Original Article

Sinensetin suppresses breast cancer cell progression via Wnt/β-catenin pathway inhibition

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Background: Although there are many treatments for breast cancer, such as surgery, radiotherapy, chemotherapy, estrogen receptor antagonists, immune checkpoint inhibitors and so on. However, safer and more effective therapeutic drugs for breast cancer are needed. Sinensetin, a safer therapeutic drugs, come from citrus species and medicinal plants used in traditional medicine, while its role and underlying mechanism in breast cancer remain unclear. Our study aimed to investigate the role and mechanism of sinensetin in breast cancer.

Methods: Cell Counting Kit-8 (CCK-8) was used to determine the safe concentration of sinensetin in MCF-10A, MCF7 and MDA-MB-231 cells; 120 μM sinensetin was used in subsequent experiments. Real time polymerase chain reaction (RT-PCR), Western blotting, Terminal Deoxynucleotidyl Transferase mediated dUTP Nick-End Labeling (TUNEL) apoptosis assay, Transwell invasion assay and Clone formation assay were used in this study to determine cell viability, mRNA expression, protein levels, apoptosis, proliferation, invasion and so on.

Results: Herein, our results showed that 120 μM sinensetin suppressed the cell viability and promoted apoptosis of MCF7 and MDA-MB-231 cells. Treatment with 120 μM sinensetin for 24 h showed no significant toxicity to normal mammary cells; 120 μM sinensetin decreased cell proliferation, invasion, and epithelial-mesenchymal transition (EMT), and downregulated β-catenin, lymphatic enhancing factor 1 (LEF1), T-cell factor (TCF) 1/TCF7, and TCF3/TCF7L1 expression in MCF7 and MDA-MB-231 cells. The Wnt agonist SKL2001 reversed the inhibitory effect of sinensetin on cell survival, metastasis, and EMT. Sinensetin-induced downregulation of β-catenin, LEF1, and TCF1/TCF7 expression were upregulated by SKL2001 in MCF7 and MDA-MB-231 cells.

Conclusions: In summary, sinensetin suppressed the metastasis of breast cancer cell via inhibition of Wnt/β-catenin pathway and there were no adverse effects on normal breast cells. Our study confirmed the role of sinensetin in breast cancer cells and provided a better understanding of the underlying mechanism.

Keywords: Breast cancer; sinensetin; epithelial-mesenchymal transition (EMT); apoptosis; Wnt

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Introduction

Compounds obtained from natural products have clinical potential as therapeutic agents. Over the past 60 years, almost 70% of the novel anti-cancer drugs derived from natural products or based on data obtained from natural products were approved (1). A large number of natural product drugs have been approved, including paclitaxel, homoharringtonine, ingenol mebutate and so on (2). With the application of traditional biological activity-guided separation strategies and advanced analytical techniques in the discovery of drugs from natural products, the discovery capabilities of modern drugs derived from natural products have been enhanced (2). These preliminary foundations and advances provide broad prospects for the application of natural products in tumors.

In recent years, a new research direction emerged in the study of flavonoids as anticancer drugs after the approval of alvocidib by the Food and Drug Administration (FDA) for the treatment of acute myeloid leukemia in 2014 (3-6). Flavonoids are widely present in the plant kingdom, and epidemiological studies have shown that dietary flavonoids have a chemopreventive effect on cancer (7-9). Polymethoxyflavones (PMFs) are flavonoids that are replaced by two or more methoxy groups. Studies of diverse medicinal plants and citrus have shown a high structural variability of PMF, such as the smaller methoxyflavones and structural isomers. The structural isomers include 5,6,7,4'-tetramethoxyflavone, tangeretin, 3,5,6,7,3',4'-hexamethoxyflavone, nobiletin, 3,5,6,7,8,3',4'-he ptamethoxyflavone, and sinensetin. While several studies indicate that composition and structure affected profoundly the antitumor capacity of flavonoids, few studies have been published on the role of these little-known compounds (7). 5,6,7,4'-tetramethoxyflavone represses the growth of triple-negative breast cancer (TNBC) by inhibiting the MAPK and Akt signaling pathways and causing cell cycle arrest (10). Nobiletin also inhibits TNBC (11). Targretin inhibits human breast carcinoma cells via a variety of mechanism (12). 3,5,6,7,3',4'-hexamethoxyflavone and 3,5,6,7,8,3',4'-heptame thoxyflavone also play an important role in breast cancer (10). Sinensetin showed strong sensitization in HepG2 cells, which caused the cell viability curve to decrease sharply (13-15). The current study suggests that if more toxicological and *in vivo* studies are conducted, sinensetin have the potential to become a candidate for the treatment of gallbladder adenocarcinoma (16). Sinensetin also targets cancer stem cells (CSCs) and enhances the anti-proliferative effect of 5-fluorouracil in a 3D cell model of colorectal cancer (17). A recent study reported that sinensetin ability to resist proliferation was enhanced by CYP1 family enzymes. These flavonoids are superior to polyhydroxylated flavonoids because they possess high metabolic stability and oral bioavailability, which increases their chemopreventive activity in cancer (18). However, the role and underlying mechanism of sinensetin in breast cancer remain unknown.

Breast cancer is one of the most common tumors in the world. The pathogenesis of breast cancer is very complicated. Many signal transduction pathways are involved (19). Cyclin-dependent kinases (CDKs) promote breast cancer cell growth by regulating breast cancer cell cycle. In breast cancer, CDKs are overactive or CDK inhibitory proteins are dysfunctional (20). In addition, Notch signaling, SHH signaling, BRK pathway, HER signaling and others pathways are reported to be involved in the occurrence and development of breast cancer (21). Wnt signaling has been shown to be active in several tumors, including breast cancer (22). The canonical Wnt protein activates transcription of β-catenin/T-cell factor (TCF)/lymphatic enhancing factor (LEF) target genes by transducing signaling β-catenin (23,24). Some researchers have demonstrated that Wnt antagonists with frequent genetic and epigenetic mutations in breast cancer led to β-catenin-mediated activation of Wnt target genes, suggesting that the Wnt-catenin-β1 (CTNNB1) signaling pathway plays an important role in the oncogenic process (25,26). Similarly, it has been reported in breast cancer that several cytosolic and nucleus components of the Wnt/β-catenin pathway is deregulated, such as DKK3, Dickkopf (DKK)-1, APC, Wnt inhibitor factor 1 (WIF1), SFRP5, MCC, glycogen synthase kinase-3β (GSK3B),

Highlight box

Key findings

- Sinensetin inhibits breast cancer cell survival, metastasis, and epithelial-mesenchymal transition via inhibiting Wnt/β-catenin pathway.

What is known and what is new?

- Researches on the antitumor activity of polymethoxyflavones (PMFs) have mostly focused on nobiletin. 5,6,7,8,3,4'-hexacycloeth oxyflavones damage several cancer cell activities *in vitro* and *in vivo*.
- This study investigated whether sinensetin suppressed the breast cancer cell progression via inhibition of Wnt/β-catenin pathway.

What is the implication, and what should change now?

