Sinomenine restrains breast cancer cells proliferation, migration and invasion via modulation of miR-29/PDCD-4 axis

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ABSTRACT
Sinomenine (Sino) is diffusely applied in heal rheumatoid arthritis and neuralgia. Howbeit, the activities of Sino in breast cancer cells remain confused. The research attempted to probe the anti-tumor function of Sino in breast cancer cells and divulge the feasible molecular mechanism. Sino at the 1–16 μM concentrations was exploited for the exposure of MDA-MB-231 or MCF7 cells, and cell growth, migration, invasion, cell cycle-relevant and apoptosis-correlative factors were estimated. Micro RNA (miR)-29 expression was evaluated via enforcing qRT-PCR, and the actions of miR-29 in MDA-MB-231 cells growth, migration and invasion were appraised after the overexpressed or suppressed vectors transfection. The functions of PDCD-4 in JNK and MEK/ERK pathways were estimated by employing western blot. We found that, Sino exposure impeded cell proliferation, provoked cell apoptosis and barricaded cell migration and invasion in MDA-MB-231 and MCF7 cells. Enhancement of miR-29 was observed in Sino-managed cells, and miR-29 overexpression further potentiated the activities of Sino in MDA-MB-231 cells. Additionally, Sino remarkably enhanced PCDC-4 expression via adjusting miR-29 in MDA-MB-231 cells. Beyond that, overexpressed PCDC-4 obstructed JNK and MEK/ERK pathways in MDA-MB-231 cells. Taken together, the explorations unveiled that Sino restrained MDA-MB-231 cells proliferation, migration, invasion, and provoked apoptosis through modulation of miR-29/PDCD-4 axis.

HIGHLIGHT
1. Sino inhibits MDA-MB-231 and MCF7 cells proliferation and provokes apoptosis;
2. Sino restrains MDA-MB-231 and MCF7 cells migration and invasion;
3. Sino ascends miR-29 expression in MDA-MB-231 and MCF7 cells;
4. Sino regulates cell growth, migration and invasion via modulating miR-29;
5. Sino up-regulates PDCD-4 expression through mediating miR-29;
6. PDCD-4 obstructs JNK and MEK/ERK pathways in MDA-MB-231 cells.

Introduction
Breast cancer is a high incidence destructive tumor in female, is also the second leading cause of death among women [1]. Like other cancers, breast cancer is caused by the interaction of multi-factors and genes, and closely related to the familial inheritance [2,3]. To date, the main management of breast cancer is surgery, which usually followed by chemotherapy or radiation therapy [4,5]. However, the five-year survival rate of breast cancer still remains dissatisfactory because of the poor prognosis, and the cancer cells diffusion [6]. The metastatic mechanisms of breast cancer are complex, and it is still a huge challenge for the clinical remedy of breast cancer. Thus, looking for a new strategy to reduce the mortality of breast cancer is extremely necessary.

Recently, the benefits of traditional Chinese medicine (TCM) in the comprehensive therapy of breast cancer have been attracted the attention of scholars. Increasing evidences have demonstrated that TCM treatment can effectively reduce postoperative complications of cancer, promote the recovery of postoperatively, and prevent recurrence and metastasis [7,8]. Additionally, the importance roles of TCM in cancer cells proliferation, metastasis and apoptosis have been extensively reported [9,10]. In breast cancer, Chen et al. declared that Curcumin restrained breast cancer cells proliferation by mediation of Nrf2 and Fen1 expression [11]. A diverting research from Liang et al. announced that Raddeanoside R13 could effectively prohibit breast cancer cell proliferation, invasion, and provoked apoptosis through modulation of miR-29/PDCD-4 axis [12].

Sinomenine (Sino, C_{19}H_{23}NO_{4}, M: 329.38), a kind of isoquinoline, is leached from the roots and stems of stems of Sinomenium actum Rehd.et wils. [13]. The proverbially pathological properties of Sino, such as anti-inflammatory and...
anti-tumor have been testified in the disparate ailments [14,15]. Recent studies have demonstrated that Sino could impede cell proliferation and trigger cell apoptosis in colon cancer cells [16], prostate cancer [17] and gastric cancer [18]. In addition, Sino prohibited lung cancer migration and invasion has also been confirmed [19]. Howbeit, it is still lack evidences for clarifying the functions of Sino in breast cancer cells.

