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Original Article

Identification of new microtubule small-molecule inhibitors and microtubule-associated genes against triple negative breast cancer

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Abstract: Breast cancer represents the leading cancer type and leading cause of cancer-related death among women in the world. Triple-negative breast cancer (TNBC) is a subset of breast cancer with the poorest prognosis and still lacking of effective therapeutic options. We recently screened a natural product library and identified 3 new hit compounds with selective and prominent anti-TNBC activities on different subtype of TNBC cell lines. Interestingly, all of these 3 hit compounds belong to "cytoskeletal drugs" that target tubulin and microtubule function. Our data also showed that these hit compounds showed consistently effective on TNBC cells which are resistant to those currently used antimicrotubule agents such as Paclitaxel. RNA-Sequencing analyses revealed the anti-TNBC mechanisms of these hit compounds and identified a subset of new cellular factors commonly affected by hit compounds in different subtypes of TNBC cells. Among them, we demonstrated AHCYL1 and SPG21 as new microtubule-associated proteins, which were required for TNBC cell survival with clinical implication through tissue array analysis. Our studies provide new insights into the mechanisms of TNBC pathogenesis and offer promising therapeutic directions for this aggressive breast cancer.

Keywords: Drug screening, natural product, breast cancer, TNBC, drug resistance

Introduction

Breast cancer, according to the American Cancer Society statistics 2024, is considered as the most frequent cancer diagnosed and remains the second leading cause of cancer-related death among women in the United States [1]. Triple-negative breast cancer (TNBC) encompasses a subset of breast cancers that lack expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). TNBC accounts for 15% to 20% of newly diagnosed breast cancer cases [2, 3], and is known for its aggressive biological behavior and poor patient outcomes compared with hormone receptor-positive breast cancer [3-5]. It has also been of special interest to breast cancer researchers due to its poor response towards standard chemotherapy, and its lack of other effective treatment options. Following the advances in sequencing technologies, TNBC has been increasingly recognized as a heterogeneous disease that exhibits substantial differences in terms of genomic and transcriptomic profiles [6, 7]. For example, cluster analysis has identified at least 6 TNBC subtypes displaying unique gene expression and ontologies, including 2 basal-like (BL1 and BL2), an immunomodulatory (IM), a mesenchymal (M), a mesenchymal stem-like (MSL), and a luminal androgen receptor (LAR) subtype [6]. The extreme heterogeneity of TNBC has led to difficulties in finding suitable molecular targets in preclinical studies and has been reflected in the limited benefit from targeted therapies observed in clinical trials for unselected TNBC patients.

Increasing evidence has supported that many natural products from plants or other resources

display anticancer activities, or enhance the efficacy of chemotherapy as well as other treatments [8-10]. One recent study reported that an extract of the plant *Amyris texana*, indigenous to Texas, was found to have selective activity against MDA-MB-453 cells, a model of the LAR subtype of TNBC [11]. Another recent study found that some natural extracts from Pacific Brittle Stars displayed anti-TNBC activities through suppression of Wnt signaling [12]. However, there is still lacking of experimental data about high-throughput screening of natural product libraries to identify hit compounds against TNBC. We recently have identified new natural products with anticancer activities to Diffuse Intrinsic Pontine Glioma (DIPG), a rare but highly aggressive pediatric brainstem tumor, via the WST-1 cell proliferation assay (Roche) based high-throughput screening methods [13]. In the current study, we screened a natural product library and identified 3 new compounds with selective and prominent anti-TNBC activities *in vitro* and *in vivo* on different subtypes of TNBC cells. Interestingly, we found that all of 3 hit compounds may act on the cytoskeleton by interfering with microtubule functions in TNBC cells. Notably, these natural compounds were effective on Paclitaxel-resistant TNBC cells. RNA-Sequencing analyses revealed the anti-TNBC mechanisms of these natural compounds and further identified a subset of new cellular factors commonly affected by these compounds in different subtypes of TNBC cell lines. Among them, we demonstrated AHCYL1 and SPG21 as new microtubule-associated proteins that are targets for these natural compounds, which were required for TNBC cell survival with clinical implication in TNBC patients through tissue array analysis.

Materials and methods

Cell culture and reagents

All of TNBC cell lines, MDA-MB-468, MDA-MB-231, HCC1806, DU4475, MDA-MB-453, BT-549, and Human primary mammary epithelial cells (HMEC) were purchased from the American Type Culture Collection (ATCC) and cultured as recommended by the manufacturer. All experiments were carried out using cells harvested at low (<20) passages. A compound library consisting of 756 natural products was purchased from Selleck Chemicals, USA. The TNBC formalin-fixed, paraffin embedded (FFPE) tissue arrays, which contained 120 cases as well as 6 normal breast tissues (Cat. #BR1301) were purchased from US Biomax, USA.

High-throughput screening

TNBC cell line MDA-MB-231 (1×10^4 cells/well) was seeded into 96-well plates for 24 h, then the natural product compounds were added into the wells at a final concentration of 10 μM for an additional 72-h treatment. The cytotoxicity against TNBC was measured using the WST-1 cell proliferation assays (Roche). Briefly, after the period of treatment of cells, 10 μL/well of cell proliferation reagent, WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate), was added and incubated for 3 h at 37°C in 5% CO_2. The absorbance of samples was measured by using a microplate reader at 490 nm. Data was normalized as the inhibition relative to the DMSO control.

Cell apoptosis and cell cycle assays

Flow cytometry was used for the quantitative assessment of apoptosis with the FITC-Annexin V/propidium iodide (PI) Apoptosis Detection Kit I (BD Pharmingen) on a FACS Calibur 4-color flow cytometer (BD Bioscience). For cell cycle analysis, TNBC cells were fixed in 70% ethanol, and incubated at 4°C overnight. Cell pellets were re-suspended in 0.5 mL of 0.05 mg/mL PI plus 0.2 mg/mL RNaseA and incubated at 37°C for 30 min prior to FACS analysis.

Soft agar assays

Anchorage-independent growth of the tumor cells was assessed using soft agar assays. Briefly, a base layer containing 0.5% agar medium and 10% FBS was poured into six-well plates. Then, 1,000 cells were mixed with compounds and 0.35% agarose in medium containing 10% FBS to form a single-cell suspension. After being seeded, the plates were incubated for 4-5 weeks. Colonies were stained with 0.005% crystal violet and photographed under a ChemiDoc Imaging system (Bio-Rad).

Immunofluorescence assays

Cells were seeded in eight-well chamber slides (Nunc) for different treatments, then fixed with 4% PFA and stained with a mouse Anti-tubulin

monoclonal antibody (Sigma), followed by a goat anti-mouse secondary antibody conjugated to 488 (Invitrogen) and DAPI. Fluorescence signal was measured using the Olympus IX83 microscope (Olympus).

Intracellular tubulin polymerization assay

Cells were treated with test drugs or vehicle for 24 h, then Hypotonic lysis buffer (VWR) was added to separate soluble and polymerized tubulin proteins as the described previously [14]. The levels of soluble and insoluble tubulin were measured by Western blot using a mouse Anti-tubulin monoclonal antibody (Sigma).

Development of Paclitaxel resistant cell line

TNBC Paclitaxel resistant cell line, MDA-MB-231^{PA}, was generated by exposing the parental cell line, MDA-MB-231, to an increasing dose of Paclitaxel (up to 100 nM) for 5 months. Clones were obtained by extracting and expanding a single colony from a colony formation assay. Cells were maintained at 100 nM Paclitaxel.

TNBC xenograft models

Cells were counted and washed once in ice-cold sterile PBS, then 1×10^6 HCC1806 cells in 50 μL PBS plus 50 μL growth factor-depleted Matrigel (BD Biosciences) were injected subcutaneously into the flank of nude mice, 6-8-week old, female (Jackson Laboratory). When tumors reached ~5 mm in diameter, the mice were randomly separated into different groups (4 mice per group) and received i.p. injection with either vehicle, Cephalomannine (1 mg/kg) or 4'-Demethylpodophyllotoxin (10 mg/kg), 3 days/week. The mice were observed and measured every 2-3 days for the size of palpable tumors for an additional 2 weeks. At the end of experiment, the tumors were excised and compared.

RNA-Sequencing and enrichment analysis

RNA-Sequencing of triplicate samples was performed by BGI Americas Corporation using their unique DNBSEQ™ sequencing technology. The completed RNA-Sequencing data was submitted to NCBI Sequence Read Archive (SRA# PRJNA1035488). Raw sequencing reads were analyzed using the RSEM software (version 1.3.0; human GRCh38 genome sequence and annotation) and gene expression was quantified as previously described [15]. The EBSeq software was utilized to call differentially expressed genes that were statistically significant using a false discovery rate (FDR) less than 0.05. Differentially expressed genes between natural compounds- and vehicle-treated TNBC cells were used as input for the GO_ enrichment analyses.

RNA interference (RNAi)

For RNAi assays, ACHYL1 or SPG21 On-Target plus SMARTpool small interfering RNA (siRNA; Dharmacon) or negative control siRNA were delivered using the DharmaFECT transfection reagent as recommended by the manufacturer.