- Importantly, sinensetin inhibited the viability of breast cancer cells and promoted their apoptosis through inhibiting the Wnt/β-catenin signaling pathway.

and CTNNBIP1 (27-29). Activation of the Wnt/β-catenin signaling pathway is required for epithelial-mesenchymal transition (EMT) to regulate breast cancer drug resistance (30). The Wnt signaling pathway is an important molecular node that maintains CSC properties and promotes epithelial-mesenchymal transformation cell programming (31). Therefore, key regulators targeting the Wnt pathway can be used as effective targets for breast cancer chemotherapy and as effective biomarkers for the prognosis of breast cancer patients.

In this study, we aimed to explore the potential anticancer role of sinensetin in breast cancer cells. We found that sinensetin inhibited the viability of breast cancer cells and promoted their apoptosis through inhibiting the Wnt/β-catenin signaling pathway. Our study was the first to demonstrate that sinensetin suppressed the malignant progression of breast cancer via inhibition of Wnt/β-catenin signaling. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1317/rc).

Methods

Cell culture

The MCF-10A (cat. no. CRL-10317, Manassas, Virginia, USA), MCF7 (cat. no. CL-0149, Manassas) and MDA-MB-231 (cat. no. CL-0150B, Manassas) cell lines were obtained from the American Type Culture Collection through commercial channels. MCF7 cells were cultured in DMEM (PM150210, Procell, Wuhan, China) containing 10% fetal bovine serum (FBS) (Procell), penicillin (50 U/mL, 10378016, Gibco, Waltham, MA, USA), and streptomycin (50 μg/mL, 10378016, Gibco) in 5% CO_2. MDA-MB-231 cells were cultured in F15 medium (PM150110, Procell) containing 15% FBS, penicillin (50 U/mL), and streptomycin (50 μg/mL) in 5% CO_2. MCF7 and MDA-MB-231 cells were cultured in sinensetin (HY-N0297, MedChemExpress, New Jersey, USA) at different concentrations (0, 30, 60, 120, 180, and 240 μM) for 24 h. Cells were treated with SKL2001 (SKL101085, MedChemExpress) for one hour before the exposure of sinensetin according to the previous study (32). The concentration of 40 μM SKL2001 was in accordance with the previous study (33).

Cell viability assay

Cell viability was analyzed according to the manufacturer's protocol using the Cell Counting Kit-8 (CCK-8) assay (C0037, Beyotime Biotechnology, Shanghai, China). Cells were seeded at a density of 5×10^3 cells/well in 96-well plates for the CCK-8 assay. After sinensetin treatment for 24 h, cells were incubated with 10 μL CCK-8 solution for 1 h at 5% CO_2 and 37 ℃ incubator. Data from the optical density (OD) at 450 nm were measured by a microplate reader (BioTek, Winooski, VT, USA). The calculating methodology was as follow: Cell viability (%) = (measured value -blank value)/(control value – blank value) × 100%. MCF7 and MDA-MB-231 cells treated with 0 μM sinensetin acted as control. MCF-10A cells treated with 120 μM sinensetin for 0 h also served as control. CCK-8 results were obtained from more than three replicate experiments performed by two operators under the same laboratory conditions. The same reagents and instrument systems were used for all experiments. All methods have mentioned the control in each experiment.

Western blotting

MCF-10A, MCF7 and MDA-MB-231 cells were lysed with radio immunoprecipitation assay (RIPA) (P0013B, Beyotime Biotechnology) containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), sodium orthovanadate, sodium fluoride, ethylene diamine tetraacetic acid (EDTA), leupeptin, phenylmethanesulfonyl fluoride (PMSF) and so on. After full lysis, the cells were centrifuged at 10,000–14,000 g for 3–5 min and the supernatant was collected. Cells without any substance acted as control. Total proteins were extracted from MCF-10A, MCF7 and MDA-MB-231 cells treated as described above. Aliquots containing 30 μg of proteins were electrophoresed on 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene fluoride (PVDF) membranes (1620256, Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% non-fat milk for 2 h, and then incubated with primary antibodies against caspase 9 (9509, CST, Danvers, MA, USA), caspase 3 (9662, CST), Bax (50599-2-Ig, Proteintech, Chicago, USA), E-cadherin (20874-1-AP, Proteintech), Bcl-2 (26593-1-AP, Proteintech), Slug (9585, CST), Snail (13099-1-AP, Proteintech), Vimentin (10366-1-AP, Proteintech), Twist (25465-1-AP, Proteintech), Zeb1 (21544-1-AP, Proteintech), Zeb2 (14026-1-AP, Proteintech), E47 (12258, CST), β-catenin (8480, CST, USA), LEF1 (14972-1-AP, Proteintech), TCF1/TCF7 (14464-1-AP, Proteintech) and

Table 1 Primers used for qPCR

Gene	Forward 5'-3'	Reverse 5'-3'
E-cadherin	CTCATGAGTGTCCCCCGGTAT	CAGCCGCTTTCAGATTTTCATC
Vimentin	GACGCCATCAACACCGAGTT	CTTTGTCGTTGGTTAGCTGGT
Snail	TCGGAAGCCTAACTACAGCGA	AGATGAGCATTGGCAGCGAG
Slug	CGAACTGGACACACATACAGTG	CTGAGGATCTCTGGTTGTGGT
twist	GCCTAGAGTTGCCGACTTATG	TGCGTTTCCTGTTAAGGTAGC
Zeb1	TGATCTGGCCATTTTCACCTGT	GACTTGCCAGGACAGCTTGC
Zeb2	CACAGGTATGAGTGACTTTGCC	TGGCTGTGTCATGCCATTTC
E47	CCACTT CACTG AGTCGC ACAG	GTCTCT CCCGAA GGAGG CATA
β-catenin	ATGTCCAGCGTTTGGCTGAA	TGGTCCTCGTCATTTAGCAGTT
LEF-1	AATGCACGTGAAGCCT	GAATCTGGTTGATAGCTGC
TCF-1	TTGATGCTAGGTTCTGGTGTACC	CCTTGGACTCTGCTTGTGTC
TCF-3	GTAACATCTGCTCCGCGTTAAT	TCGAGGGAGACCCAACTTCA
GAPDH	AACAGGAGGTCCCTACTCCC	GCCATTTTGCGGTGGAAATG

qPCR, quantitative polymerase chain reaction.

β-actin (81115-1-RR, Proteintech). Membranes were then incubated in secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit IgG antibody (A0216 and A0208, Beyotime Biotechnology). Protein expressions from Western blotting images were quantitatively analyzed by image J (Version 1.52v). ChemiDocTM Touch Imaging System (Bio-Rad) was used to image and chemiluminescence detection (34080; Thermo Fisher Scientific, Waltham, MA, USA and 1705061; Bio-Rad). Western blotting results were obtained from more than three replicate experiments performed by two operators under the same laboratory conditions. The same reagents and instrument systems were used for all experiments. All methods have mentioned the control in each experiment.

