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Research Communication

Cardamonin, a chalcone, inhibits human triple negative breast cancer cell invasiveness by downregulation of Wnt/β-catenin signaling cascades and reversal of epithelial–mesenchymal transition

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Abstract

Cardamonin (CD), an active chalconoid, has shown potent anticancer effects in preclinical studies; however, the effect and underlying mechanism of CD for the treatment of triple negative breast cancer (TNBC) is unclear. This study aims to examine the cytotoxic effects of CD and investigate the underlying mechanism in human TNBC cells. The results show that CD exhibits cytotoxicity by inducing apoptosis and cell cycle arrest in TNBC cells via modulation of Bcl-2, Bax, cyt-C, cleaved caspase-3, and PARP. We find that CD significantly increases expression of the epithelial marker E-cadherin, while reciprocally decreasing expression of mesenchymal markers such as snail, slug, and vimentin in BT-549 cells. In parallel with epithelial–mesenchymal transition (EMT) reversal, CD down regulates invasion and migration of BT-549 cells. CD markedly reduces stability and nuclear translocation of β-catenin, accompanied with downregulation of β-catenin target genes. Using the TopFlash luciferase reporter assay, we reveal CD as a specific inhibitor of the Wnt3a-induced signaling. These results suggest the involvement of the Wnt/β-catenin signaling in the CD-induced EMT reversion of BT-549 cells. Notably, CD restores the glycogen synthase kinase-3β (GSK3β) activity, required for β-catenin destruction via the proteasome-mediated system, by inhibiting the phosphorylation of GSK3β by Akt. These occurrences ultimately lead to the blockade of EMT and the invasion of TNBC cells. Further antitumor activity of CD was tested in 4T1 (TNBC cells) induced tumor and it was found that CD significantly inhibited the tumor volume at dose of 5 mg/kg-treated mice. © 2016 BioFactors, 43(2):152–169, 2017

Keywords: cardamonin; triple negative breast cancer; epithelial–mesenchymal transition; β-catenin; glycogen synthase kinase-3β

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1. Introduction

Triple negative breast cancer (TNBC) accounts for approximately 15–20% of breast cancer cases. TNBC lacks the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression [1,2]. Because of a high propensity for metastasis and poor prognosis, TNBC is considered the most aggressive of all breast cancers [3]. Current treatment of patients with TNBC is limited to chemotherapy because of the absence of specific targeted therapies [4,5]. Invasiveness and metastasis are considered as a main reason for TNBC death. In the past few years, an increasing number of studies reported that epithelial–mesenchymal transition (EMT) plays an important role in cancer progression and metastasis [6–8].

EMT is a process that is characterized by the loss of epithelial characteristics and acquisition of a mesenchymal phenotype to produce enhanced motility, resistance to apoptosis, and the capacity to invade the surrounding tissues [9]. EMT is a physiologic process that is critical for embryonic development and that plays an important role in wound healing and tissue morphogenesis, but which becomes activated during pathological conditions, including fibrosis and cancer [10,11]. EMT is characterized by decreased adhesion to neighboring epithelial cells and the basement membrane, a specialized network of extracellular matrix (ECM) proteins that underlie epithelial tissues; increased association with interstitial ECM molecules; as well as increased cellular motility [12]. Activation of EMT is associated with altered expression of many genes, including downregulation of epithelial markers, such as E-cadherin and epithelial cytokeratins, and; upregulation of mesenchymal marker genes such as N-cadherin and vimentin [13–15]. Decrease in the expression of E-cadherin, a cell adhesion receptor found in the basolateral membrane of polarized epithelia, is a hallmark of epithelial tumor cells undergoing EMT. The loss of E-cadherin expression and function has frequently been observed in breast cancer, and its expression is often repressed by several EMT-related transcription factors, such as snail, slug, and ZEB1 [16]. Furthermore, dysregulation of Wnt/β-catenin signaling has been proved to play a significant role in the progression of both the EMT and cancer metastasis. It also plays a critical role in cancer progression in TNBC [17,18]. β-Catenin is one of the adherent junction components anchored with E-cadherin to regulate cell–cell adhesion and cell migration [19,20], and also a key transducer of the Wnt pathway [21]. The stabilization and nuclear accumulation of β-catenin led to stimulation of its target genes—cyclin D1, slug, c-Myc and VEGF, stimulation of EMT, stem cell maintenance, and self-renewal. In addition, accumulation of nuclear β-catenin is a significant marker of enhanced Wnt signaling [22,23].

Cardamonin (CD), (2,4-dihydroxy-6-methoxychalcone; [C16H14O4]; molecular mass 270.28) is a naturally occurring chalcone (Fig. 1A) [24]. Several mechanisms of actions of CD were proposed to explain its diverse biological activities, such as anticancer [25,26], anti-inflammatory [27,28], antioxidant [24], vasorelaxant [29], hypoglycemic [30], anti-infective [31]. Notably, the mechanisms involved in the anti-EMT effects of CD in TNBC are unknown. We hypothesize that CD will exert EMT inhibitory effects through modulating Wnt/β-catenin signaling pathway in TNBC. In this study, we investigated the role of CD in inhibiting EMT and correlated its regulatory effects on the EMT with Wnt/β-catenin signaling pathway in TNBC.