Western blot

Total cell lysates (20 μg) were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies to ACHYL1 or SPG21 (Abcam), cleaved Caspase 3, BAX, p21, Cyclin B1 (Cell Signaling). GAPDH served as the loading control (Cell Signaling). Immunoreactive bands were identified using an enhanced chemiluminescence reaction (Perkin-Elmer) and visualized by autoradiography.

Immunohistochemistry

Immunohistochemistry was performed using the Avidin-Biotin-Peroxidase complex, according to the manufacturer's instructions (Vector Laboratories) as described previously [16, 17]. The rabbit polyclonal anti-ACHYL1 or anti-SPG21 (Abcam) was used at 1:100 dilution. Tissue array slides were then scanned with an Aperio CS2 digital pathology scanner. Images were obtained with Aperio ImageScope software (Leica) at 40× magnification. The percentage of DAB stained pixels were determined by analyzing the raw images with the QuPath software (version 0.2.3) [18].

RT-qPCR

Total RNA was isolated by using the RNeasy Mini kit (Qiagen), and cDNA was synthesized using a SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen). Specific primers used for amplification of individual target gene:

ACHYL1 sense, 5' GGCCTGAAGAGGACCACA 3'; *ACHYL1* antisense, 5' CTTTACCACCCTGAACCC 3'; *SPG21* sense, 5' GTCCATTCCCTAATCCTC 3'; *SPG21* antisense, 5' GGTAAGTCTTGAAGCCAGT 3'. The amplification was carried out using an iCycler IQ Real-Time PCR Detection System, and cycle threshold (Ct) values were tabulated in triplicate for each gene of interest in each experiment. "No template" (water) controls were used to ensure minimal background contamination. Using mean Ct values tabulated for each gene, and the paired Ct values for β-actin gene as a loading control, the fold changes for experimental groups relative to assigned control groups were calculated by using automated iQ5 2.0 software (Bio-rad).

Statistical analysis

Significant differences between experimental and control groups were determined using the two-tailed Student's *t*-test. The 50% Cytotoxicity Concentrations (CC_{50}) were calculated from the dose-response curves using GraphPad Prism 9.

Results

High-throughput screening and identification of new natural compounds displaying anti-TNBC activities

One of TNBC cell lines, MDA-MB-231, was used for our initial screening assays. After screening a chemical library containing 756 natural products, we found 13 compounds induced prominent cytotoxicity (>60%) at 10 μM concentration (**Figure 1A**, **1B**). After searching published literature, we then excluded molecules with known anti-BC activities (e.g., Palmatine, Baohuoside I) which yielded 9 novel compounds. After calculating the 50% Cytotoxicity Concentrations (CC_{50}) using drug-killing curves on different TNBC cell lines, including MDA-MB-468, MDA-MB-231, HCC1806, DU4475, MDA-MB-453, BT-549, we ultimately identified 3 compounds with CC_{50}<1 μM (**Figure 1C-E** and **Table 1**). Actually, most of their CC_{50} on different TNBC cell lines are at nM levels, demonstrating satisfied cell line relevance. These 3 natural compounds are 4'-Demethylepipodophyllotoxin (isolated from *Dysosma versipellis*), 4'-Demethylpodophyllotoxin (isolated from *Dysosma pleiantha*), Cephalomannine (isolated from *Taxus yunnanensis*), and the first two compounds share very similar chemical structure (**Figure 1C-E**). Notably, all of the 3 natural compounds showed almost no cytotoxicity on normal human primary mammary epithelial cells (HMEC, CC_{50}>90 μM), making them highly selective towards TNBC cells and suitable for drug development with a selective index (SI) of ~30-10,000 (**Table 1**).

By doing a time-course assay, we found that all of the 3 natural compounds treatments effectively blocked TNBC cell growth (e.g., reducing the number of TNBC cells but not HMEC) when compared to the vehicle control (**Figure 2A**, **2B**). By using the soft agar assays, we observed all of the 3 natural compounds treatments dramatically inhibited anchorage-independent growth of TNBC cell lines, MDA-MB-231 or HCC1806 (e.g., having some smaller spheroids formation or only having some cell debris left) when compared to the vehicle control (**Figure 2C**, **2D**).

The new natural compounds induce apoptosis and cell cycle arrest of TNBC cells

Using FITC-Annexin V/propidium iodide (PI) staining combined with flow cytometry analysis, we found that all of the 3 natural compounds treatments significantly induced TNBC cell apoptosis, including the increased subpopulation of both early (Annexin V+/PI-) and late (Annexin V+/PI+) apoptotic cells, when compared to the vehicle control (**Figure 3A**, **3B**). We also found that these compounds treatments mainly caused cell cycle G2/M arrest in TNBC cell lines (**Figure 3C**, **3D**). By using Western blot, we found that these natural compounds treatments affected the expression of several apoptosis or cell cycle regulators, such as increasing levels of cleaved Caspase 3, BAX, p21 and Cyclin B1 proteins in different TNBC cell lines (**Figure 3E**).

The new natural compounds target microtubule functions in TNBC cells

Interestingly, previous study has reported that one of the 3 natural compounds, 4'-demethylepipodophyllotoxin, can bind to monomeric tubulin and prevent microtubule polymerization [19]. Another compound, Cephalomannine is an active agent obtained from *Taxus yunnanensis* and found as one of yew alkaloids taxoids [20]. These data raise the possibility that the

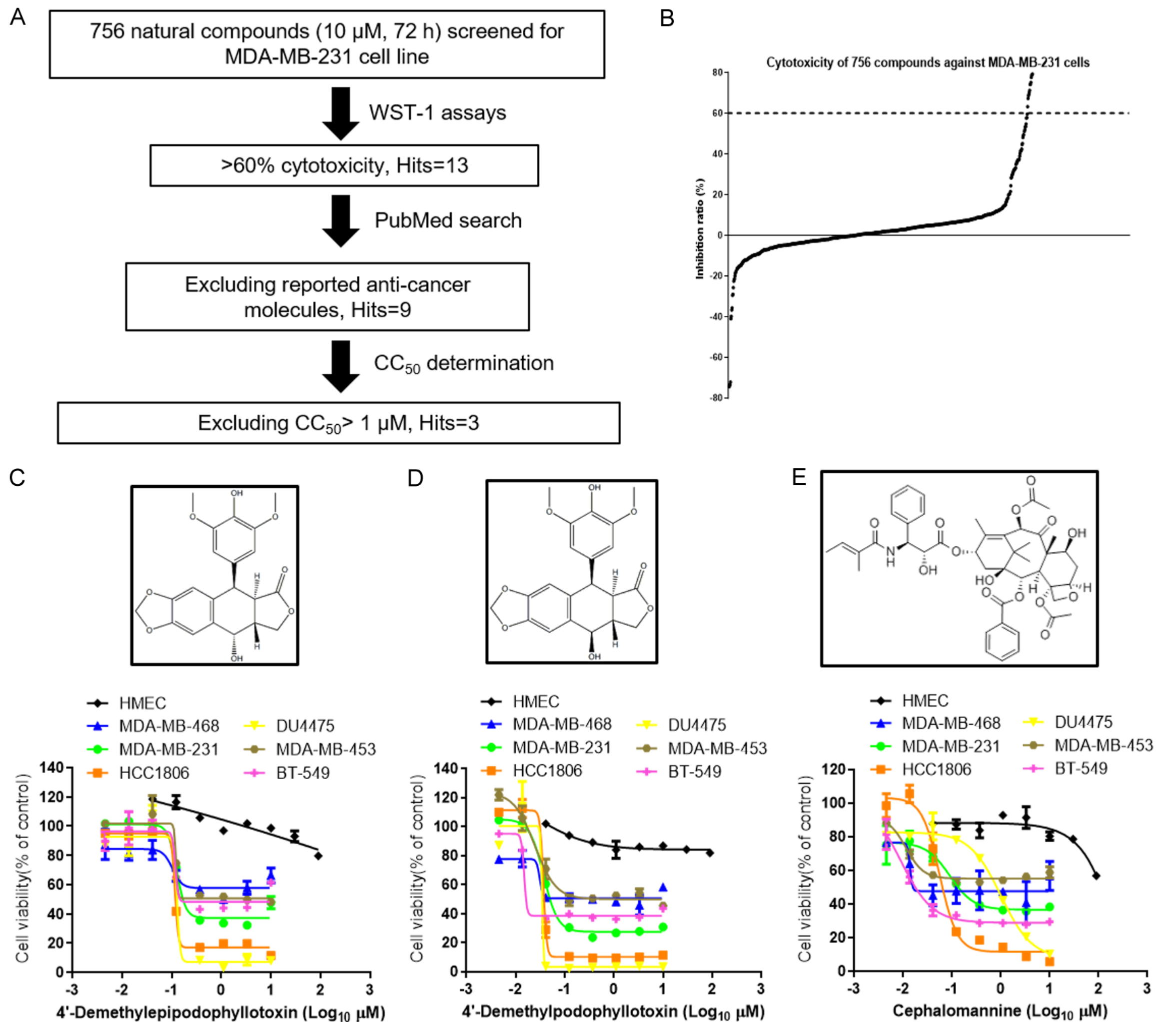


Figure 1. High-throughput screening and identification of new natural compounds against TNBC cell lines. A, B. Primary screening results of 756 natural compounds against TNBC, which are arranged in order of inhibition rate. The natural compounds in source plates were delivered at 10 μM (final concentration) to 96-well plates seeded with TNBC cell line MDA-MB-231 for 72 h treatment, then cell proliferation was examined using the WST-1 cell proliferation assays (Roche). C-E. The chemical structures of final 3 hit compounds and their dose-dependent inhibition of growth curves on different subtypes of TNBC cell lines as well as human primary mammary epithelial cells (HMEC).