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

Total RNA was extracted according to the manufacturer's instructions using Trizol (R0016, Beyotime Biotechnology). Trizol volume was added to the cells at a ratio of 10 cm^2/mL. They were allowed to dissociate fully at room temperature for 5 min. After collecting the lysate, the precipitate was discarded by centrifugation at 12,000 rpm for 5 min. Chloroform was added at 200 μL (chloroform)/mL (Trizol), mixed with shaking and left for 15 min at room temperature. The cells were centrifuged at 4 ℃ and 12,000 g for 15 min. Drain the upper aqueous phase into another centrifuge tube. Isopropanol was added and mixed, and the mixture was placed at room temperature for 10 min. We centrifuged the mixture at 12,000 g for 10 min at 4 ℃, and the supernatant was discarded; 75% ethanol was added at 1 mL of 75% (ethanol)/mL (Trizol), the centrifuge tube was gently shaken, and the precipitate was suspended. The samples were centrifuged at 4 ℃ 8,000 g for 5 min, and the supernatant was discarded as much as possible. Allow to dry at room temperature for 5–10 min. RNA samples were dissolved using pure water. The final measurement quantified RNA concentration. RNA was converted into cDNA using the one-step qRT-PCR SYBRGreen Kit (DBI, Ludwigshafen, Germany) and the ABI 7500 Fast Real-Time PCR System (Life Tech, Carlsbad, CA, USA). Cells without any substance acted as control. Primers are listed in *Table 1*. The $2^{-\Delta\Delta Ct}$ method was used to quantify gene expression normalized to GAPDH. RT-PCR results were obtained from more than three replicate experiments performed by two operators under the same laboratory conditions. The same reagents and instrument systems were used for all experiments. All methods have mentioned the control in each experiment.

Clone formation assay

Approximately 1,000 cells were seeded in 6-well plates and cultured in medium without sinensetin for the first

2 days, then in medium containing 120 μM sinensetin for the experimental group until 2 weeks at 5% CO_2 and 37 ℃ incubator. Cells without any substance acted as control. Briefly, cells were fixed in 4% paraformaldehyde (P0099, Beyotime Biotechnology) and then washed three times. Cells were cultured with 0.1% crystal violet (C0121, Beyotime Biotechnology) and washed with water. Photographs were taken to assess the relative number of clones. The results of clone formation assay were obtained from more than three replicate experiments performed by two operators under the same laboratory conditions. The same reagents and instrument systems were used for all experiments. All methods have mentioned the control in each experiment.

Transwell invasion assay

The experiment were carried out by 24-well Transwell plates (3422-ND, Corning, NY, USA) Matrigel® (C0372, Beyotime Biotechnology) with a membrane pore size of 8 μm. Approximately 1×10^5 MCF7 and MDA-MB-231 cells were cultured with serum-free medium in the upper chamber. Medium supplemented with 10% FBS and 120 μM sinensetin in the lower chamber served as the chemo-attractant. Cells above the Matrigel layer were swabbed after incubation for 24 h, and 4% paraformaldehyde were used to fix MCF7 and MDA-MB-231 cells below the membrane. Subsequently, cells were stained with 0.1% crystal violet for 15 min and photographs were taken to assess the relative number of invaded cells for each well. Cells without any substance acted as control. The results of Transwell invasion assay were obtained from more than three replicate experiments performed by two operators under the same laboratory conditions. The same reagents and instrument systems were used for all experiments. All methods have mentioned the control in each experiment.

TUNEL apoptosis assay

Cells were seeded into coverslips and exposed to various treatments. Briefly, cells were fixed with 4% paraformaldehyde, washed with phosphate buffered saline (PBS) (C0221A, Beyotime Biotechnology) twice for 3 min, and then treated with 0.3% Triton X-100 for 10 min. Aliquots of 50 μL TUNEL solution (C1090, Beyotime Biotechnology) were placed in coverslips and cells were cultured for 1 h. Coverslips were washed with tris buffered saline tween (TBST) (ST673, Beyotime Biotechnology) three times. Cells without any substance acted as control. Photographs were taken by microscopy (Olympus BX51). The results of TUNEL were obtained from more than three replicate experiments performed by two operators under the same laboratory conditions. The same reagents and instrument systems were used for all experiments. All methods have mentioned the control in each experiment.

Statistical analysis

Data were presented as the mean ± standard deviation, and statistical analyses were performed using SPSS 20.0 (SPSS, Chicago, IL, USA). The Student's test was used to calculate the differences between two groups. One-way analysis of variance (ANOVA) was used for comparison between groups, followed by Tukey multiple comparisons. All P values were two-sided, and ***, P<0.001, **, P<0.01, *, P<0.05 was considered to be statistically significant. All experiments were performed in triplicate.

Results

Sinensetin significantly inhibits breast cancer cell viability and promotes apoptosis of breast cancer cell

The CCK-8 assay was performed to investigate the inhibitory impact of sinensetin in MCF7 and MDA-MB-231 cells. As shown in *Figure 1A*, sinensetin showed a concentration-dependent inhibitory effect on MCF7 and MDA-MB-231 cell viability. The IC_{50} (half maximal inhibitory concentration) of sinensetin in MCF7 and MDA-MB-231 cells were 131.5 and 97.45 μM, respectively. Therefore, a concentration of 120 μM sinensetin was used to treat MCF7 and MDA-MB-231 cells in subsequent experiments. CCK-8 was used to assess the impact of sinensetin on normal breast cells (MCF-10A) before subsequent experiments. The results showed that 120 μM sinensetin began to suppress the activity of MCF-10A cells after 72 h. This slight toxicity after 72 h might be related to the purity of sinensetin. Importantly, treatment with 120 μM sinensetin for 24 h showed no significant toxicity to normal mammary cells (*Figure 1B*).

After treatment of MCF7 and MDA-MB-231 cells with 120 μM sinensetin for 24 h, the expression of apoptotic markers was detected by Western blotting. Sinensetin upregulated the protein level of cleaved-caspase 9 and cleaved-caspase 3 in MCF7 and MDA-MB-231 cells. The ratio of Bcl-2/Bax was also decreased after sinensetin treatments. Decreased ratio of Bcl-2/Bax indicated an