2. Materials and Methods

2.1. Chemicals and Antibodies

CD (≥97% purity) was purchased from Sigma-Aldrich (St. Louis, MO). Compounds were dissolved in dimethyl sulfoxide (DMSO) and stored as aliquots (20 mM) at −20 °C. Acridine orange, ammonium acetate, agaropectin, acrylamide, caspase-3-substrate, 2,7-dichlorofluorescein diacetate (DCFDA), dithiothreitol, dihydrogen sodium phosphate, EGTA, HEPEs, lithium chloride (LiCl), methylthiazolylidiphenyl-tetrazoliumbromide (MTT), Nonidet P-40, N1,1-dimethyl bis acrylamide, NADH, propidium iodide, proteinase K, RNase A, sodium pyrophosphate, Tween 20, Triton X-100 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Dubecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Penicillin, Streptomycin, Trypsin/EDTA were purchased from Invitrogen Life Technologies, USA. Primary antibodies included Bcl-2 (28 kDa), Bax (20 kDa), cytochrome c (14 kDa), p53 (53 kDa), caspase-3 (17, 19, 35 kDa), poly(ADP-ribose) polymerase (PARP) (116/89 kDa), total Akt (60 kDa), Ser 473 phospho Akt (p-Akt) (60 kDa), phospho-GSK-3β (46 kDa), GSK-3β (46 kDa), Cyclin D1 (37 kDa), c-Myc (57–70 kDa), phospho-p65NFκB (65 kDa), p65NFκB (65 kDa), E-cadherin (135 kDa), N-cadherin (140 kDa), vimentin (57–50 kDa), β-catenin (92 kDa), slug (30 kDa), snail (29 kDa), and Actin (42 kDa), horseradish peroxidase-conjugated secondary anti-mouse and anti-rabbit antibodies were purchased from Cell Signaling Technology, Danvers, MA.

2.2. Cell Lines

The cell lines used were: MCF-7 (ER+ve), MDA-MB-231 (ER−ve), and BT-549 (ER−ve) (Kind gift samples by Dr. Radha, Centre for Cellular and Molecular Biology, Hyderabad, Telangana, India) whereas MCF-10A (human normal breast epithelial cell) cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin–streptomycin whereas MCF-10A were cultured in base medium for this cell line (MEBM) along with 100 ng/mL cholera toxin. The cells were maintained in a 5% CO2/95% air humidified incubator at 37 °C. CD was dissolved in dimethyl sulfoxide (DMSO) with a stock concentration of 100 mM and was freshly diluted to predetermined concentrations with culture medium. The final concentration of DMSO was at 0.05% (v/v). The control cells received the vehicle only. The cells were maintained in a humidified incubator at 37 °C with 5% CO2. Cell lines were subcultured by enzymatic digestion with 0.25% trypsin/1 mM. Shrivastava et al. 153
EDTA solution when they reached approximately 70–80% confluency.

2.3. Cytotoxicity Studies

Cell viability was determined by MTT colorimetric assay [32]. The breast cancer cells (MCF-7, MDA-MB-231, BT-549, and MCF-10A) were seeded at a density of $1 \times 10^4$ cells per well, in 100 µL of respective medium and were allowed to attach for overnight in a CO$_2$ incubator. Cells were treated with 0–100 µM CD for 24 and 48 h. After the treatment, 10 µL of MTT (5 mg/mL) in 100 µL medium was added and incubated at 37 °C for 4 h after aspirating the medium with CD. Then the media with MTT was removed and the formed purple formazan crystals were dissolved in 200 µL of dimethyl sulfoxide and read at 570 nm in a multidetection plate reader (Spectramax M4, Molecular devices, USA). Cytotoxicity was expressed as the concentration of CD inhibiting cell growth by 50% (IC$_{50}$ value).

2.4. Colony Formation Assay

BT-549 cells (250 cells/well) were cultured in six well plates overnight and then exposed to designated concentration of CD for 24 h. After rinsing with fresh medium, cells were allowed to grow for 12 days to form colonies. The resulting colonies were fixed and stained with 1% crystal violet (in methanol) for 4 h. All the values are expressed as Mean ± SEM of three experiments in which each treatment was performed in triplicate wells. ***$P<0.001$, **$P<0.01$, and *$P<0.05$ versus control.

2.5. Assessment of Cell Morphology

Cells (1× 10$^6$ cells/well) were grown in 6-well plates and treated with or without CD at concentrations ranging from 0.5 to 1.5 µM for 24 h. Morphological changes were observed with an inverted phase contrast microscope (Model: Nikon, Japan) and photographs were taken with digital camera (Nikon, Japan) at 200× magnification. The acridine orange/ethidium
bromide (AO/EtBr) staining procedure was followed to differentiate the live, apoptotic and necrotic cells [34]. Briefly, treated or untreated cells were stained with acridine orange (10 μg/mL) and ethidium bromide (10 μg/mL) and analyzed under fluorescence microscope with excitation at 488 nm and emission (550 nm) at 200× magnification. To detect nuclear damage or chromatin condensation, treated and untreated cells were washed twice with PBS and stained with 10 μg/mL of DAPI and observed under fluorescence microscope with excitation at 359 nm and emission at 461 nm using DAPI filter at 200× magnification [35].

2.6. Annexin V Binding Assay

It was performed by the method given by Kloesch et al. with slight modifications [36]. Briefly, 1 × 10⁶ cells were seeded in a six well plate and treated with different concentrations of CD for 24 h. Then cells were analyzed by Muse™ Cell Analyzer (Merck-Millipore, Germany) according to the manufacturer instructions using Muse™ Annexin-V and Dead Cell reagent (Merck-Millipore, Germany). Apoptosis and necrosis were analyzed with quadrant statistics on propidium iodide-negative cells, fluorescein positive cells and propidium iodide (PI)-positive cells, respectively.