Herein, the present research attempted to delve the anti-tumor activity of Sino in breast cancer cells, in the meantime divulge the feasible molecular mechanisms. The explorations might furnish a theoretical foundation for the remedy of breast cancer and innovative perspective for clinical therapy.

Materials and methods

Cell culture and stimulation

MDA-MB-231 (ATCC® HTB-26™) and MCF7 cells were respectively attained from American Type Culture Collection (ATCC, Rockville, MD, USA) and Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 cells were trained in Leibovitz’s L-15 medium (ATCC® 30–2008™) containing 10% FBS (GIBCO, CA, USA) with 100% air. Additionally, MCF7 cells were cultured in MEM (#41500034, GIBCO), and 10% FBS was replenished into above media, and was co-cultured with MCF7 cells at 37°C in a 5% CO2 incubator.

Sino gained from Sigma-Aldrich, which was dissolved in dimethylsulfoxide (DMSO), and watered down the diverse dosages for utilizing this study. MDA-MB-231 and MCF7 cells were then managed with Sino for 24 h at a series of concentrations, respectively.

Cell transfection

MiR-29 mimic, inhibitor and associative control were compounded by GenePharma Co. (Shanghai, China). The entire length of PDCD-4 and short-hairpin RNA (shRNA) directed against PDCD-4 (sh-PDCD-4) were concatenated into the pcDNA3.1 and U6/GFP/Neo plasmids (GenePharma), respectively. All above-involved recombination plasmids were transfected into MDA-MB-231 or MCF7 cells via implementing Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) pursuant to the explanatory memorandum.

Cell viability

After Sino (0, 1, 2, 4, 8 and 16 μM) stimulation, a Cell Counting Kit-8 (CCK-8, Dojindo, Gaithersburg, MD) experiment was worked out for the estimation of cell viability. The managed MDA-MB-231 and MCF7 cells were inoculated in a 96-well plate, and then CCK-8 solution (10 μL) was supplied into the culture medium and co-cultivated for 1 h at 37°C. A Microplate Reader (Bio-Rad, Hercules, CA, USA) instrument was applied for the evaluation of the absorbance at 450 nm.

Cell proliferation

After management with 4 μM Sino for 24 h, 10 μM bromodeoxyuridine (BrdU) was supplemented into the culture plate, in the meantime co-fostered with MDA-MB-231 or MCF7 cells for 4 h at 37°C. Afterward, the above-mentioned stimulated cells were rinsed 3 times with PBS (Sigma-Aldrich), meanwhile fastened with methyl alcohol for 10 min. MDA-MB-231 or MCF7 cells were then co-cultivated with a suited anti-BrdU antibody (ab1893, Abcam, Cambridge, UK, dilution of 1:1000) overnight at surrounding temperature. The BrdU positive cells were ultimately counted via adopting a microscope (Olympus, Tokyo, Japan).

Cell apoptosis

The Annexin V-Phycoerythrin (PE) Apoptosis Detection Kit (Beyotime, Shanghai, China) was executed for the measurement cell apoptosis pursuant to the manufacturer’s recommendations. After manipulation with Sino (4 μM) for 24 h, MDA-MB-231 or MCF7 cells were rinsed 2 times with PBS (Sigma-Aldrich), and subsequently reacted with 5 μL Annexin V-PE for 30 min without of light at indoor temperature. Flow cytometry facility (Beckman Coulter, Brea, California) was executed for the evaluation of the percentage of apoptotic cells.

Cell migration and invasion

After stimulation with 4 μM Sino for 24 h, the migratory and invasive capacities of MDA-MB-231 or MCF7 cells were estimated through executing 24-well Transwell chamber (8 μM pore size, Becton Dickinson, Mountain View, CA, USA). The managed MDA-MB-231 or MCF7 cells were suspended in 200 μL serum-free medium and were supplied into the upper compartment. The lower compartment was synchronously suffused with 600 μL of complete medium. After incubation, the methanol (Sigma-Aldrich) was employed for the immobilization of MDA-MB-231 cells or MCF7 cells for 30 min. The non-traversed cells were then cleaned up with a drippy cotton swab, and the traversed cells were dyed with 0.1% crystal violet (Merck, Darmstadt, Germany) for 20 min, and calculated through applying a microscope (Leica Microsystems, Wetzlar, Germany). For the assessment of cell invasion, the inserts were wrapped with BD MatrigelTM Matrix (BD Biosciences). The residual experimental steps were executed same as the cell migration assay.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The Trizol reagent (Life Technologies Corporation) was exploited for isolating the total RNA from MDA-MB-231 or MCF7 cells after management with Sino at 1–4 μM or MDA-MB-231 cells transfection with miR-29 mimic/inhibitor. The MultiScribe RT kit (ABI, Foster City, California) was executed for compounding the cDNA. The Taqman Universal Master Mix II (ABI) was exploited for qRT-PCR analysis to determine miR-29 expression. U6 was regarded as a reference gene for
examining miR-29 expression. The correlative data were reckoned by the $2^{-\Delta\Delta CT}$ method [20].