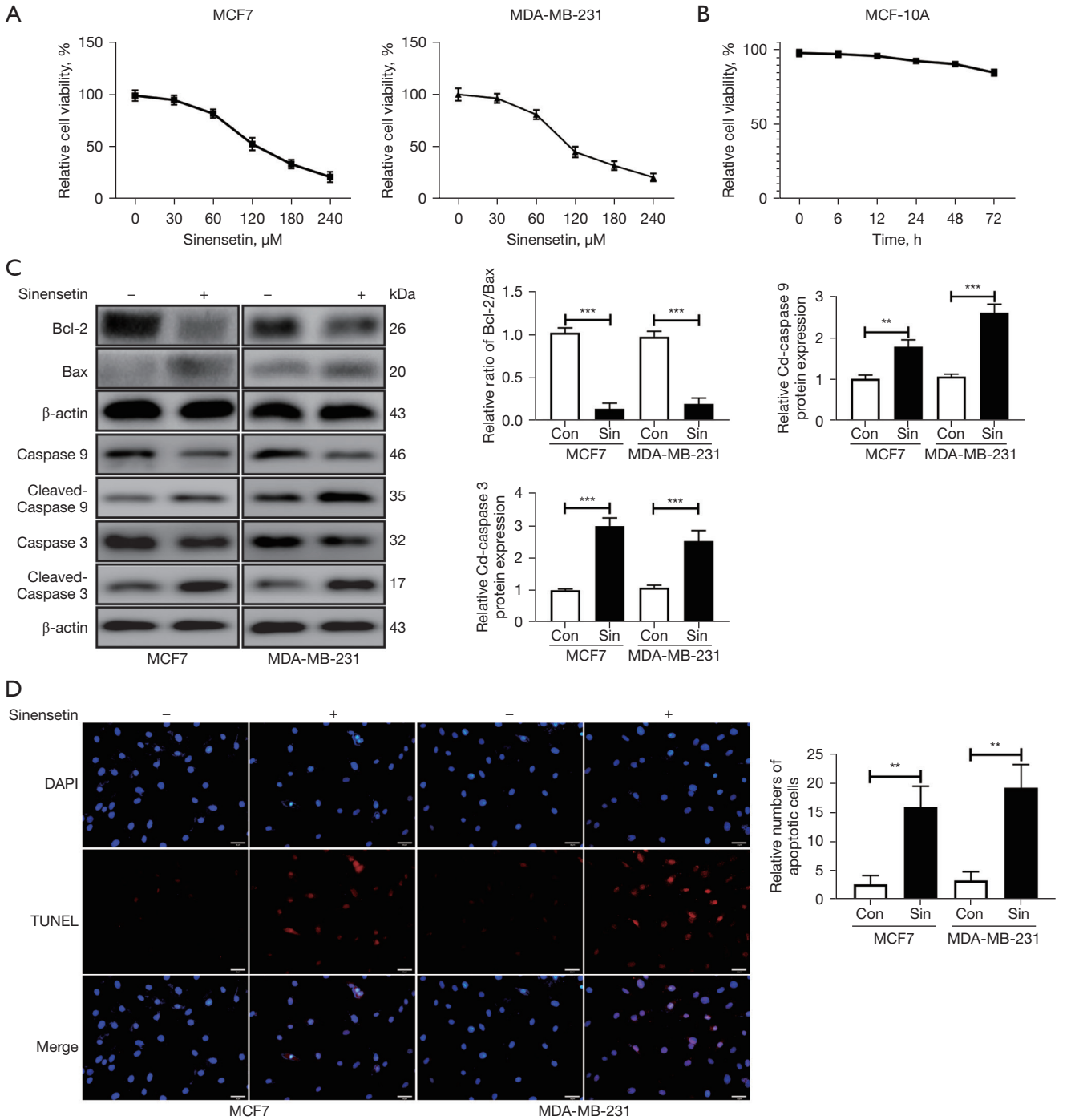

Figure 1 Sinensetin reduces breast cancer cell viability and increases breast cancer apoptosis. (A) MCF7 and MDA-MB-231 cell viability was measured by CCK-8 after sinensetin treatment. (B) MCF-10A cell viability was measured by CCK-8 after 120 μM sinensetin treatment. (C) Caspase 9, Caspase 3, Cleaved-caspase 9, Cleaved-caspase 3, Bcl-2, Bax and β-actin protein expression in MCF7 and MDA-MB-231 cells were determined by Western blotting. Quantitative analysis for Cleaved-caspase 9, Cleaved-caspase 3 and Bcl-2/Bax protein expression in MCF7 and MDA-MB-231 cells. (D) MCF7 and MDA-MB-231 cell apoptosis were measured by TUNEL assay. Scale bar =50 μm. Briefly, cells were fixed with 4% paraformaldehyde, washed with PBS twice for 3 min, and then treated with 0.3% Triton X-100 for 10 min. Aliquots of 50 μL TUNEL solution were placed in coverslips and cells were cultured for 1 h. Coverslips were washed with TBST three times. Cells without any substance acted as control. Quantitative analysis for MCF7 and MDA-MB-231 cell apoptosis. ***, P<0.001; **, P<0.01. Sin, sinensetin; DAPI, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride; TUNEL, TdT-mediated dUTP Nick-End Labeling; CCK-8, Cell Counting Kit-8; PBS, phosphate buffered saline; TBST, tris buffered saline tween.

increase in apoptosis. These results demonstrated that sinensetin increased apoptosis of MCF7 and MDA-MB-231 cells (*Figure 1C*). The results of the TUNEL assay confirmed that sinensetin increased apoptosis of MCF7 and MDA-MB-231 cells (*Figure 1D*). These results showed that sinensetin inhibited the viability and increased apoptosis of MCF7 and MDA-MB-231 cells.

Sinensetin decreases metastasis and epithelial-mesenchymal transition (EMT) of MCF7 and MDA-MB-231 cell

To explore the inhibitory effect of sinensetin on metastasis and EMT, Western blotting, Clone formation assay, and Transwell assay were performed. The results of the Clone formation assay showed that sinensetin suppressed obviously the proliferation property, tumor formation and self-renewal of MCF7 and MDA-MB-231 cells (*Figure 2A*). Moreover, sinensetin inhibited MCF7 and MDA-MB-231 cell invasion as determined by the Transwell assay (*Figure 2B*). Sinensetin downregulated the protein expression of Vimentin, Slug, Snail, and Twist in MCF7 and MDA-MB-231 cells, while E-cadherin protein expression was upregulated (*Figure 2C*). Moreover, the results of qRT-PCR showed that sinensetin remarkably decreased the mRNA expression of Vimentin, Slug, Snail, and Twist in MCF7 and MDA-MB-231 cells. E-cadherin mRNA expression was increased after sinensetin treatments (*Figure 2D*). Other EMT markers including Zeb1, Zeb2, and E47 were also downregulated by sinensetin in MCF7 and MDA-MB-231 cells (*Figure 3A,3B*). The results of qRT-PCR showed that sinensetin remarkably decreased the mRNA expression of Zeb1, Zeb2, and E47 in MCF7 and MDA-MB-231 cells (*Figure 3C*). These results indicated that sinensetin suppressed the proliferation, migration, invasion, and EMT of MCF7 and MDA-MB-231 cells.

Sinensetin may exert anticancer effects on breast cancer cells through inhibiting the Wnt/β-catenin signaling pathway

The activation of Wnt/β-catenin signaling pathway can promote malignant phenotype of cancer (34,35). The Wnt/β-catenin signaling pathway is involved in regulating the effect of drugs derived from natural products (36,37). To determine whether sinensetin had anticancer effects in breast cancer cell via inhibiting the Wnt/β-catenin signaling pathway, we performed Western blotting to detect the expression of related proteins in MCF7 and MDA-MB-231 cells exposed to sinensetin. Sinensetin downregulated the protein expression of β-catenin, LEF1, TCF1/TCF7, and TCF3/TCF7L1 in MCF7 and MDA-MB-231 cells (*Figure 4A,4B*). Since the Wnt/β-catenin pathway plays a pivotal role in processes like cell fate determination, proliferation, and migration during mammary gland development and tissue homeostasis in adult tissue (38), alterations in its target genes could affect normal cells. Therefore, the inhibitory effect of sinensetin to Wnt/β-catenin on normal breast cell (MCF-10A) was investigated. There was not no significant difference in the protein expression of β-catenin, LEF1, TCF1/TCF7, and TCF3/TCF7L1 of MCF-10A cells (*Figure 4C*). Gene expression analysis of markers related to the Wnt/β-catenin pathway by qRT-PCR showed that β-catenin, LEF1, TCF1/TCF7, and TCF3/TCF7L1 mRNA expression were significantly downregulated by sinensetin (*Figure 4D*). These results indicated that sinensetin suppressed the Wnt/β-catenin signaling pathway in MCF7 and MDA-MB-231 cells.