2.7. Cell Cycle Analysis

Cell cycle analysis was carried out using method given by Lee et al., 2012 with slight modifications [37]. To determine the effect of CD on the cell cycle, cells were seeded in six-well plates at a density of 1 × 10⁵ cells/mL for 24 h. After incubation, cells were treated with 0.5–1.5 μM for 24 h. After treatment period, cells were collected, washed and fixed overnight in 70% ethanol in PBS at −20 °C. Fixed cells were pelleted and stained with cell cycle analysis reagent at 30 min at 37 °C in dark according to the manufacturer instruction, and about 5000 events were analysed on BD FACSVerse™ cell Analyzer (BD Biosciences, NJ).

2.8. Analysis of Mitochondrial Membrane Potential (MMP)

The mitochondrial specific cationic dye JC-1 (Invitrogen, Carlsbad, CA), which undergoes potential-dependent accumulation in the mitochondria was used to detect effect of CD on mitochondrial potential. JC-1 is a monomer when the membrane potential is lower than 120 mV and emits a green light (540 nm) following excitation by blue light (490 nm). At higher membrane potentials, JC-1 monomers convert to J-aggregates that emit a red light (590 nm) following excitation by green light (540 nm) [38]. Briefly, cells were plated at a seeding density of 2 × 10⁵ cells/well in a 12-well plate. After 24 h of treatment with CD (0.5, 1.0, and 1.5 μM), cells were incubated with 5 μM JC-1 for 30 min in dark at room temperature. Fluorescence was monitored with the Multimode Plate Reader at excitation wavelengths of (540 nm) and emission wavelength (590 nm). Changes in the ratio between the measurement at wavelengths of 590 nm (red) and 540 nm (green) fluorescence intensities are indicative of changes in the mitochondrial membrane potential.

2.9. Determination of Apoptosis by DNA Fragmentation Analysis

The detection of DNA ladder in agarose gel electrophoresis is used as a biochemical marker for the measurement of apoptosis. It is used to determine the presence of internucleosomal DNA cleavage. Exponentially growing cells (6 × 10⁶) treated with different concentrations of CD for 24 h were collected, washed in PBS and DNA was isolated according to the method given by Giri et al. with slight modifications [39]. Briefly, 100 μL of lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris–HCl, pH 7.5) was added to the cell pellet for 10 sec. After digestion, the supernatant was collected by centrifugation at 3000 rpm for 10 min. Then supernatant was treated with sodium dodecyl sulfate (final concentration 1%) and RNase A (final concentration 0.1 mg/mL) for 2h at 56 °C, followed by proteinase K (final concentration 0.1 mg/mL) for 2 h at 37 °C. DNA was precipitated by adding half volume of 10 M ammonium acetate and 2.5 volumes of 100% ethanol and the samples were stored for overnight at −20 °C. The DNA was recovered by centrifugation at 13,000 rpm for 15 min at 4 °C. The DNA was resolved by 1.5% agarose electrophoresis at 80–100 V and finally visualized by a UV transilluminator (BIO-RAD, USA).

2.10. Wound Healing Assay

A cell-based wound healing assay was performed following well-established methods [40]. Briefly, serum-starved BT-549 cells were grown to 90% confluency and a linear wound was created in the confluent monolayer using a 200 μl micropipette tip. The cells were then washed with PBS to eliminate detached cells and diluted in serum-free DMEM. Then, various concentrations of CD were added for 24 h and 48 h of incubation, and the wound edge closure was monitored with a microscope.

2.11. Cell Adhesion Assay

Cell adhesion assay was carried out by a colorimetric method based on staining cells with the dye crystal violet [41]. Briefly, a 24-well culture plate was coated with 10 μg/mL of type I collagen for 24 h at 4 °C, washed twice with PBS, and then plates were blocked with 1% BSA in DMEM for 1 h. BT-549 cells treated with various concentrations of CD in DMEM containing 10% FCS were plated at a density of 1 × 10⁶ cells/mL micropipette tip. The cells were then washed with PBS, adherent cells were fixed in 50% ethanol, stained with 0.1% crystal violet, and then photographed under a microscope at 200×.

2.12. Preparation of Total, Cytoplasmic, and Nuclear Protein Extracts

Cells from different experimental groups were homogenized in ice-cold RIPA buffer (Sigma-Aldrich) containing 1% protease inhibitor cocktail (Sigma-Aldrich) and 1% phosphatase inhibitor cocktail (Sigma-Aldrich) to get total protein extracts. Similarly, cytosolic and nuclear extracts were prepared by using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Rockford, IL) containing 1% protease inhibitor cocktail.
7.8, 1% Triton X-100, 15 mM MgSO₄, 4 mM EGTA, 1 mM DTT) were removed, and 15 mL mouse Wnt3a purified as described [19] with or without manufacturer’s protocol. After 24 h cells were collected and washed twice in 1X PBS, then lysed in RIPA buffer with protease and phosphatase inhibitors on ice for 1 h. Cell lysates were then centrifuged for 15 min at 13,000 rpm at 4 °C. Proteins were separated using SDS–PAGE and subsequently transferred to PVDF (polyvinylidene difluoride) membrane. The blots were blocked with 5% non-fat milk in TBST or 1% BSA (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, and 0.05% Tween-20) at room temperature for 1 h and incubated overnight with the appropriate primary antibody at 4 °C. After washing with TBST, the blots were incubated with peroxidase conjugated secondary antibody for 1 h. To ensure equal protein loading, β-actin was used as an internal control. Bands were monitored using enhanced chemiluminescence reagent (Millipore, USA). The strength of western blotting bands was determined by Image J density measurement program (http://imagej.en.softonic.com).