targets of these natural compounds are probably related to interfere with microtubule dynamicity and functions. Using immunofluorescence assay with tubulin specific antibody, we found that 4'-Demethylepipodophyllotoxin and 4'-Demethylpodophyllotoxin treatments caused many paracrystals, spirals, and tubules formation within TNBC cells, which was similar with Vinblastine, a classical microtubule destabilizer binding free tubulin heterodimers to inhibit microtubule assembly. In contrast, Cephalomannine treatment caused shorter and highly polymerized tubules formation in TNBC cells, which was similar with Paclitaxel, a classical microtubule stabilizer (**Figure 4A**). Next, using the tubulin polymerization assay, we found that Cephalomannine or Paclitaxel treatments decreased soluble fraction of tubulin while increased tubulin polymerization with most of the tubulin in the insoluble fraction. However, 4'-Demethylepipodophyllotoxin, 4'-Demethylpodophyllotoxin or Vinblastine treatments caused depolymerization of tubulin with most of the tubulin in the soluble fractions (**Figure 4B**).

The new natural compounds overcome the resistance to Paclitaxel by TNBC cells

One of potential problems for cytoskeletal drugs such as Paclitaxel used in the treatment

Table 1. The prominent anti-TNBC activities of hit natural products *in vitro*

Compounds	CC_{50} (μM)[a]						
	MDA-MB-231	MDA-MB-468	HCC1806	DU4475	MDA-MB-453	BT-549	HMEC[b]
4'-Demethylepipodophyllotoxin	0.12	0.10	0.12	0.13	0.12	0.12	>90
4'-Demethylpodophyllotoxin	0.03	0.03	0.03	0.03	0.02	0.01	>90
Cephalomannine	0.08	0.04	0.05	1.01	0.01	0.01	>90

[a]CC_{50}: the 50% cytotoxic concentration determined by using the WST-1 assay. [b]HMEC: Human primary mammary epithelial cells.

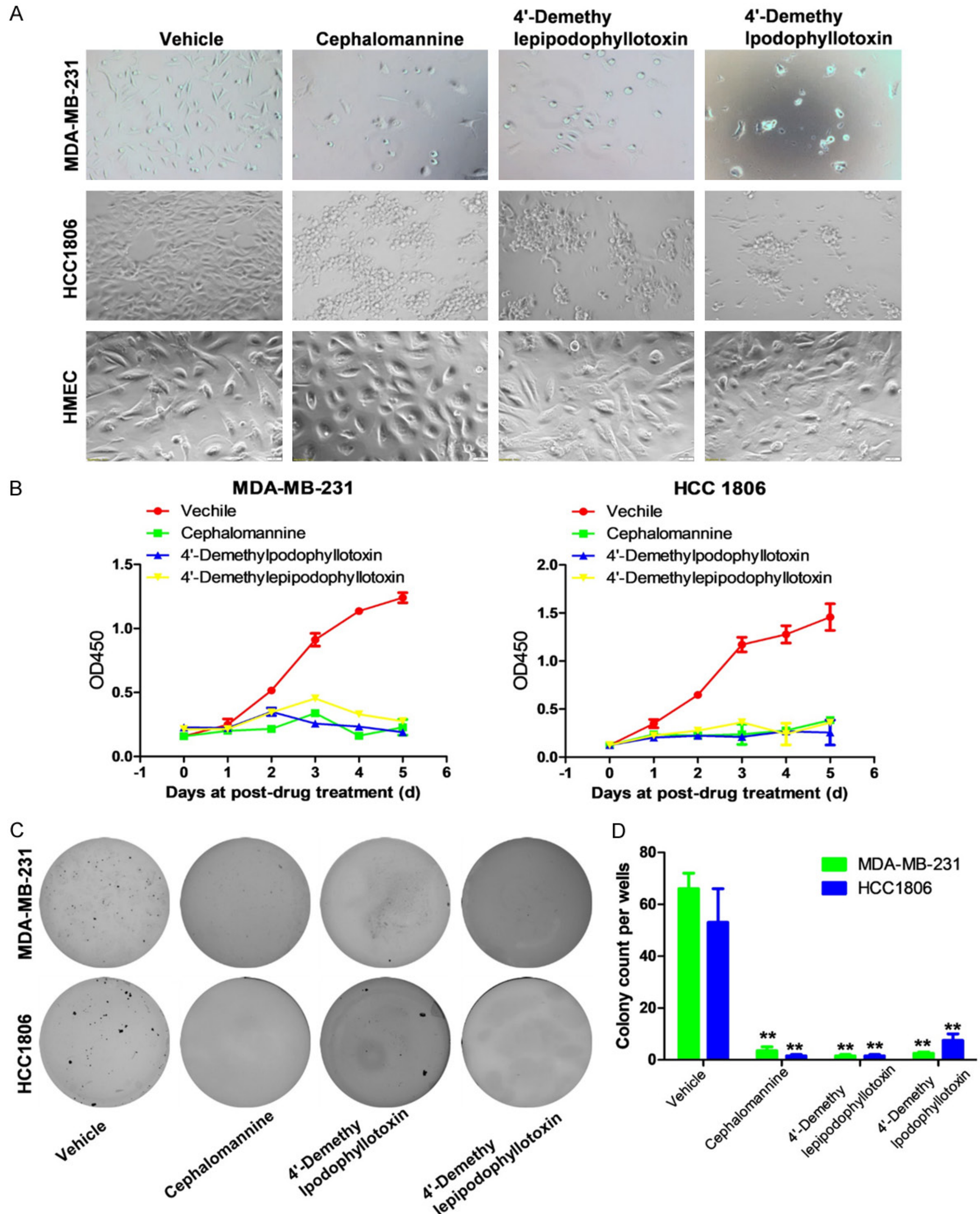


Figure 2. The new natural compounds prominently inhibit TNBC cell growth. A. TNBC cell lines, MDA-MB-231, HCC1806 as well as normal cells HMEC were treated with 2× CC_{50} of hit compounds for 72 h, then cell morphology were imaged under the microscope. B. The time-course inhibition of growth curves by hit compounds for MDA-MB-231 and HCC1806. C, D. The inhibition of TNBC anchorage-independent growth ability by natural compounds were tested using soft agar assays as described in Methods. Error bars represent S.D. for 3 independent experiments, ** = P<0.01 (*vs* the vehicle control).

of cancer patients is the development of resistance by cancer cells. Here we developed a new Paclitaxel-resistant TNBC cell line, MDA-MB-231^{PA}, through exposing the parental cell line to an increasing dose of Paclitaxel (up to 100 nM) for 5 months. MDA-MB-231^{PA} showed ~10 folds of CC_{50} increased to Paclitaxel when compared to its parental cell line (8.48 *vs* 0.81 μM) (Figure S1A and **Table 2**). In contrast, all of the 3 natural compounds (4'-Demethylepipodophyllotoxin, 4'-Demethylpodophyllotoxin, Cephalomannine) displayed similarly high efficacy between MDA-MB-231^{PA} and its parental cell line (CC_{50} at 0.03-0.09 μM), which are much better than Paclitaxel (Figure S1B-D and **Table 2**).

The new natural compounds effectively repress TNBC cell growth in vivo

To assess *in vivo* efficacy of our new natural compounds, we tested 4'-Demethylpodophyllotoxin and Cephalomannine in an established TNBC xenograft mice model. Our results indicated that both compounds treatments significantly repressed HCC1806 tumor growth in mice (**Figure 5A**), while the compounds did not cause obvious animal body weight changes during the treatment (data not shown). At the end of treatments, the tumors were excised for size and weight comparison. We found that the mice from both Cephalomannine and 4'-Demethylpodophyllotoxin treated groups formed much smaller tumors (especially the latter) when compared to the vehicle treated mice (**Figure 5B**, **5C**). Together, these data demonstrate significant *in vivo* efficacy of the two new natural compounds against TNBC, positing them as promising therapeutic agents.