The Wnt agonist SKL2001 reverses the anticancer effect of sinensetin on breast cancer cells

To confirm the role of the Wnt/β-catenin signaling pathway in the anticancer effect of sinensetin on breast cells, cells were treated with the Wnt agonist SKL2001 (40 μM) (33) for 1 h before exposure to sinensetin (32). The results showed that SKL2001 increased the protein levels of β-catenin, LEF1, and TCF1/TCF7 in MCF cells (*Figure 5A*). SKL2001 increased the Bcl-2/Bax ratio (*Figure 5B*) and inhibited breast cancer cell apoptosis induced by sinensetin (*Figure 5C*). In addition, SKL20001 upregulated Vimentin and downregulated E-cadherin expression in MCF7 cells (*Figure 5B*). Transwell assay results showed that SKL20001 promoted the invasion of MCF7 cells exposed to sinensetin (*Figure 5D*). These results demonstrated that the Wnt agonist SKL20001 reversed the anticancer effect of sinensetin on breast cancer cells, suggesting that sinensetin suppressed breast cancer cell growth by inhibiting the Wnt/β-catenin signaling pathway.

Discussion

Despite significant advances in the diagnosis and treatment of breast cancer, metastasis to distant organs remains the leading cause of death (39,40). Metastatic breast cancer spreads to other parts of the body. Because of its non-specific effect, it can only be treated by systemic chemotherapy, which is associated with toxicity and other side effects. Drugs targeting the estrogen receptor, progesterone receptor, or human epidermal growth factor

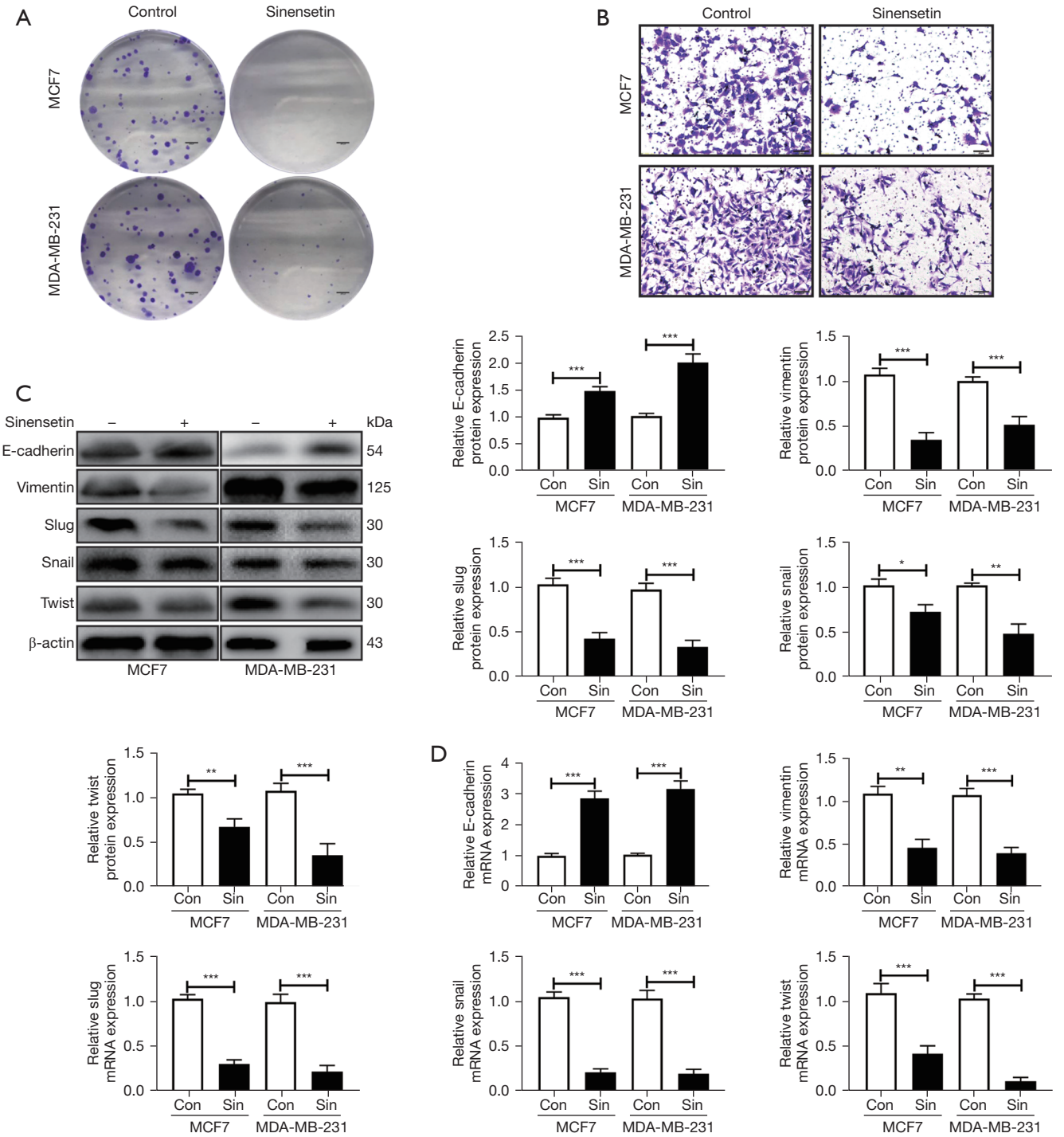

Figure 2 Sinensetin inhibits MCF7 and MDA-MB-231 cell proliferation, invasion and migration through EMT process. (A) MCF7 and MDA-MB-231 cell proliferation were determined by Clone formation assay. Scale bar =1,000 μm. Cells without any substance acted as control. Briefly, cells were fixed in 4% paraformaldehyde (P0099, Beyotime Biotechnology) and then washed three times. Cells were cultured with 0.1% crystal violet and washed with water. (B) MCF7 and MDA-MB-231 cell invasion were determined by Transwell invasion assay. Scale bar =100 μm. Cells below the membrane were fixed with 4% paraformaldehyde. Subsequently, cells were stained with 0.1% crystal violet for 15 min and photographs were taken to assess the relative number of invaded cells for each well. Cells without any substance acted as control. (C) Vimentin, E-cadherin, Slug, Snail, Twist and β-actin protein expression in MCF7 and MDA-MB-231 cells were determined by Western blotting. Quantitative analysis for Vimentin, E-cadherin, Slug, Snail and Twist protein expression in MCF7 and MDA-MB-231 cells. (D) Gene expression analysis to Vimentin, E-cadherin, Slug, Snail and Twist in MCF7 and MDA-MB-231 cells were performed by qRT-PCR. ***, P<0.001; **, P<0.01; *, P<0.05. Sin, sinensetin; EMT, epithelial-mesenchymal transition; qRT-PCR, quantitative real-time polymerase chain reaction.