2.13. Western Blotting
After treatment with the tested agents as indicated in the figure legends, BT-549 cells were collected and washed twice in 1X PBS, then lysed in RIPA buffer with protease and phosphatase inhibitors on ice for 1 h. Cell lysates were then centrifuged for 15 min at 13,000 rpm at 4 °C. Proteins were separated using SDS–PAGE and subsequently transferred to PVDF (polyvinylidene difluoride) membrane. The blots were blocked with 5% non-fat milk in TBST or 1% BSA (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, and 0.05% Tween-20) at room temperature for 1 h and incubated overnight with the appropriate primary antibody at 4 °C. After washing with TBST, the blots were incubated with peroxidase conjugated secondary antibody for 1 h. To ensure equal protein loading, β-actin was used as an internal control. Bands were monitored using enhanced chemiluminescence reagent (Millipore, USA). The strength of western blotting bands was determined by Image J density measurement program (http://imagej.en.softonic.com).

2.14. Luciferase-Based Assay of the Wnt-Dependent Transcriptional Activity
HTB19 (BT-20) triple-negative breast cancer cells (ATCC) were stably transfected with M50 Super 8× TOPFlash plasmid (Addgene, plasmid 12456) at the 20:1 ratio with pcDNA3.1 to provide antibiotic resistance. The best-responding clone was selected and expanded; this line is referred to as HTB19-Tf throughout the text. A luciferase reporter activity measurements were performed essentially as described with several modifications [42]. The HTB19-Tf cells were seeded in 100 μL DMEM medium containing 10% FCS at ~10,000 cells/well. After overnight attachment the cells were additionally transfected by the pCMV-RL plasmid for constitutive expression of Renilla luciferase (kindly provided by Konrad Basler) [43] using X-tremeGENE 9 reagent (Roche) according to the manufacturer’s protocol. After 24 h cells were stimulated by 0.5μg/mL mouse Wnt3a purified as described [19] with or without drug compound for 12 h. The medium was subsequently removed, and 15 μL of the lysis buffer (25mM glycylglycine pH 7.8, 1% Triton X-100, 15 mM MgSO₄, 4 mM EGTA, 1 mM DTT) were pipetted into each well. After incubation for 5 minutes at room temperature the 96-well plate was analyzed in the Victor3 Multilabel Counter (PerkinElmer) with two-channel dispensing unit primed with the buffer solutions for firefly and Renilla luciferase activity measurements prepared as described in [44]. The final volumes dispensed per well were 50 μL of firefly and 50 μL of Renilla solutions.

2.15. Determination of Tumor Inhibition in a Murine Model of Mammary Carcinoma
All animal studies were performed according to the guidelines and approval of the Institutional. Six- to seven-week-old female Balb/c mice were used for all experiments. The mice were housed in groups of four to five animals per cage. To establish tumor grafts, subconfluent breast cancer cells 4T1 were dispersed with 0.1% trypsin/EDTA and washed once with medium containing 5% calf serum to remove the trypsin. The cells were resuspended at a concentration of 8 × 10⁶ cells/mL in phosphate-buffered saline. A total of 8 × 10⁶ breast cancer cells were injected into the mammary fat pad in mice under the anesthesia and permitted to grow until palpable. At that time, the mice were randomly assigned into control and treatment groups and chemotherapy was initiated. The doses and route of administration for CD was chosen. CD doses (30 mg/kg) was delivered intraperitoneally everyday every 2 days. Control animals received an injection of 0.9% saline solution in volumes equivalent to those used for injection of the drug.

Two-dimensional measurements were taken with calipers during the treatment period, and tumor volume was calculated with the use of the following formula: tumor volume (mm³) = a × b² × 0.52, where a is the longest diameter and b is the shortest diameter.

2.16. Statistical Analysis
All results were expressed as mean ± SEM. The intergroup variation between various groups was measured by one way analysis of variance (ANOVA) using the Graph Pad Prism, version 5.0 and the comparison between groups was conducted by “Bonferroni’s Multiple Comparison Test.” Results were considered statistically significant when *P < 0.05.

3. Results
3.1. CD Treatment Induces Cell Death in TNBC but Not in Normal Breast Cells
Towards understanding the antiproliferative potential of CD against breast cancer cells, firstly we examined the effect of CD on cell proliferation and clonogenic survival on breast-derived cells, including malignant and nonmalignant cell lines. Cultured cells were exposed to 3.25–100 μM concentrations for 24 and 48 h (Figs. 1B and 1C). The IC₅₀ value was determined by the concentration of the drug, which reduces the absorbance to half that of the control and the cell viability. CD has demonstrated significant antiproliferative properties in multiple cells in a dose-and time-dependent manner. Sensitivity of CD for cell lines decreased in the control and the cell viability. CD has demonstrated significant antiproliferative properties in multiple cells in a dose-and time-dependent manner. Sensitivity of CD for cell lines decreased in the following order: BT-549 > MDA-MB-231 > MCF-7 (Figs. 1D and 1E). We performed in vitro clonogenic assays that correlated very well with cell viability. Figures 1F and 1G show the effects of CD on the relative clonogenicity of the control and the CD-treated MDA-MB-231 and BT-549 cells. Clonogenicity of MBA-MB-231 and BT-549 cells were reduced in a dose-dependent manner after exposure to CD. To examine the selectivity of CD-mediated cell proliferation inhibition, we also evaluated the effect of CD on the
normal breast epithelial cell line, MCF-10A. Interestingly, CD had very little effect on cell proliferation in this case. These findings indicate that CD is selectively killing TNBC cells, without affecting nonmalignant breast epithelial cells.