Transcriptomic analysis of gene profiling in different subtypes of TNBC cell lines altered by natural compounds

To determine the global cellular changes induced by these natural compounds, we compared the gene profiles of vehicle- to compound-treated tumor cells (Cephalomannine and 4'-Demethylpodophyllotoxin, respectively) by using RNA-Sequencing analyses. Here we tested different subtypes of TNBC cell lines, including HCC1806 (BL2 subtype), MDA-MB-468 (BL1 subtype), BT-549 (M subtype), MDA-MB-231 (MSL subtype), MDA-MB-453 (LAR subtype). After intersection analysis, the heat map indicated 59 genes commonly upregulated and 28 genes commonly downregulated (FDR<0.05) in all of subtypes of TNBC cell lines treated by Cephalomannine (**Figure 6A**). Meanwhile, the heat map indicated 85 genes commonly upregulated and 38 genes commonly downregulated (FDR<0.05) in all of subtypes of TNBC cell lines treated by 4'-Demethylpodophyllotoxin (**Figure 6B**). The GO_enrichment analysis of these common candidate genes altered by Cephalomannine or 4'-Demethylpodophyllotoxin from different subtypes of TNBC cell lines identified several major functional categories potentially involved. The Biological process module analysis indicated many of these genes belong to pathways important for mitotic cell cycle phase transition, anaphase-promoting complex-dependent catabolic process, type I interferon signaling pathway, integrin-mediated signaling pathway and cytokine-mediated signaling pathway (**Figure 6C**, **6D**). Not surprisingly, the Cellular component module analysis showed the majority of the genes were associated with microtubule cytoskeleton, mitotic spindle, condensed chromosome and focal adhesion (Figure S2).

Identification of AHCYL1 and SPG21 as new microtubule-associated factors and potential therapeutic targets for TNBC

We propose that certain gene candidates identified from RNA-Sequencing analysis are potentially related to TNBC survival and/or pathogenesis. Thus, we selected AHCYL1 (S-adenosylhomocysteine hydrolase-like protein 1, also named as IRBIT) and SPG21 (Spastic paraplegia 21, also named as Maspardin) for subsequent functional validation, since both proteins expression was dramatically downregulated by Cephalomannine or 4'-Demethylpodophyllotoxin in all the subtypes of TNBC cell lines we tested.

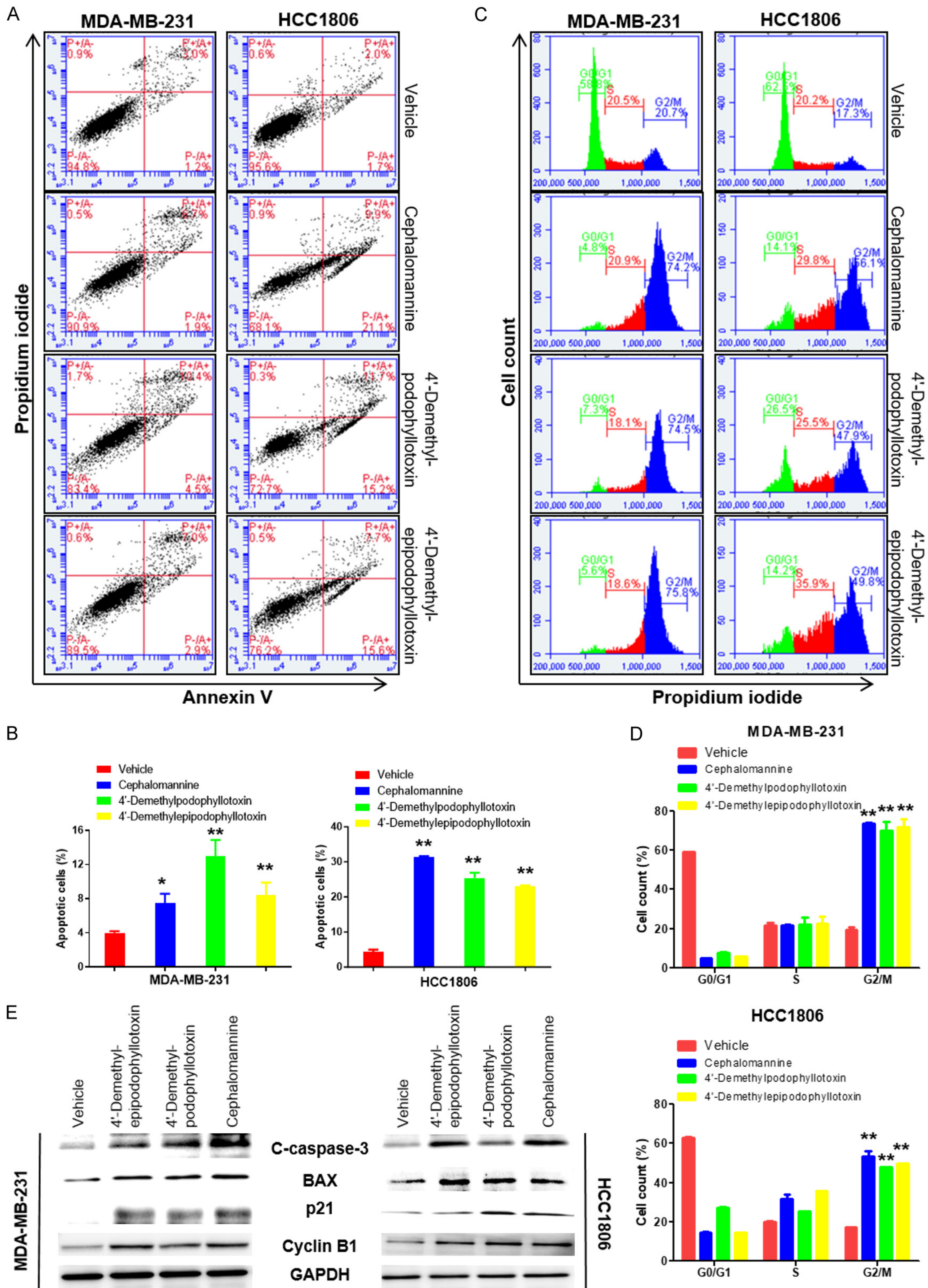


Figure 3. The new natural compounds induce TNBC cell apoptosis and cell cycle arrest. A, B. TNBC cell lines, MDA-MB-231 and HCC1806, were treated with natural compounds or vehicle for 48 h, then cell apoptosis was measured by Annexin V-PI staining and flow cytometry analysis. C, D. Cell cycle was measured by PI staining and flow cytometry analysis. Error bars represent S.D. for 3 independent experiments, * = P<0.05, ** = P<0.01 (*vs* the vehicle control). E. Protein expression was measured by using Western blot.