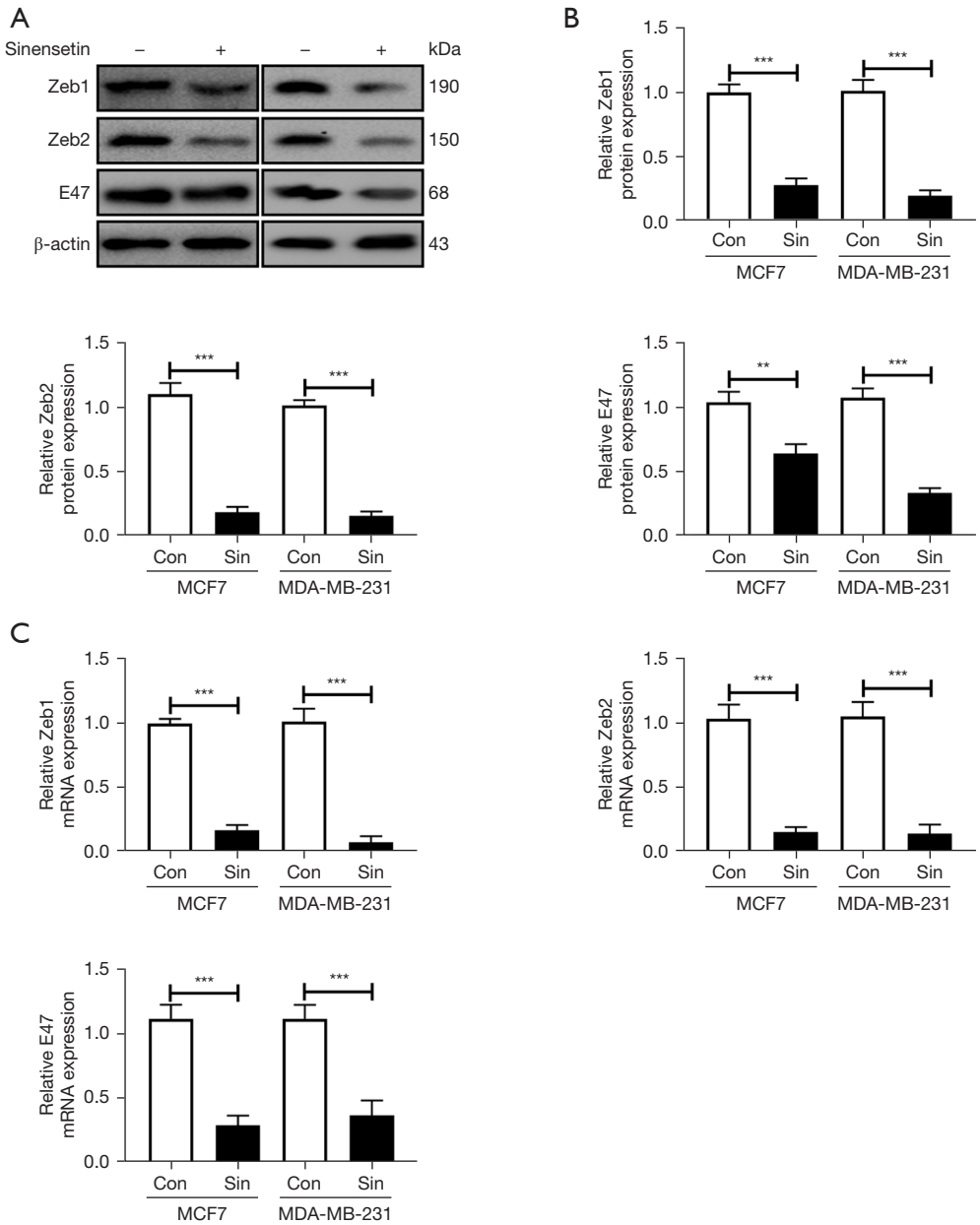

Figure 3 Sinensetin reduces mRNA expression of EMT marker. (A) Zeb1, Zeb2, E47 and β-actin protein expression in MCF7 and MDA-MB-231 cells were determined by Western blotting. (B) Quantitative analysis for Zeb1, Zeb2 and E47 protein expression in MCF7 and MDA-MB-231 cells. (C) Gene expression analysis to Zeb1, Zeb2 and E47 in MCF7 and MDA-MB-231 cells were performed by qRT-PCR. ***, P<0.001; **, P<0.01. Sin, sinensetin; EMT, epithelial-mesenchymal transition; qRT-PCR, quantitative real-time polymerase chain reaction.

receptor 2 (HER2) are only used for tumors that express the corresponding receptors (40). However, a common breast cancer subtype, TNBC, lacks these three receptors and is not suitable for existing targeted therapies. TNBC patients account for 12% of all breast cancer patients. Although this disease initially responds to primary chemotherapy, the recurrence rate is high and the survival rate is low (39,40).

The present study demonstrated that sinensetin derived from *citrus* species repressed the viability of breast cancer cells (MCF7 and MDA-MB-231 cell lines). Sinensetin is a