3.2. CD Induces Apoptosis and Cell Cycle Arrest in TNBC Cells

After incubation with 0, 5, 10, and 20 μM of CD, morphological alterations in BT-549 and MDA-MB-231 cells were observed (Figs. 2A and 2B) comparing with control cells. Majority of untreated or control cells were cuboid and polygonal. Exposure of cells to CD for 24 h led to retraction, rounding and significant detachment from the surface. Membrane blebbing and apoptotic bodies were observed by phase contrast inverted microscope. Dual staining with AO/EB was examined under a fluorescent microscope (Figs. 2C and 2D). No significant apoptosis was detected in control group. Early-stage apoptotic cells, marked by crescent-shaped or granular yellow-green AO nuclear staining, were detected in the experimental group. Staining was localized asymmetrically within the cells. With increasing concentrations of CD, the number of early-stage apoptotic cells increased. Late-stage apoptotic cells, with concentrated and asymmetrically localized orange nuclear EB staining, were also detected. Necrotic cells were identified as those increased in volume and showing uneven orange-red fluorescence at their periphery. Nuclear fragmentation and nuclear shrinking of BT-549 and MDA-MB-231 cells visualized by AO/EB staining were also visible by DAPI staining (Figs. 2E and 2F). Resistance against apoptosis is critical for survival and contributes to drug resistance in many cancers, including TNBC. Next, we assessed the integrity of cellular plasma membrane and the exposure of inner phosphatidylserine by a combinational assay of propidium iodide staining and Annexin V binding. CD treatment of both BT-549 and MDA-MB-231 cells induced a significant increase of propidium iodide/Annexin V dual positive population in TNBC cells in a concentration-dependent manner (Figs. 3A and 3B). In addition, cell cycle distribution of TNBC cells was accessed following 24 h treatment of increasing concentration of CD using flow cytometry. The results showed that treating cells with CD caused a significant inhibition of cell cycle progression in both TNBC cell lines (Figs. 3C and 3D), resulting in a clear increase of the percentage of cells in the G2/M phase compared to control.

3.3. CD Induces the Execution of Apoptosis Through Activation of the Mitochondrial Pathway

Towards understanding the mitochondrial apoptotic events involved in CD-induced apoptosis, we first analyzed the changes in the levels of proapoptotic proteins Bax antiapoptotic proteins Bcl-2. Western blot analysis showed that treatment of BT-549 and MDA-MB-231 cells with CD increased Bax protein levels in contrast, CD decreased Bcl-2 levels, which led to an increase in the proapoptotic/antiapoptotic Bax/Bcl-2 ratio (Figs. 4A–4D). Cytosolic extracts were prepared and cytosolic cytochrome c protein levels were measured by immunoblotting analysis. Figure 4E shows that the cytosolic fraction from untreated BT-549 and MDA-MB-231 cells contained very low amounts of cytochrome c, whereas it amounts significantly increased after 24 h of 20 μM CD treatment. Apoptosis was also evident upon examination of common molecular markers of apoptosis, including the caspase-3 activation (Fig. 4F) and cleavage of the caspase substrate PARP into the 89 kDa cleavage product (Fig. 4A). Both mitochondrial depolarization and the loss of cytochrome c from the mitochondrial intermembrane space have been proposed as the early events during apoptotic cell death. Mitochondria damage is often reported in chemodrug-induced apoptosis [45]. Therefore, we investigated whether CD treatment could cause any mitochondrial damage. We assessed the integrity of mitochondrial membrane permeability with a fluorescent dye JC-1, which exerts an orange color in healthy mitochondria but a green color when mitochondria are damaged [46]. The results shown in Figs. 5A and 5B revealed that in the majority of CD-treated BT-549 and MDA-MB-231 cells appeared as green color whereas vehicle-treated cells were stained red, which was a clear sign of mitochondrial membrane potential transition. We next assessed the effect of CD on the induction of apoptosis in TNBC cells by DNA fragmentation assay. The results showed that CD treatment results in the formation of DNA fragments in TNBC cells, as determined by agarose gel electrophoresis in a dose-dependent manner at 24 h (Figs. 5C and 5D). Taken together, these data demonstrated that CD mainly induced apoptotic cell death in TNBC cells.

3.4. CD Exerts Reversion Effect on EMT Markers in BT-549 Cells

We started our investigation by looking at the expression of markers of EMT, which is one of the characteristics that define aggressiveness of breast cancer cells. We chose two breast cancer cell lines that represent mesenchymal phenotype, MDA-MB-231 and BT-549 [47], for understanding the mechanism of anticancer effects of CD. β-Catenin expression was more in BT-549 control cells compared to MDA-MB-231 cells (Figs. 6A and 6B). Treatment with CD for 24 h resulted in upregulation of epithelial marker E-cadherin in BT-549 cells (Figs. 6C and 6D) with concomitant downregulation of mesenchymal markers, N-cadherin, vimentin (Figs. 6C–6F), which was found to be highly significant. E-cadherin expression is repressed by various transcription factors, including snail and slug [48]. Thus, we investigated whether these transcription factors could be involved in the effects of CD on EMT of BT-549 cells. Interestingly, CD downregulated the induction of N-cadherin, slug and snail, in parallel with increased E-cadherin expression (Figs. 6C, 6E, 6H, and 6I). These results suggested an effective switch from MET phenotype of breast cancer cells when exposed to CD.