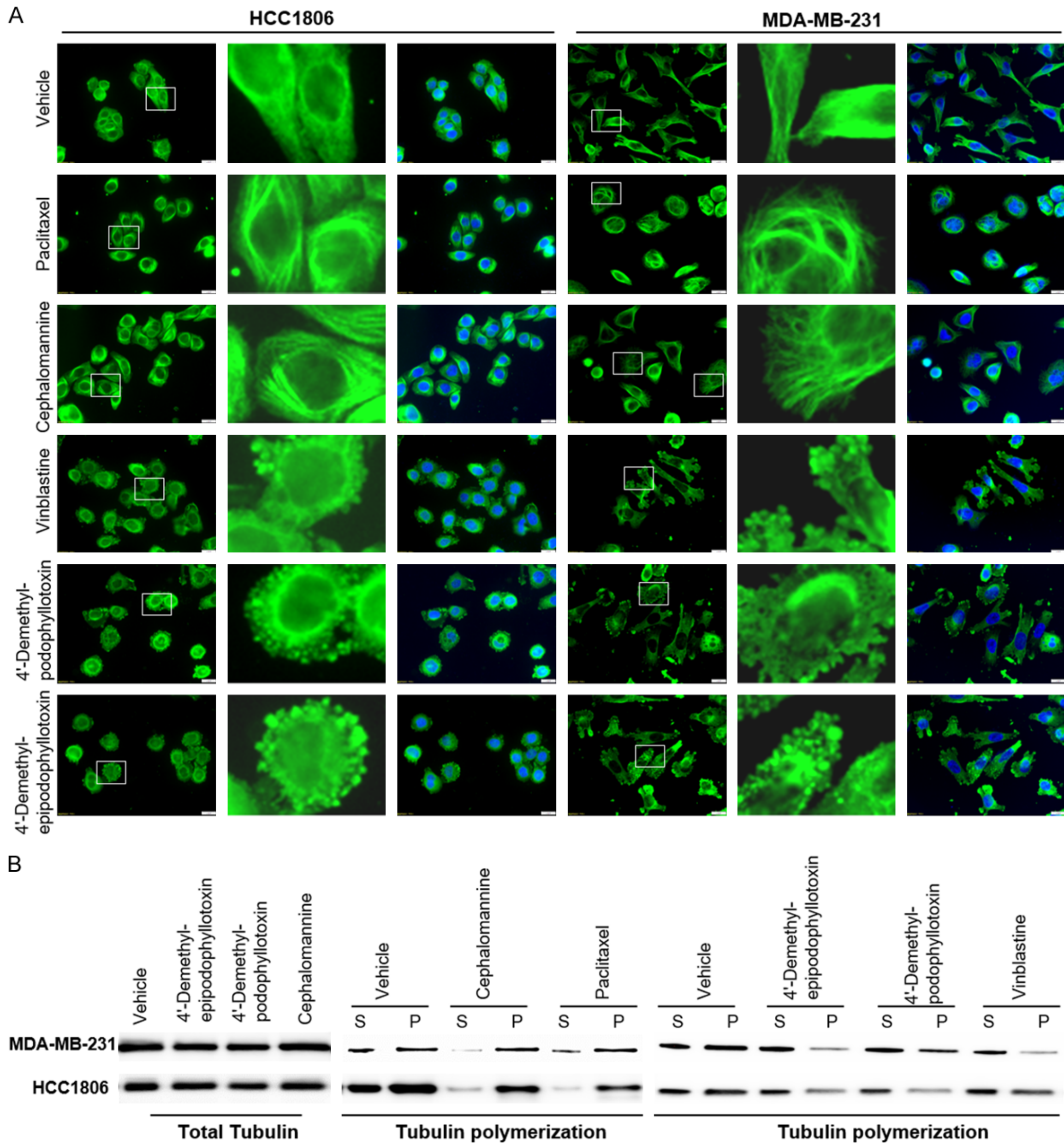


Figure 4. The new natural compounds target microtubule functions in TNBC cells. A, B. MDA-MB-231 and HCC1806 were treated with natural compounds or vehicle for 24 h, then the morphology of microtubule and tubulin polymerization were measured using immunofluorescence and immunoblots with specific antibody for tubulin, respectively. The Paclitaxel and Vinblastine were used as positive controls. S: soluble fraction; P: polymerization.

Table 2. The anti-TNBC activities of hit natural products compared between Paclitaxel-sensitive and resistant TNBC cell line *in vitro*

	MDA-MB-231 (μM)	MDA-MB-231^{PA} (μM)
4'-Demethylepipodophyllotoxin	0.12	0.09
4'-Demethylpodophyllotoxin	0.03	0.03
Cephalomannine	0.08	0.04
Paclitaxel	0.81	8.48

AHCYL1/IRBIT is a member of AHCY family of proteins involved in metabolism of S-adenosyl-L-homocysteine [21]. AHCYL1 plays important role in the inositol phospholipid (IP) signaling pathway by interacting with the inositol 1,4,5-trisphos-

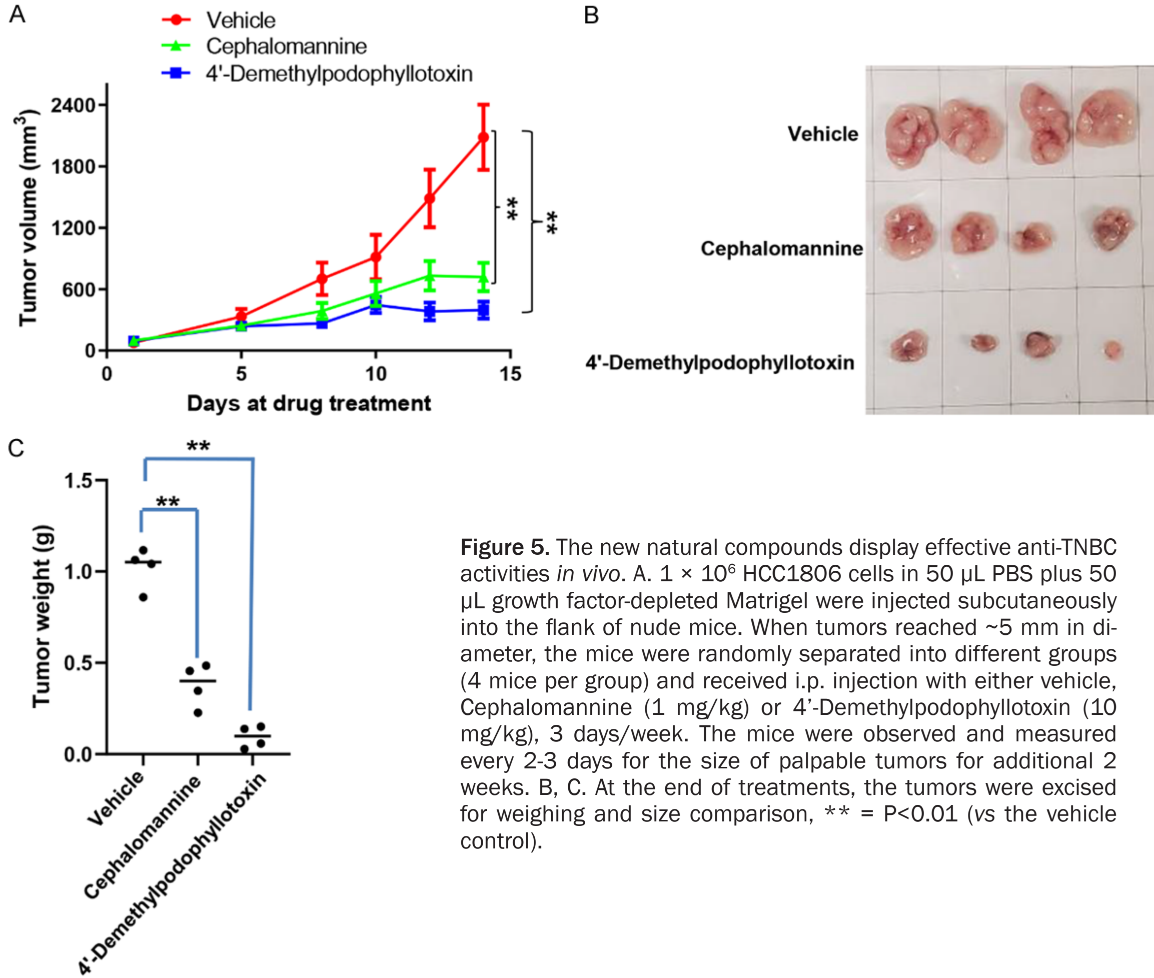


Figure 5. The new natural compounds display effective anti-TNBC activities *in vivo*. A. 1 × 10^6 HCC1806 cells in 50 μL PBS plus 50 μL growth factor-depleted Matrigel were injected subcutaneously into the flank of nude mice. When tumors reached ~5 mm in diameter, the mice were randomly separated into different groups (4 mice per group) and received i.p. injection with either vehicle, Cephalomannine (1 mg/kg) or 4'-Demethylpodophyllotoxin (10 mg/kg), 3 days/week. The mice were observed and measured every 2-3 days for the size of palpable tumors for additional 2 weeks. B, C. At the end of treatments, the tumors were excised for weighing and size comparison, ** = P<0.01 (*vs* the vehicle control).

phate (IP3) receptor, which is an intracellular Ca^{2+} release channel located on the endoplasmic reticulum. Therefore, this protein influences the IP3-induced Ca^{2+} signaling cascade essential for numerous cellular and physiological processes such as organ development, fertilization, and cell death [22-24]. Another candidate, SPG21/Maspardin is responsible for human Mast syndrome, a complicated form of human hereditary spastic paraplegias [25]. SPG21 presents similarity to the α/β-hydrolase superfamily, but might lack enzymatic activity and rather be involved in protein-protein interactions. However, the functional roles of AHCYL1 and SPG21 in cancer cells especially TNBC remain largely unknown. By using Western blot, we first confirmed that all of 3 natural compounds (4'-Demethylepipodophyllotoxin, 4'-Demethylpodophyllotoxin, Cephalomannine) treatments significantly reduced the expression of AHCYL1 and SPG21 from TNBC cell lines in a dose-dependent manner, which are consistent with our RNA-Sequencing results. Interestingly, other cytoskeletal drugs such as Paclitaxel and Vinblastine treatments also reduced AHCYL1 and SPG21 expression, although with a less extent (**Figure 7A**).

We then found that successful knockdown of either *AHCYL1* or *SPG21* by RNAi effectively repressed TNBC cell growth (Figure S3), indicating that these genes are required for TNBC cell survival. We also found that knockdown of either *AHCYL1* or *SPG21* obviously impaired microtubule normal structure in TNBC cells through immunofluorescence analysis (**Figure 7B**). These data together indicate AHCYL1 and SPG21 as new microtubule-associated proteins in TNBC cells, which may represent attractive therapeutic targets for TNBC.