**Western blot**

The total proteins from MDA-MB-231 or MCF7 cells with 4 μM Sino management were elicited through exploiting RIPA (Beyotime) lysis buffer. The BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA) was executed for quantifying the protein concentrations. Subsequently, 25 μg protein samples were loaded into 10% SDS-PAGE and then transfected to PVDF membranes, which were next co-fostered with the extraordinary primary antibodies at 4°C overnight. The utilized primary antibodies included PCNA (ab18197), CyclinD1 (ab134175), CDK4, (ab199728), p16 (ab118457), pro-Caspase-3 (ab32499), cleaved-Caspase-3 (ab2302), pro-Caspase-9 (ab138412), cleaved-Caspase-9 (ab2324), PDCD-4 (ab80590), t-JNK (ab199380), p-JNK (ab47337), t-MEK (ab32091), p-MEK (ab138662), t-ERK (ab32537), p-ERK (ab131438), and β-actin (ab16039, Abcam). After washing with PBS, the appropriate second antibody (ab205718, 1:2000, Abcam) was hatched (ab16039, Abcam). After washing with PBS, the appropriate second antibody (ab205718, 1:2000, Abcam) was hatched with the PVDF membranes for another 1 h at indoor temperature. The ECL reagent (Pierce, IL, USA) was utilized for capturing the chemiluminescence signals, and the blot images were obtained via adopting Image Lab™ Software (Bio-Rad).

**Statistical analysis**

The statistical analyses were figured out via employing Graphpad statistical software (San Diego, California, USA), which were emerged as mean ± SD. The common statistical analysis methods of Student t-test and ANOVA were adopted for reckoning the $p$ values. A $p$ values of <.05 was passed for a discrepant consequence.

**Results**

**Sino suppressed breast cancer cells proliferation and induced apoptosis**

Sino is an alkaloid monomer, its chemical structure was presented in Figure 1. To uncover the functions of Sino in breast cancer cells, MDA-MB-231 and MCF7 cells were firstly stimulated with diverse dosages of Sino (0, 1, 2, 4, 8 and 16 μM). CCK-8 experiment was subsequently utilized for the assessment of cell viability. We observed that cell viability was memorably restrained in MDA-MB-231 cells after manipulation with an increasing dosage of Sino (1 and 2 μM, $p < .05$; 4 and 8 μM, $p < .01$; 16 μM, $p < .001$, Figure 2(A)). The 4 μM Sino was taken as a befitting dosage for the further evaluation of cell proliferation and apoptosis. Figure 2(B) divulged that the positive BrdU cells were decreased by Sino management ($p < .01$). Meanwhile, the repressions of PCNA, CyclinD1, CDK4 ($p < .01$ or $p < .001$) were observed after Sino management, and the elevation of p16 was also discovered after Sino stimulation ($p < .001$, Figure 2(C) and 2(D)). The apoptotic experiment results disclosed that Sino dramatically induced cell apoptosis, and raised cleaved-Caspase-3/-9 expression ($p < .001$, Figure 2(E,F)). Likewise, the similarly results were emerged in MCF7 cells (Supplementary Figure S1A–1F). All the observations corroborated that Sino hindered breast cancer cells proliferation and evoked apoptosis.

**Sino suppressed breast cancer cells migration and invasion**

Transwell experiment was carried out for the assessment of cell migratory and invasive activities of MDA-MB-231 or MCF7 cells after management with Sino (4 μM). We observed that Sino administration markedly repressed the percentage of migrated cells, similarly suppressed the ability of cell invasion in MDA-MB-231 cells ($p < .05$, Figure 3(A,B) or MCF7 cells ($p < .01$, Supplementary Figure S2A and 2B) as contrasted to the corresponding controls. The outcomes declared that Sino could affect the migratory and invasive capacities of breast cancer cells.