Integrin-mediated adhesion to extracellular proteins, such as fibronectin and collagen, is a key mechanism by which mesenchymal cells acquire increased motility [49,50]. We hypothesized that CD-induced EMT inhibition might affect cell adhesion to the ECM. To this end, cell–matrix adhesion assay was
performed. The data show that BT-549 cells adhesion to the matrix decreased in a dose-dependent manner in response to CD treatment (Figs. 6J and 6K). Cancer cells undergoing EMT have been considered as an early event that leads to local invasiveness and distant metastasis [51]. Thus, to validate whether EMT conversion by CD is closely linked to the downregulation of

FIG 2

Effect of CD on morphological changes. Phase-contrast images (A) BT-549 and (B) MDA-MB-231. Cells (C) BT-549 and (D) MDA-MB-231 treated with CD (0, 5, 10, and 20 μM) for 24 h, and then exposed to 100 μg/mL AO/EB. Chromatin condensation and nuclear fragmentation were considered to indicate apoptotic cells. In experiments, green cells were indicated as live cells; yellow cells were indicated as early apoptotic cells; orange cells were indicated as late apoptotic cells; and red cells were indicated as dead cells. Nuclear morphology determined by DAPI staining, (E) BT-549 and (F) MDA-MB-231 cells treated with CD (0, 5, 10, and 20 μM) for 24 h were stained with 10 μM DAPI dye for 10 min. The cells with bright, fragmented, condensed nuclei were identified as apoptotic cells.

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Bt-549 cell invasion, we performed a scratch wound healing assay. This procedure enables the assessment of whether CD affects cell motility. Figures 6L and 6M illustrated that the stimulation of BT-549 cells with Wnt-3a produced a marked wound closure at the leading edge of the wound area at 24 h. Contrastingly, the wounds treated with CD exhibited significant delays in healing after the same treatment period. Collectively, the findings confirm that EMT-reversal mediated by CD is closely associated with the downregulation of the aggressive invasion phenotype of BT-549 cells.
3.5. CD Restores Cytosolic β-Catenin and Suppresses the Nuclear Localization and Function of β-Catenin in BT-549 Cells

We further studied the effect of CD treatment on Wnt signaling pathway to elucidate the mechanism of anticancer action of CD. β-catenin linked to E-cadherin plays a crucial role in the maintenance of adherens junctions and functional epithelia. By contrast, free β-catenin functions as a transcriptional coactivator of the canonical Wnt signaling pathway, which contributes to EMT during tumorigenesis [22]. The dissociation of β-catenin from the cytoplasmic tail of E-cadherin may increase the nonjunctional pool of β-catenin in cytosol, thereby leading to epithelial cell migration [12]. Therefore, to elucidate whether the increase in E-cadherin in response to CD in BT-549 cells is accompanied by the reconstitution of the E-cadherin-β-catenin complex and the suppression of Wnt/β-catenin signaling, the expression, cellular distribution, and function of β-catenin were studied. An inhibition of β-catenin protein levels was observed in the CD-treated BT-549 cells in a dose-dependent manner (Figs. 7A and 7B). To further verify an effective inhibition of β-catenin by CD, we evaluated the expression of cyclin D1, c-Myc, VEGF, and CDK-4, downstream targets of β-catenin. We found a significant downregulation of cyclin D1, c-Myc, VEGF, and CDK-4 expression in BT-549 cells after CD treatment (Figs. 7A and 7C–7F). These observations indicate a potent inhibitory action of CD on Wnt signaling.
pathway. We next examined whether CD treatment was associated with changes in the cellular contents and localization of β-catenin protein. Following 24 h treatment with CD, both nuclear and cytosolic cell lysates were collected and used for western blot analysis to determine the amount of β-catenin in each cellular fraction. As shown in Figs. 7G–7I, we found that the amount of β-catenin in the cytoplasmic fraction was not altered by CD treatment at lower concentration, whereas that in the nuclear fraction was markedly decreased by CD. At lower concentration, CD inhibited only translocation of β-catenin to the nucleus whereas at higher concentration CD treatment also diminished cytoplasmic β-catenin levels. To further confirm it, we performed western blotting of β-catenin in the presence Bortezomib (BTZ, proteasome inhibitor). We observed that CD treatment significantly decreased the levels of total β-catenin, as well as phospho-β-catenin (S33/37/T41) (supplementary information). Whereas, the proteasome inhibitor BTZ abolishes the CD-induced degradation of β-catenin. From these results, it is clear that CD inhibits Wnt/β-catenin signaling by proteasomal degradation of β-catenin. In next array of experiments, we treated BT-549 cell with Wnt-3a, a known canonical Wnt, which activate the β-catenin-dependent pathway. Stimulation with Wnt-3a enhanced proliferation in a concentration dependent manner at Wnt-3a concentration of 200 ng/mL for 24 h, cell viability of BT-549 was increased 38.2 ± 2.15% compared to untreated cells, as shown in Fig. 8A. Treatment with CD at 10, 20 μM considerably decreased proliferation of BT-549 cells when stimulated with Wnt-3a ligand (Fig. 8B). We further confirm that Wnt-3a induced β-catenin activation was inhibited in CD treated BT-549 cells through the inhibition of nuclear translocation. Indeed, Western blot analysis showed that β-catenin protein abundance was increased with Wnt-3a ligand stimulation in a time-dependent manner (Figs. 8C and 8D). Treatment with Wnt-3a ligand increased β-catenin and decreased E-Cadherin protein expression whereas CD at 20 μM considerably decreased β-catenin and increased E-cadherin protein expression in BT-549 cells when stimulated with Wnt-3a ligand (Figs. 8E–8G). To determine whether the suppression of β-catenin by CD corresponded to the suppression of β-catenin-mediated transcriptional activities, we investigated its effect on the model of a Wnt dependent cancer—the TNBC cell line HTB19 [42].
subtype of cancer is known to be especially reliant on the canonical Wnt pathway and disruption of its components is fatal for these cancer cells. By using Top Flash Luciferase reporter assay, we determined β-catenin mediated transcriptional activity in CD treated and control cells. CD showed a concentration-dependent inhibition of Wnt-3a specific Wnt-dependent transcription, and not on transcription in general (Fig. 8H). Therefore, the reduction of β-catenin transcriptional activity was in turn to cause the decrease of its transcriptional target gene expression, in terms of the downregulated expression of c-myc, cyclin D1 and cdk4 protein, suggesting that the modulation of Wnt/β-catenin signaling may be a possible course of action through which CD may elicit the EMT reversal of BT-549 cells.
(A) Protein expression levels of β-catenin and its targeted genes in BT-549 cells were determined by western blotting after 24 h of treatment with the indicated concentrations of CD. Representative bar diagram showing quantitative results for relative levels of (B) β-catenin, (C) Cyclin D1, (D) c-Myc (E) VEGF, and (F) CDK-4 proteins for CD treated cells. Values are the means ± SEM (n = 3). (G) Western blots for β-catenin in the nuclear and cytosolic fractions. The cells were treated with 20 μM of CD for 24 h. BT-549 cells were pretreated with CD (20 μM) for 1 h followed by stimulation with Wnt-3a (200 ng/mL) for 24 h. Subsequently, cytosolic and nuclear fractions were prepared and analyzed by Western blotting. β-actin and Lamin B1 were used as the internal control for the cytosolic and nuclear extracts, respectively. Representative bar diagram showing quantitative results for relative levels of (H) cytosolic β-catenin, and (I) nuclear β-catenin proteins for CD treated cells. Values are the means ± SEM (n = 3).
3.6. CD Elevates Degradation of β-Catenin by Employing the Akt/GSK3β-Dependent Pathway