Clinical implications of AHCYL1 and SPG21 in TNBC patients

TNBC tissue arrays containing 120 cases as well as 6 normal breast tissues were used to

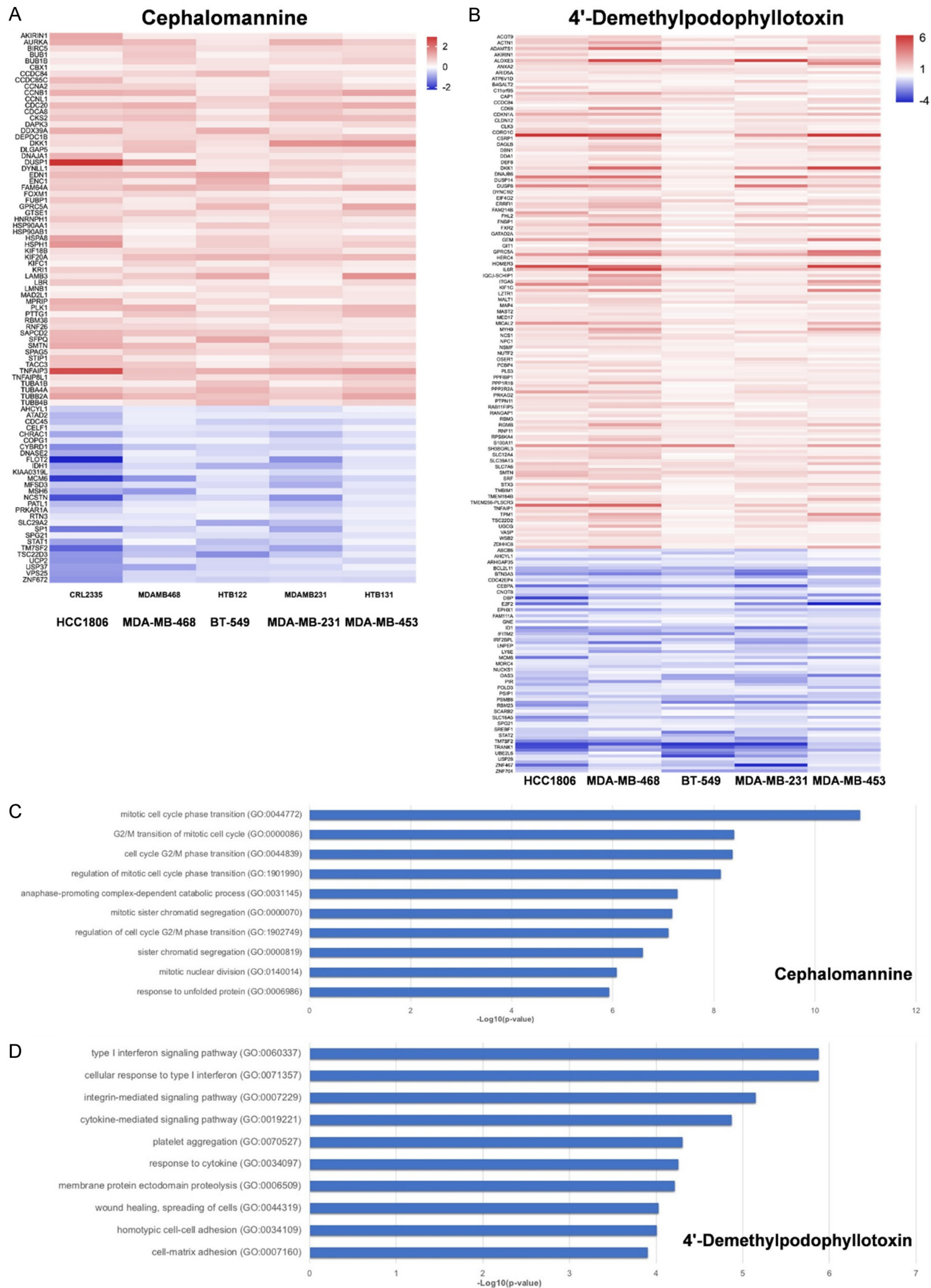


Figure 6. Transcriptome analysis of new natural compounds treated TNBC cells. A, B. RNA-Sequencing was used to investigate changes in the transcriptome between natural compounds and vehicle treated different subtypes of TNBC cell lines, including HCC1806 (BL2 subtype), MDA-MB-468 (BL1 subtype), BT-549 (M subtype), MDA-MB-231 (MSL subtype), MDA-MB-453 (LAR subtype). The heat maps showed candidate genes commonly upregulated or

downregulated (FDR<0.05) in all of subtypes of TNBC cell lines treated by Cephalomannine or 4'-Demethylpodophyllotoxin. C, D. The GO_enrichment (Biological Process module) analysis of the commonly changed cellular genes by hit compounds in different subtypes of TNBC cell lines.

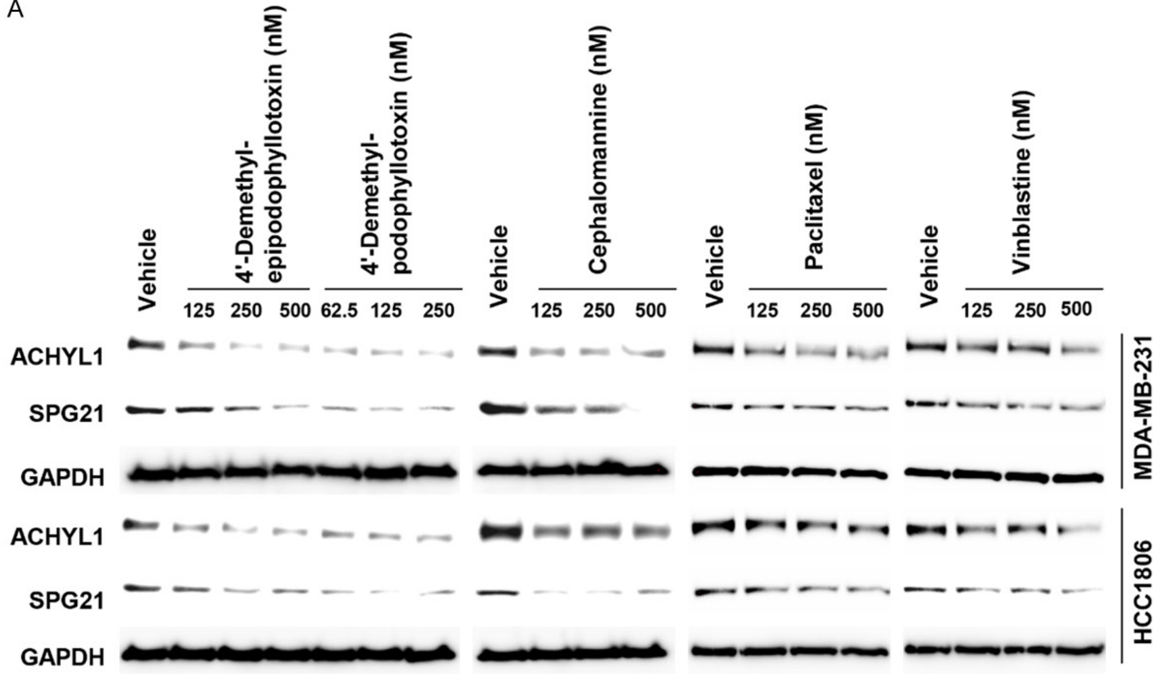
explore the clinicopathological role of AHCYL1 and SPG21 in TNBC progression through immunohistochemistry (IHC) staining. The IHC results indicated that the expressional levels of both AHCYL1 and SPG21 proteins were significantly upregulated in TNBC tumor tissues when compared to normal breast tissues (**Figure 8A**), although their expression were variable among tumor tissues from different TNBC patients. Based on clinical characteristics of the patient cases, we observed the expressional levels of AHCYL1 and SPG21 were significantly increased in different stages of TNBC, especially advanced stages such as Stage II and III (**Figure 8B**, **8D**). Furthermore, based on TNM scores, the expression of AHCYL1 and SPG21 were significantly higher in different TNM groups, especially T2-T4 groups (**Figure 8C**, **8E**). Taken together, these clinical data strongly support a role of AHCYL1 or SPG21 in TNBC development and progression as potential tumor biomarkers.

Discussion

Although TNBC accounts for about 10-15% of all breast cancers, this subset of breast cancer tends to grow and spread faster, has fewer treatment options, and tends to have a worse prognosis. In addition, TNBC tends to be more common in women younger than age 40, who are African American, or who have a *BRCA1* mutation [26]. In the current study, we identified 3 natural compounds with prominent anti-TNBC activities while showing almost no cytotoxicity on normal breast cells, using high-throughput screening methods. Notably, all of the 3 natural compounds (4'-Demethylepipodophyllotoxin, 4'-Demethylpodophyllotoxin, Cephalomannine) have no known association with breast cancer including TNBC treatment. Based on chemical structure and functional assays, we identified three compounds that each belong to "cytoskeletal drugs" classification that target tubulin and microtubule function. More importantly, our data showed that these compounds showed consistently effective on TNBC cell line which is resistant to those currently used anti-microtubule agents such as Paclitaxel *in vitro*. Thus, future work will focus on the underlying mechanisms of resistance overcome and testing their efficacy in resistant cancer cells xenograft models.

One of the compounds, Cephalomannine, has been found as a novel potential agent against malignant pleural mesothelioma after a drug screening [27]. Another recent study has reported that Cephalomannine can inhibit hypoxia-induced cellular function via the suppression of APEX1/HIF-1α interaction in lung cancer [28]. In details, Cephalomannine can significantly inhibit cell viability, ROS production, intracellular pH, and migration in hypoxic lung cancer cells. Zhou *et al.* recently reported to design and synthesize two tertiary amine-derived 4'-demethylepipodophyllotoxin conjugates (DC and DP), using N,N,N'-trimethyl-N'-(4-carboxyl benzyl)-1,3-propanediamine (CPDM) and 4-(4-methylpiperazinomethyl)benzoic acid (PBA) as the targeting ligands [29]. They found that both DC and DP exhibited strong *in vitro* cytotoxicity against small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) cell lines. In addition, DC and DP remarkably reduced the lung weight and the number of lung metastases of B16 melanoma in mice, and further prolonged the survival of tumor-bearing mice.