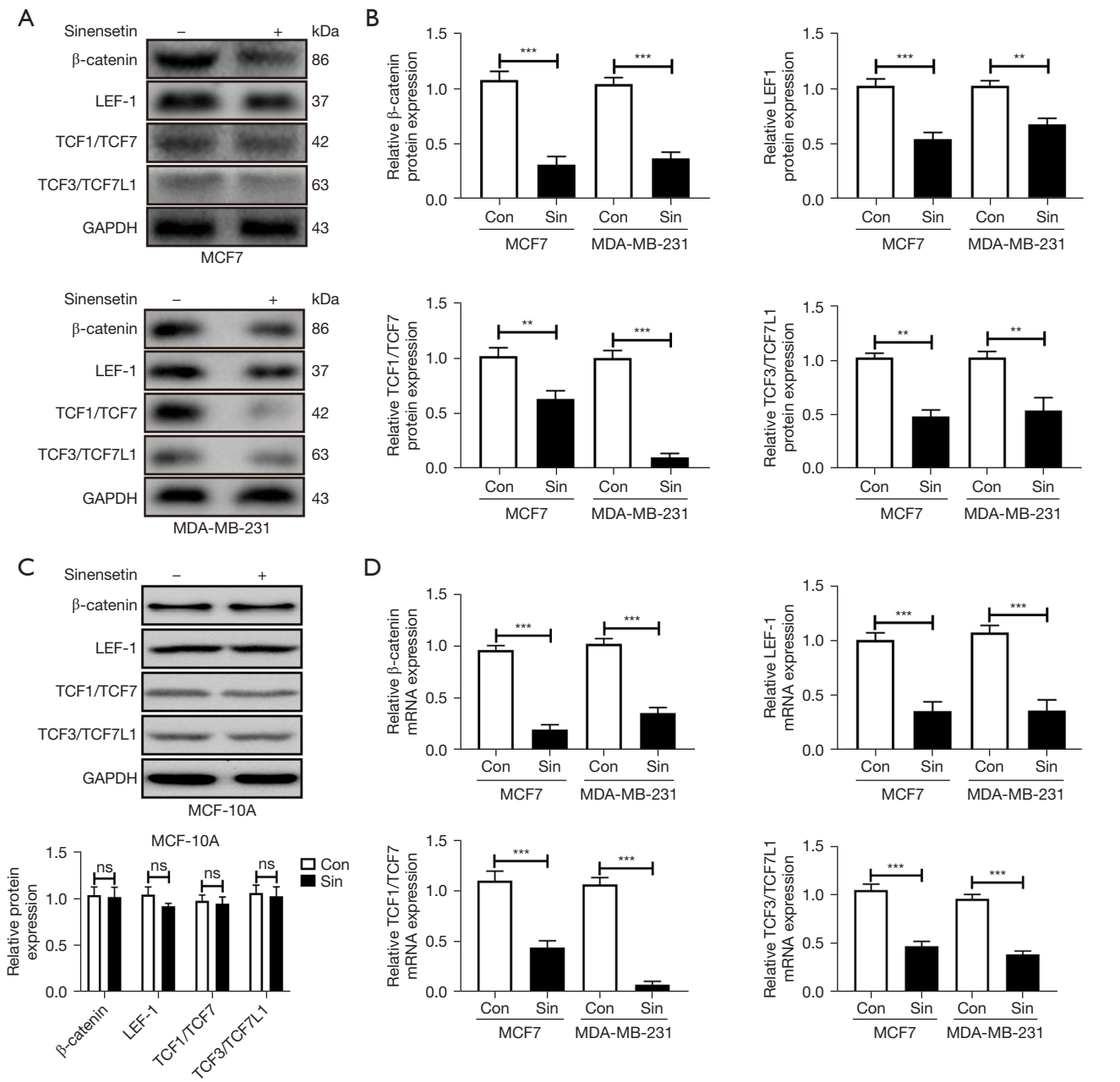

Figure 4 Sinensetin results in a decreased protein expression of Wnt/β-catenin signaling pathway. (A) β-catenin, LEF-1, TCF1/TCF7, TCF3/TCF7L1 and β-actin protein expression in MCF7 and MDA-MB-231 cells were determined by Western blotting. (B) Quantitative analysis for β-catenin, LEF1, TCF1/TCF7 and TCF3/TCF7L1 protein expression in MCF7 and MDA-MB-231 cells. (C) β-catenin, LEF-1, TCF1/TCF7, TCF3/TCF7L1 and β-actin protein expression in MCF-10A cells were determined by Western blotting. Quantitative analysis for β-catenin, LEF-1, TCF1/TCF7 and TCF3/TCF7L1 protein expression in MCF-10A cells. (D) Gene expression analysis to β-catenin, LEF-1, TCF1/TCF7 and TCF3/TCF7L1 in MCF7 and MDA-MB-231 cells were performed by qRT-PCR. ***, P<0.001; **, P<0.01. Sin, sinensetin; qRT-PCR, quantitative real-time polymerase chain reaction.