**Elevation of miR-29 evoked by Sino was emerged in breast cancer cells**

The qRT-PCR experiment was implemented for the measurement of miR-29 expression in MDA-MB-231 or MCF7 cells after manipulation with Sino. The result showed that enhancement of miR-29 was observed after Sino management at the dosages of 2 ($p < .05$) and 4 μM in MDA-MB-231 cells ($p < .001$, Figure 4) or in MCF7 cells (Supplementary Figure S3). These verdicts imparted that miR-29 might join in influencing the functions of Sino in breast cancer cells growth, migration and invasion.

**Sino restrained the biological processes of MDA-MB-231 cells through modulation of miR-29**

We next further probed the influences of miR-29 in the biological processes of MDA-MB-231 cells after management with Sino. The qRT-PCR experiment displayed that the enhancement of miR-29 ($p < .01$) was observed after miR-29 mimic transfection, meanwhile the repression of miR-29 ($p < .001$) was observed after miR-29 inhibitor transfection compared (Figure 5(A)). The percentage of BrdU positive cells was further repressed in miR-29 mimic transfected cells after Sino.
management compared with Sino stimulation alone \((p < .01, \text{Figure 5(B)})\). Additionally, overexpressed miR-29 declined PCNA, CyclinD1 and CDK4 expression \((p < .05 \text{ or } p < .01)\), but upgraded p16 expression in Sino-stimulated cells \((p < .001, \text{Figure 5(C,D)})\). Outside of this, compared with Sino management alone, miR-29 overexpression and Sino manipulation...
triggered cell apoptosis, and further enhanced cleaved-Caspase-3/9 expression ($p < .001$, Figure 5(E,F)). Furthermore, the migratory and invasive abilities of MDA-MB-231 cells were also restrained in miR-29 overexpression-transfected and Sino-stimulated cells compared with in Sino-stimulated cells alone ($p < .01$, Figure 5(G–H)). All these above-mentioned consequences were both inverted by the suppression of miR-29 in MDA-MB-231 cells (Figure 5(B–H)). Taken together, these results suggested that Sino affected the biological processes of MDA-MB-231 cells through ascending miR-29 expression.

**PDCD-4 obstructed JNK and MEK/ERK pathways in MDA-MB-231 cells**

Finally, we investigated the effect of PDCD-4 on JNK and MEK/ERK pathways. Firstly, the elevation of PDCD-4 was discovered in MDA-MB-231 cells after management with Sino ($p < .05$), the accelerative action of Sino in PDCD-4 expression was further upgraded by overexpressed miR-29, and abated by repressed miR-29 ($p < .05$, Figure 6(A)), indicating that Sino increased PDCD-4 expression might via mediation of miR-29 expression. Then, the expression plasmids of pc-PDCD-4, sh-PDCD-4 and the corresponding controls were transfected into MDA-MB-231 cells to alter PDCD-4 expression. As shown in Figure 6(B), PDCD-4 expression was notably upgraded by overexpression of PDCD-4, and overexpressed miR-29 further ascended PDCD-4 expression in Sino-managed cells. Finally, the results showed that overexpressed PDCD-4 obstructed JNK and MEK/ERK pathways in MDA-MB-231 cells.

**Discussion**

The diverting results from the present research imparted that Sino prohibited cell proliferation, migration, invasion simultaneously triggered cell apoptosis in MDA-MB-231 and MCF7 cells. Additionally, we observed that the expression level of miR-29 was increased in MDA-MB-231 and MCF7 cells after manipulation with Sino, and overexpressed miR-29 further enhanced the anti-tumor activity of Sino in MDA-MB-231 cells. Besides, we found that Sino elevated PDCD-4 expression in MDA-MB-231 cells, and overexpressed miR-29 further ascended PDCD-4 expression in Sino-managed cells. Finally, the results showed that overexpressed PDCD-4 obstructed JNK and MEK/ERK pathways in MDA-MB-231 cells.