β-catenin stability is controlled by the action of the so-called destruction complex, of which glycogen synthase kinase 3β (GSK-3β) is one of the key components [52]. The phosphorylation of β-catenin by glycogen synthase kinase-3β (GSK3β) targets it for proteasomal degradation. GSK3β activity, in turn, is regulated by its phosphorylation status, with phosphorylation at Ser⁹ rendering the protein functionally inactive. One of the upstream regulators of GSK3β is Akt, an upstream kinase directly responsible for the phosphorylation and inhibition of GSK-3β [53]. We wondered if the effects of CD on β-catenin and...
β-catenin-dependent transcription described above may be due to the action of the drug on Akt-GSK3β. As shown in Figs. 9A, 9C, and 9D markedly decreased the levels of GSK-3β phosphorylation, similarly, the phosphorylated status of Akt was blocked by CD in a dose-dependent manner (Fig. 9B). To further prove that the effect of CD on β-catenin is indirect but mediated by its action on Akt-GSK3β, we assessed the effect of CD on β-catenin stability in combination with the known GSK3β inhibitor, LiCl. Immunoblot analysis showed that LiCl could protect BT-549 cells from CD-mediated suppression of β-catenin abundance (Figs. 9E and 9F), indicating that GSK3β had a key role in CD-induced β-catenin degradation. These findings provide strong evidence that CD adversely affects the nuclear translocation and transcriptional activity of β-catenin, as well as the main events of Wnt/β-catenin signaling activation, by inhibiting Akt signaling and liberating GSK-3β activity, which then destabilizes β-catenin.

3.7. CD-Inhibited Breast Cancer Growth In Vivo
To investigate in vivo anti-tumor effects of CD, murine breast cancer (4T1) model in Balb/c mice was built to evaluate whether CD could suppress tumor growth in vivo. When tumors were palpable, therapy with CD was initiated at 5 and 2.5 mg/kg body weight every day. Control mice received 0.9% saline solution. Representative tumors are shown in Fig. 10A. It was found that treatment with CD significantly led to suppression of 4T1 tumor volumes when compared with the control group treated with vehicle at a dose of 5 mg/kg (Fig. 10B), suggesting that CD could inhibit tumor growth in vivo.
the tumor cell proliferation by inducing apoptosis, results of present study were consistent with previously reported results [25,26,55,56]. Apoptosis is characterized by distinct morphological features such as cell shrinkage, chromatin condensation, plasma membrane blebbing, oligonucleosomal DNA fragmentation, and finally breakdown of the cell into smaller units (apoptotic bodies) [57–59]. Anticancer agents with different modes of action have been reported to trigger apoptosis in chemosensitive cell.

Our findings showed that CD induced the apoptotic cell death associated with activation and cleavage of caspase-3 in TNBC cells. Next we demonstrated the effect of CD on Bcl-2 family proteins, including the anti-apoptotic (e.g., Bcl-2) and pro-apoptotic proteins (e.g., Bax). The balance between these two groups could profoundly affect cellular response to undergo apoptosis or not [60,61]. This interaction ablates prosurvival function and activates the Bax which render the cells to undergo apoptosis by permeabilizing the mitochondrial outer membrane. Mitochondria are important organelles which are involved to release of apoptotic signals during an intrinsic pathway for the execution of apoptosis [62]. Dysfunction of mitochondria leads to the dissipation of mitochondrial transmembrane potential and subsequently release of pro-apoptotic proteins, such as cytochrome c, from inner membranes of mitochondria, leading to activation of executioner caspase-3 to induce apoptosis [63]. It is one of the mechanisms of caspase activation in a mainly apoptotic cell death. Fragmentation of cellular DNA at the internucleosomal linker regions has been observed in cells undergoing apoptosis. Because of their characteristic patterns revealed by agarose gel electrophoresis, these nucleosomal DNA ladders are widely used as biochemical markers of apoptosis [64]. Consistently, cleavage of caspase-3 and poly-(ADP-ribose) polymerase (PARP) were enhanced by treatment of CD in TNBC cells.

4. Discussion

Aberrant activation of the Wnt/β-catenin signaling pathway promotes a myriad of cellular activities such as proliferation, migration, invasion and cell cycle disruption, as well as suppresses apoptosis in many human malignancies including TNBC [22,51]. Inhibitors of Wnt/β-catenin signaling clearly hold great promise for the delay of TNBC tumorigenesis and metastasis. For the past decade, the intense effort to search for Wnt/β-catenin signaling antagonists among natural products has led to the discovery of numerous effective candidates. In the current study, we investigated the potential of CD in the inhibition of EMT in breast cancer cells and elucidated the underlying molecular mechanisms.