By using RNA-Sequencing analyses, we identified a subset of gene candidates which were commonly changed by these hit compounds in different subtypes of TNBC cells. Among them, we demonstrated AHCYL1 and SPG21 as new microtubule-associated proteins, which were required for TNBC cell survival with clinical implication. However, how these proteins are involved in microtubule functions in TNBC cells still require further investigation. S-adenosyl-l-homocysteine (SAH) is a key intermediate metabolite in methionine metabolism, which is normally considered as a harmful by-product and hydrolyzed quickly once formed. AHCYL1 can function as a SAH sensor to inhibit macroautophagy/autophagy through PIK3C3 [30]. In cancer research, one recent study has reported AHCYL1 as a novel biomarker for predicting prognosis and immunotherapy response in colorectal cancer (CRC) [31]. They found that CRC tissues without AHCYL1 have a weaker


A
Vehicle
4'-Demethyl-epipodophyllotoxin (nM)
4'-Demethyl-podophyllotoxin (nM)
Vehicle
Cephalomannine (nM)
Vehicle
Paclitaxel (nM)
Vehicle
Vinblastine (nM)
125 250 500 62.5 125 250
125 250 500
125 250 500
125 250 500
ACHYL1
SPG21
GAPDH
ACHYL1
SPG21
GAPDH
MDA-MB-231
HCC1806

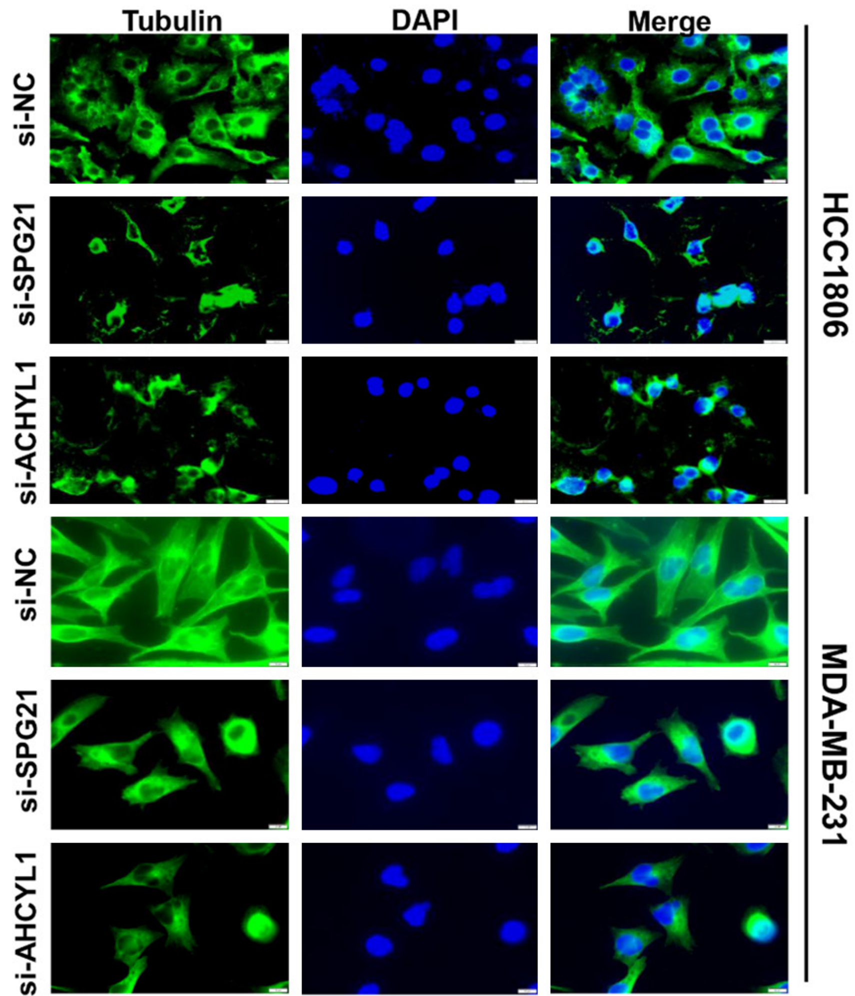

B
Tubulin
DAPI
Merge
si-NC
si-SPG21
si-ACHYL1
si-NC
si-SPG21
si-AHCYL1
HCC1806
MDA-MB-231

Figure 7. AHCYL1 and SPG21 expression is downregulated by cytoskeletal drugs from TNBC cells and required for microtubule formation. A. MDA-MB-231 and HCC1806 cells were treated with 4'-Demethylepipodophyllotoxin, 4'-Demethylpodophyllotoxin, Cephalomannine, Paclitaxel or Vinblastine, respectively, then protein expression was measured by using Western blot. B. HCC1806 and MDA-MB-231 cells were transfected with either AHCYL1-siRNA, SPG21-siRNA or non-target control siRNA (si-NC) for 48 h, then the morphology of microtubule was observed using immunofluorescence with specific antibody for tubulin.

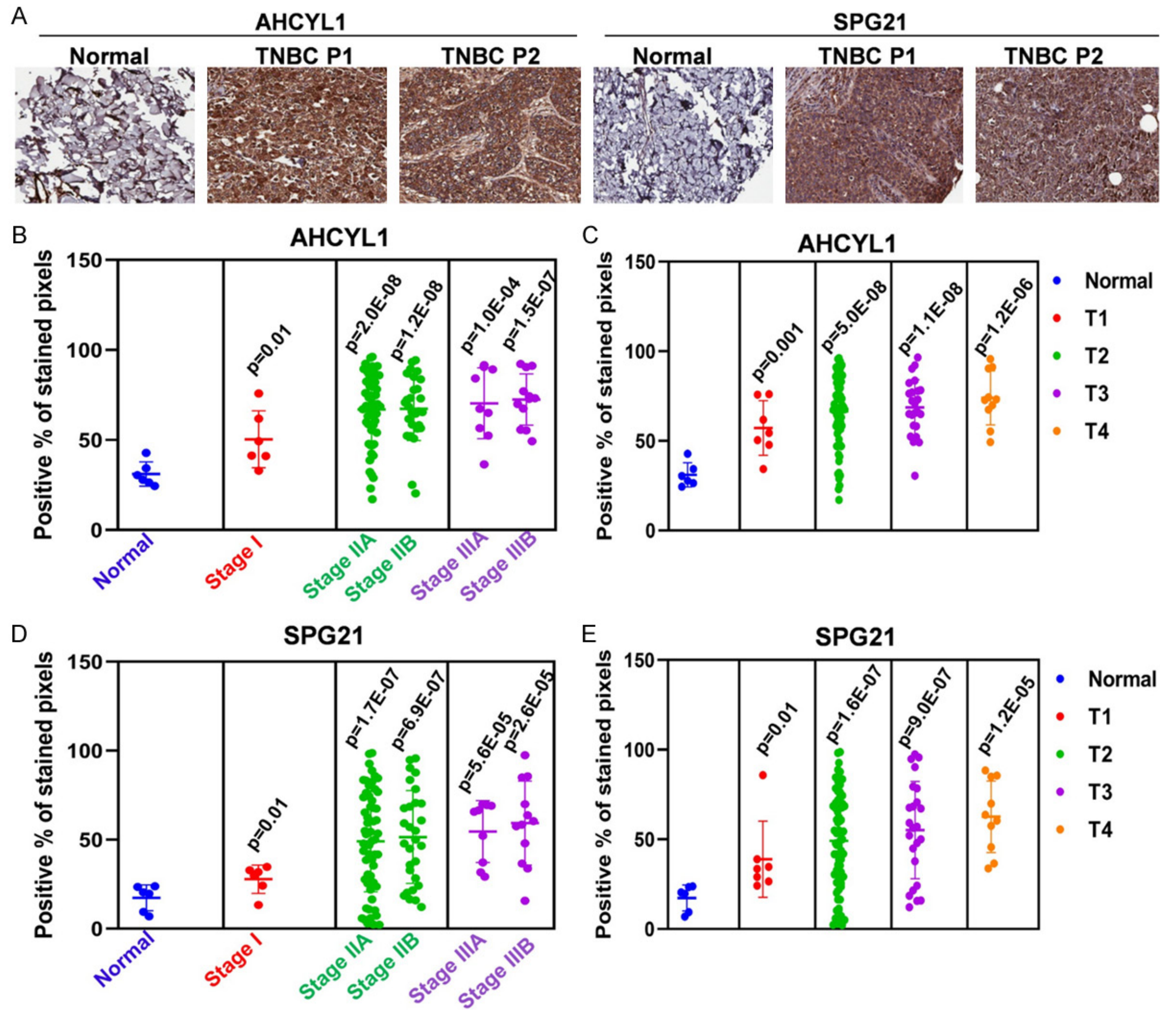


Figure 8. The clinical relevance of AHCYL1 and SPG21 in TNBC. Expression of AHCYL1 and SPG21 in formalin-fixed paraffin-embedded (FFPE) TNBC and normal breast tissue arrays were determined using immunohistochemistry (IHC). A. The IHC images from representative cases. B-E. The percentage of DAB stained pixels were determined by analyzing the raw images with the QuPath software (version 0.2.3). The nested graphs show expressional difference among between these groups and normal breast tissue group.

ability to recruit the natural killer (NK) cell, CD8+ T cells, and tumor-infiltrating lymphocytes (TILs) and response to immunotherapy. Additionally, knockdown of AHCYL1 promoted tumor growth in the CRC mouse model and recruited lower CD8+ T cells in CRC tissues. In contrast, here we found that the expressional levels of AHCYL1 were significantly increased in advanced stages or higher TNM scores of TNBC patients. Moreover, knockdown of AHCYL1 effectively repressed TNBC cell growth. These findings suggest that AHCYL1 mediated cellular functions and consequence are probably dependent on cancer type.