For a long time, Sino is considered as a specific agent for healing rheumatic and arthritic ailments because of the anti-inflammatory and immunosuppressive effects [21]. Recently, increasing studies have demonstrated that Sino exhibited anti-tumor capacity in multitudinous cancer cells, encompassing breast cancer [22,23]. It is reported that Sino could restrain MDA-MB-231 and 4T1 cells migration and invasion [24]. Another study demonstrated that Sino suppressed breast cancer cells growth and metastasis via suppressing the SHh signaling pathway [25]. Similar with these researches, our study found that Sino significantly impeded MDA-MB-231 and MCF7 cells proliferation, migration, invasion, and induced apoptosis, which indicated that Sino might emerge the anti-tumor ability in breast cancer cells.

Mounting evidences have demonstrated the importance of miRNAs in breast cancer, embracing miR-155, miR-21 and miR-10b, which are implicated in affecting cell growth and metastasis [26,27]. MiR-29 is an important regulator, that plays diverse roles in the dissimilar cancers [28]. Wu et al. hinted that miR-29b functioned as a tumor suppressor in breast cancer cells by down-regulation of B-Myb [29]. Liu et al. announced that miR-29b accelerated breast cancer cells cell growth and metastasis [30]. Nonetheless, whether miR-29 affected the biological processes of breast cancer cells under Sino administration remains vague. Our research showed an interesting result that miR-29 expression level was up-regulated in Sino-treated cells, as well as overexpression of miR-29 exhibited an inhibitory function in MDA-MB-231 cells growth, migration and invasion after management with Sino. All above data disclosed that miR-29 might be a vital mediator for participating in the modulation of the anti-tumor action of Sino in breast cancer cells.

PDCD-4 is a newly discovered tumor suppressor gene in recent years, which can inhibit tumor cells growth through regulation of the transcription and translation of proteins [31]. Recent literatures have proven that abnormally expressed PDCD-4 is linked to the development of breast cancer, and presents an indispensable role in breast cancer cells growth and invasion [32,33]. Study from Huang et al. discovered the elevation of PDCD-4 in MDA-MB-231 cells, which was triggered by resveratrol (RSV) through repression of AKT signaling [34]. Partly similar with the study, our consequences demonstrated that Sino upgraded PDCD-4 expression in MDA-MB-231 cells, in the meantime overexpressed miR-29 further ascended the accelerative functions of Sino in PDCD-4 expression.

To further uncover the molecular mechanism, the interrelated overexpressed or suppressed expression plasmids were applied, and the regulatory effect of PDCD-4 on JNK and MEK/ERK pathways was delved. As important MAPK
pathways, JNK and MEK/ERK pathways have been proverbially reported to join in modulating the momentous biological processes in diverse cancers, comprising breast cancer [35,36]. Evidence from Wen et al. reported that activation or inactivation of JNK and MEK/ERK pathways took part in mediating breast cancer cells apoptosis and invasion [35]. In our study, we found that PDCD-4 remarkably abated JNK and MEK/ERK pathways in MDA-MB-231 cells, which might be
linked to the anti-tumor activity of Sino. Further experiments are still necessary for the confirmation of this hypothesis in the future.

Taken together, these investigations delineated that Sino prohibited breast cancer cells cell proliferation, migration, invasion, and aggrandized apoptosis via adjusting miR-29/PDCD-4 axis. Moreover, JNK and MEK/ERK pathways were barricaded by PDCD-4 in breast cancer cells. These findings corroborated that Sino exhibited anti-tumor effects on breast cancer cells, and might supply an innovative perspective for the clinical therapy of breast cancer.

**Disclosure statement**
Authors declare that there is no conflict of interests.

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**Author contributions**
Conceived and designed the experiments: Guanglei Gao and Wenyan Ma; Performed the experiments and analyzed the data: Guanglei Gao, Xiaolin Liang and Wenyan Ma; Contributed reagents/materials/analysis tools: Xiaolin Liang; Wrote the manuscript: Guanglei Gao and Wenyan Ma.

**Data availability statement**
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Figure 6.** Impacts of PDCD-4 on JNK and MEK/ERK pathways in MDA-MB-231 cells. (A) PDCD-4 expression in Sino-managed and miR-29 mimic/inhibitor transfected MDA-MB-231 cells was evaluated through applying western blot and qRT-PCR experiments. The pc-PDCD-4, sh-PDCD-4 and the correlative controls were transfected into MDA-MB-231 cells, (B) PDCD-4 expression was measured by western blot ans qRT-PCR experiments; (C) p/t-JNK, and (D) p/t-MEK and p/t-ERK protein levels were then appraised via adopting western blot experiment. *p < .05; **p < .01; ***p < .001.
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