Anti-cancer effects of CD have been reported in many types of cancer [25,26,54,55]. The present study demonstrated that CD exhibited antiproliferative effect and apoptosis-against triple-negative MDA-MB-231 and BT-549 cells without harming the normal breast epithelial MCF10A cells. Apoptosis is one of the modes of cell death and induction of apoptosis is the key characteristic of anticancer drugs as it plays an imperative role in the elimination of damaged cells and the maintenance of homeostasis and many of the natural chemopreventive agents exert their effect via induction of apoptosis in cancer cells. Previous studies revealed that CD inhibited the growth of the tumor cell proliferation by inducing apoptosis, results of present study were consistent with previously reported results [25,26,55,56]. Apoptosis is characterized by distinct morphological features such as cell shrinkage, chromatin condensation, plasma membrane blebbing, oligonucleosomal DNA fragmentation, and finally breakdown of the cell into smaller units (apoptotic bodies) [57–59]. Anticancer agents with different modes of action have been reported to trigger apoptosis in chemosensitive cell.

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EMT has been implicated in cancer initiation, invasion, migration, metastasis, and drug resistance [65]. A number of previous studies have shown that regulation of EMT may represent an emerging therapeutic approach to fight against TNBC. EMT depends on a reduction in expression of cell adhesion molecules and loss of tight junctions. E-cadherin can act as an active suppressor of invasion and growth of many epithelial cancers are able to form a continuous barrier to fluids across the epithelium and endothelium [66]. In the present study, our findings demonstrated that CD treatment significantly increased the expression level of E-cadherin in BT-549 cells. In fact, E-cadherin mediates calcium-dependent cell–cell adhesion, which is also suppressed by snail/slug [67], and our data showed that CD treatment inhibited the expression of slug, which in turn might result in the increased expression of E-cadherin. Furthermore, β-catenin can act as an integral component of a protein complex in adherent junctions, which helps cells maintain epithelial layers, and β-catenin participates in the Wnt signaling pathway as a downstream target [50,68]. CD treatment decreased the expression of β-catenin in TNBC cells. In the presence of BTZ which is a proteasomal inhibitor
CD induced degradation of β-catenin was inhibited in BT-549 cells. It confirmed that CD inhibits wnt/β-catenin signaling by proteasomal degradation of β-catenin. Another important finding of our study was the downregulation of slug by CD treatment, as slug/snail axis has been proposed to be repressor of E-cadherin in breast carcinomas. Increased expression of E-cadherin is suggestive of reduced EMT, which clearly has implications in tumor invasion and metastasis [70]. In BT-549 cells, we showed that CD modulated the expression of E-cadherin by significantly downregulating the levels of slug and snail protein, supporting the potential use of CD in anti-cancer metastasis therapy of TNBC.

Given the increased E-cadherin expression by CD, we hypothesize that CD modulates the EMT program by targeting Wnt/β-catenin signaling. Concomitantly, CD regulated the expression, cellular distribution, and transcriptional activity of β-catenin. The patterns strongly correlate with the increased protein levels of E-cadherin with a reduced invasive BT-549 cell phenotype. The accumulation of both cytosolic and nuclear β-catenin was reduced. Several Wnt target genes such as cyclin D1, c-Myc, VEGF, and CDK-4 play important roles in tumorigenesis: cyclin D1 and c-Myc regulate cell proliferation and cell cycle progression. Downregulation of these apoptotic genes represent an advantage in controlling metastatic behavior in TNBC [71]. For instance, c-myc is reported to induce a more aggressive phenotype and metastatic features in TNBC. Altogether, our results suggested that the suppression of cyclin D1, c-Myc, VEGF, and CDK-4 by CD may collectively delayed tumorigenesis and metastasis in TNBC cell lines.

The other important component of Wnt pathway investigated in our study was GSK3β. Normally, cytoplasmic pool of β-catenin is maintained by the activity of GSK3β and other components of destruction complex. Wnt signaling facilitates Dvl-mediated phosphorylation of GSK3β thereby facilitating the increase of cytoplasmic and nuclear pool of β-catenin [72–74]. Our results showed that CD attenuated the GSK-3β phosphorylation levels at Ser 9 and down-regulated β-catenin abundance. We further provide evidence suggesting that this activation of GSK-3β is due to inhibition of Akt activity caused by CD. Interestingly, inhibition of GSK-3β activity by LiCl decreased the downregulation of β-catenin protein induced by CD. On CD treatment, the expression of phosphorylated GSK3β was decreased in a concentration-dependent manner and we cognize that this unphosphorylated GSK3β (active) leads to the degradation of β-catenin through ubiquitin-dependent mechanism. The diagrammatic representation depicting the possible mechanisms involved in CD mediated protection against human triple breast cancer cell invasiveness by downregulation of Wnt/β-catenin signaling cascades and reversal of EMT is illustrated in (Fig. 11).
In conclusion, to our knowledge this is the first evidence that CD reverses the EMT process with the downregulation of Wnt/β-catenin signaling, as well as invasive and migratory phenotypes. Our explorations of the underlying mechanisms reveal that CD maintains E-cadherin expression through the limitation of β-catenin nuclear localization. Collectively, this study gives insight into the specific pathways that are required for CD mediated EMT inhibition and put forth CD as a rational therapeutic strategy for TNBC treatment.

**Conflicts of Interest**

The authors declare that there are no conflicts of interest.

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