis of Nar binding to recombinant human STAT3 ($K_d=15\text{ }\mu\text{M}$). (M) The CETSA assay determined the thermal stabilization of STAT3 interaction with Nar at a series of temperatures from 50 to 68 °C. (N) The thermal stabilization of STAT3 by increasing concentrations of Nar was assayed at 62 °C. (O) Cell lysates were incubated with Nar-conjugated Sepharose 4B beads or Sepharose 4B beads alone, and then the pulled-down proteins were analyzed by western blotting. Data are shown as mean $\pm$ S.D. ** $p < 0.01$, *** $p < 0.001$.

Figure 3

Nar binds to SH2 domain, decreases STAT3 dimerization and inhibits STAT3 translocation into the nucleus. (A) The predicted binding complex structure of Nar in the SH2 domain of STAT3, where Nar interacts with the SH2 domain via four pronounced hydrogen bonds (red lines). (B) MCF-7 cells were treated with different concentrations of Nar for 24 h, separated using native PAGE, and STAT3 analyzed by Western blotting. (C) 293T cells were transfected with STAT3-luc, and then treated with Nar and statistic

in the presence of 50 ng/ml IL-6 for 24 h. (D) Immunofluorescence staining for STAT3, p-STAT3 and the nucleus (DAPI, blue) after Nar treatment (100 nM, 24 h) in MCF-7 cells. (E) After treatment with Nar, the nuclear and cytoplasmic proteins were extracted to determine the levels of STAT3 and p-STAT3. (F) After treatment with Nar, STAT3 downstream target proteins (i.e., c-Myc, cyclin D1, survivin and P53) were analyzed by Western blotting. Data are shown as mean $\pm$ S.D. ** $p < 0.01$, *** $p < 0.01$, ### $p < 0.001$.

Figure 4

Nar specifically targets degradation of STAT3 via a ROS-dependent proteasome pathway in MCF-7/TR cells. (A) Protein expression of p-STAT3, STAT3, ER α and ER β in MCF-7 and MCF-7/TR cells were determined by Western blotting. (B) MCF-7/TR cells were treated with different concentrations of Nar for 24 h followed by western blot analysis of total and phosphorylated STAT3. (C) MCF-7/TR cells were treated with Nar at 100 nM for various time points. (D) The expression of STAT3 target genes cyclin D1 and c-Myc were determined by Western blotting. (E) MCF-7/TR cells were treated with Nar at 100 nM for various time points followed by real-time RT-PCR analysis of mRNAs encoding STAT3 and cyclin D1. (F) STAT3 protein levels in MCF-7/TR cells treated with Nar in the absence or presence of proteasome inhibitor bortezomib (BTZ) determined by Western blotting. (G, H) MCF-7/TR cells treatment with Nar and cycloheximide (CHX) alone or in combination for the indicated time points, STAT3 protein were determined by Western blotting and quantified by degradation kinetic curves. (I) MCF-7/TR and MCF-7 cells with Nar treatment for 1 and 3 h, and cellular ROS levels were detected. (J) MCF-7/TR cells were treated with the ROS scavenger N-acetylcysteine (NAC) for 1 h, followed treatment with or without Nar, and the images of cellular ROS levels were captured. (K) MCF-7/TR cells were treated with NAC for 1 h, followed treatment with or without Nar, and STAT3 protein were determined by western blotting. Data are shown as mean $\pm$ S.D. **p < 0.01, ##p<0.01.

Figure 5

Nar inhibits cell proliferation, promotes cell cycle arrest in G2/M, and suppresses the migration in MCF-7/TR cells. (A) MCF-7 and MCF-7/TR cells viability were measured using the CCK-8 assay. (B, C) Colony formation assay was used to validate the effect of Nar on MCF-7/TR cells growth. (D-G) The cell cycle distribution and percentage of apoptotic cells were analyzed by flow cytometry. (H) Wound-healing assay was performed to determine the effect of Nar on migration. (I, J) Transwell migration assay was used to

validate the effect of Nar on cell invasion, and quantitative analysis of cells migration. Data are shown as mean $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6

Nar suppresses the growth of MCF-7/TR xenograft tumors. (A) TEM image of Nar-LP nanoparticles. (B, C) The cytotoxicity of Nar-LP and Nar in MCF-7 cells and MCF-7/TR cells were determined using the CCK-8 assay. (D) Schematic plan for the administration of Nar-LP and Nar. (E) Representative image of MCF-

7/TR xenograft tumors after treatment. Nar (2 mg/kg) was administered daily via intraperitoneal injection, and Nar-LP (5 mg/kg) was administered daily via tail vein injection. (F) Measurement of tumor volume at indicated time points after different treatments. (G) Weights of xenografted tumors were summarized. (H) The body weights of nude mice in each group during the treatment. (I, J) Total proteins were extracted from excised tumors, and the expression levels of STAT3, p-STAT3 and cyclin D1 were determined by western blotting. (K, L) The IHC staining analyzed Ki67, STAT3 and p-STAT3 in MCF-7/TR xenograft tumors. Data are shown as mean $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 7

Hypothetic model illustrating the antitumor research of Nar. (A) Predicting potential target of Nar. (B) Narciclasine targets STAT3 via distinct mechanisms in breast cancer MCF-7 and tamoxifen-resistant MCF-7 cells. (C) Nar-LPs suppress the growth of MCF-7/TR xenograft tumors.

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