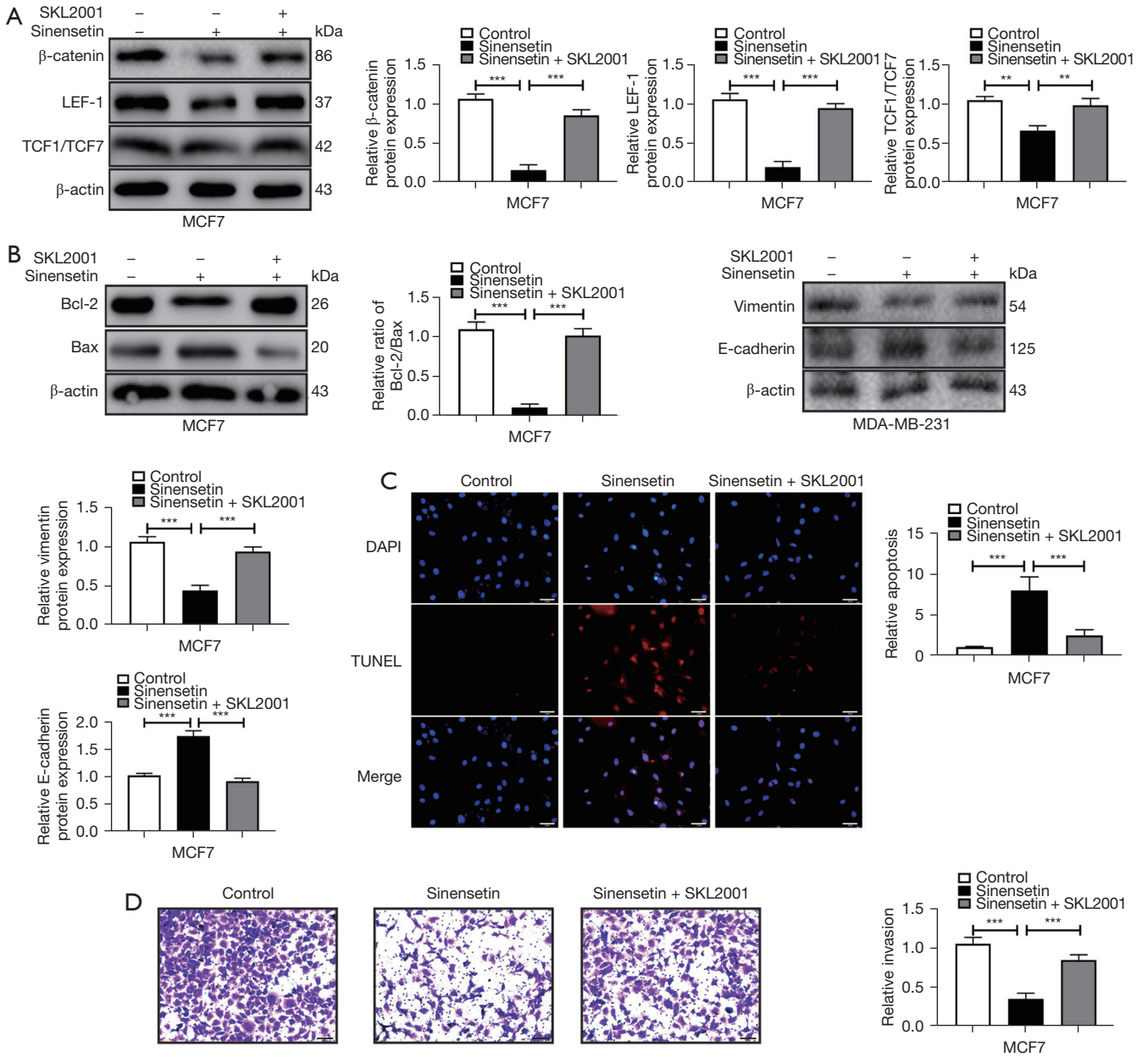

Figure 5 SKL20001 reverses the anti-cancer effect of sinensetin on breast cancer cells. (A) β-catenin, LEF-1, TCF1/TCF7 and β-actin were measured by Western blotting. Quantitative analysis for β-catenin, LEF-1 and TCF1/TCF7MCF7 in MCF cells. (B) Bcl-2, Bax and β-actin were measured by Western blotting. Quantitative analysis for Bcl-2/Bax in MCF cells. (C) MCF7 cell apoptosis were determined by TUNEL assay. scale bar =50 μm. Briefly, cells were fixed with 4% paraformaldehyde, washed with PBS twice for 3 min, and then treated with 0.3% Triton X-100 for 10 min. Aliquots of 50 μL TUNEL solution were placed in coverslips and cells were cultured for 1 h. Coverslips were washed with TBST three times. Cells without any substance acted as control. Quantitative analysis for MCF7 cell apoptosis. (D) MCF7 cell invasion were measured by Transwell invasion assay. Scale bar =100 μm. Cells below the membrane were fixed with 4% paraformaldehyde. Subsequently, cells were stained with 0.1% crystal violet for 15 min and photographs were taken to assess the relative number of invaded cells for each well. Cells without any substance acted as control. Quantitative analysis for MCF7 cell invasion. ***, P<0.001; **, P<0.01. Sin, sinensetin; DAPI, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride; TUNEL, TdT-mediated dUTP Nick-End Labeling; PBS, phosphate buffered saline; TBST, tris buffered saline tween.

PMF isomer that plays an important role in various cancers. Therefore, we explored the potential anticancer effect of sinensetin on breast cancer. The results showed that sinensetin increased apoptosis of MCF7 and MDA-MB-231 cells. Sinensetin upregulated the expression of apoptotic markers including cleaved-caspase 3 and cleaved-caspase 9 and decreased the Bcl-2/Bax ratio. To investigate whether sinensetin could inhibit the metastasis of breast cancer cells, we performed Western blotting, Clone formation assays, and Transwell invasion assays to analyze proliferation, migration, invasion, and EMT. The results showed that sinensetin suppressed proliferation, migration, and invasion by affecting EMT.

The IC_{50} of a drug is generally related to the purity of the drug, experimental conditions, cell type, culture medium type and other factors. Compared with the study by Androutsopoulos *et al.* (41), different cell lines were used in our study. Sinensetin used in this study also come from a different manufacturer. Meanwhile, Manthey *et al.* have reported that the IC_{50} of flavonoids occurred below 10 μM in many cases including breast cancer cells (42). Compared to their study (41,42), CCK-8 was used to determine IC_{50} rather than MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide, Thiazolyl Blue Tetrazolium Bromide] in this study. Interestingly, Kim *et al.* demonstrated that the IC_{50} of sinensetin was more than 100 μM in Hepatocellular Carcinoma HepG2 Cells (43). Sinensetin used in their study also come from MedChemExpress. These might be the reasons why our IC_{50} differed from previous studies. The result obtained from the clone formation assay could be due to an effect on the cell survival rather than an effect on cell proliferation. In fact, the results in *Figure 2A* showed that cell proliferation was suppressed obviously by 120 μM sinensetin under these conditions. Therefore, we hypothesized that sinensetin was able to inhibit the proliferation of breast cancer cells. Due to experimental conditions, we could not perform flow cytometry to detect breast cancer cell cycle analysis. These results of proliferation needed to be confirmed by follow-up studies.

Natural drugs and compounds can act against the aggressiveness of breast cancer, inhibiting cancer cell proliferation and regulating cancer-related pathways (44). For example, 3,3'-diindolylmethane, which is obtained from broccoli, cauliflower, and cabbage, can decrease hypoxia inducible factor (HIF)-1α expression and activity in breast cancer under a hypoxic environment (45). 3,3'-diindolylmethane causes breast cancer apoptosis by downregulating Bcl-2 and cdc25A and upregulating p21 (WAF1) expression (46). Jasmonates restrain the growth of breast cancer cells though the mTOR signaling pathway (47). Xanthohumol inhibits breast cancer cell survival by decreasing the activity of the Notch signaling pathway (48). The Wnt/β-catenin signaling pathway is involved many cancer processes. Its involvement in cell migration, embryonic development, and polarization has been reported in recent years (49). The activity of this pathway is closely related to various aspects of human carcinogenesis, such as cell viability, cell cycle regulation, EMT, and stem cell maintenance. β-catenin accumulation in the nucleus of cancer cells results in the continuous activation of TCF/LEF transcription factors (50). The Wnt/β-catenin signaling pathway is activated in breast cancer, which increases the metastasis of breast cancer (41). Small molecules derived from natural products can be used to target the Wnt/β-catenin pathway to inhibit the metastasis of cancer (51). Astragaloside, a natural drug, attenuates Wnt/β-catenin signaling through frizzled-related protein-4 secreted by Wnt antagonists, thereby inhibiting breast CSC-like cells (52). To explore whether sinensetin inhibits breast cancer cells by suppressing Wnt/β-catenin signaling, we detected the expression of proteins related to the Wnt/β-catenin signaling pathway. The results showed that sinensetin decreased the mRNA and protein expression of β-catenin, LEF1, TCF1/TCF7, and TCF3/TCF7L1 in MCF7 and MDA-MB-231 cells. Furthermore, treatment of MCF7 cells with the Wnt agonist SKL20001 for 1 h before sinensetin treatment increased the protein expression of β-catenin, LEF1, and TCF1/TCF7 in MCF7 cells exposed to sinensetin. SKL20001 also increased the survival and invasion of MCF7 cells exposed to sinensetin. These results indicated that sinensetin exerted anticancer effects on breast cancer cells through the Wnt/β-catenin pathway.

At present, there are many treatments for breast cancer including surgery, radiotherapy, chemotherapy, estrogen receptor modulators, estrogen receptor antagonists, sulfatase inhibitors, immune checkpoint inhibitors, and so on (53-58). Task of treating breast cancer remains daunting. On the other hand, compounds obtained from natural products have clinical potential as therapeutic agents (59). Androutsopoulos *et al.* (41) have firstly reported that sinensetin inhibited strongly *in vitro* proliferation of MDA-MB-468 cells at submicromolar and micromolar concentrations by CYP1. Compared with their study, our study demonstrated that sinensetin also suppressed the progression of MCF7 and MDA-MB-231 cells via Wnt/β-catenin pathway inhibition.

Two studies complement each other and together confirm the inhibitory effect of sinensetin on breast cancer.

Conclusions

In summary, the present study demonstrates that sinensetin inhibits breast cancer cell viability and decreases cell proliferation and invasion by affecting EMT. The effects of sinensetin on breast cancer cells may be mediated by the inhibition of the Wnt/β-catenin signaling pathway. The present study is the first to demonstrate that sinensetin exerts anticancer effects on breast cancer cells, suggesting the therapeutic potential of sinensetin for the treatment of breast cancer.

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Footnote

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Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1317/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1317/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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