Another gene candidate, SPG21, its functions in cancer cells remain completely unknown. Davenport *et al.* reported that loss of SPG21

attenuated the growth and maturation of mouse cortical neurons [32]. They found that SPG21$^{-/-}$ mice demonstrated significantly less agility and coordination compared to wild-type mice. The SPG21$^{-/-}$ mice exhibited symptoms of mast syndrome at 6 months which worsened in 12-month-old cohort, suggesting progressive dysfunction of motor neurons.

In summary, we have identified new natural compounds with prominent anti-TNBC activities *in vitro* and *in vivo*, and identified new cellular genes required for TNBC cell survival and tumor progression which may represent promising therapeutic targets or tumor biomarkers.

Acknowledgements

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Disclosure of conflict of interest

None.

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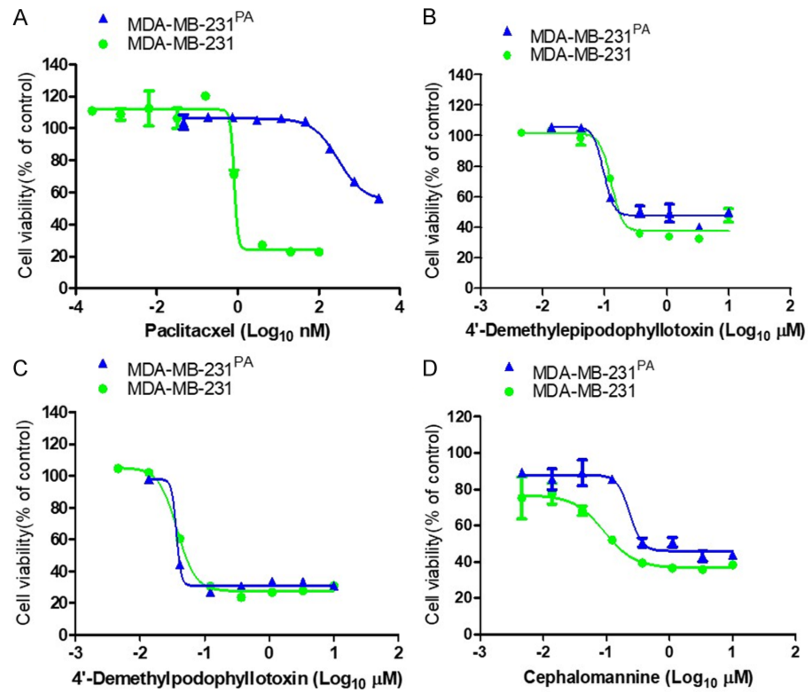

Figure S1. The hit natural compounds display still effective on Paclitaxel-resistant TNBC cells *in vitro*. A-D. The MDA-MB-231 derived Paclitaxel-resistant cell line (MDA-MB-231[PA]) and parental cell line were treated with a range of doses of natural compounds or Paclitaxel for 48 h, then the cell proliferation was assessed using the WST-1 assays.

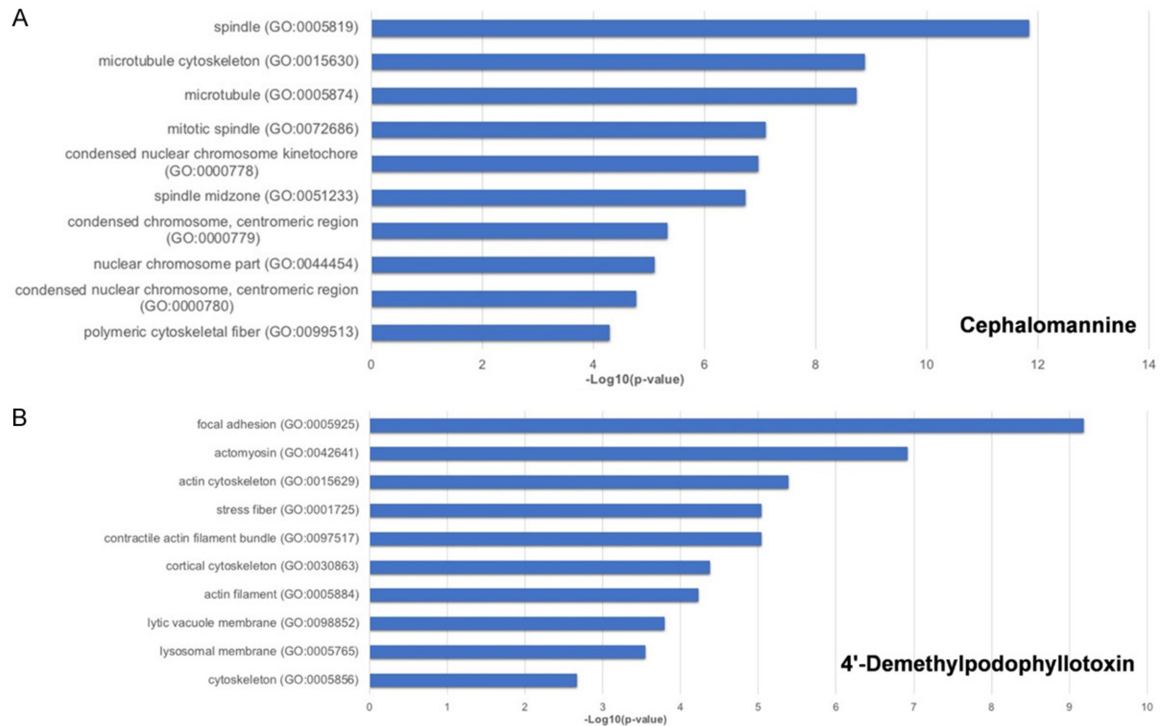

Figure S2. The GO_enrichment analysis of the commonly changed cellular genes by hit compounds. A, B. The GO_enrichment (Cellular Component module) analysis of the commonly changed cellular genes by hit compounds in different subtypes of TNBC cell lines.

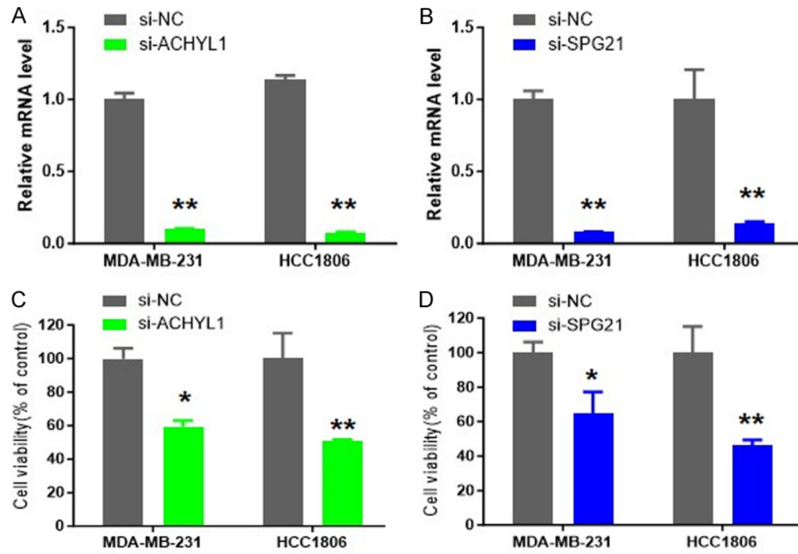

Figure S3. AHCYL1 and SPG21 are required for TNBC cell survival. A, B. MDA-MB-231 or HCC1806 cells were transfected with either AHCYL1-siRNA, SPG21-siRNA or non-target control siRNA (si-NC) for 72 h, then gene transcription were quantified by using RT-qPCR. C, D. The cell proliferation was assessed using the WST-1 assays. Error bars represent S.D. for 3 independent experiments, * = P<0.05; ** = P<0.01 (*vs* the si-NC group).