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Curcumin analog B14 has high bioavailability and enhances the effect of anti-breast cancer cells in vitro and in vivo

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Abstract

Curcumin has a variety of anticancer properties, but low bioavailability prevents its use in chemotherapeutic applications. To address this problem, we tested the efficacy of the synthetic curcumin analog B14 in breast cancer cells and explored the mechanism by which B14 inhibits proliferation and metastasis of breast cancer cells. We used the breast cancer cell line MCF-7, MDA-MB-231 to study the anticancer effects of B14 and assessed cell viability, cell migration and invasion, cell cycle, and apoptosis, in addition, the antitumor effect of B14 in vivo was examined in mice bearing MDA-MB-231 cells. We found that, as the concentration of B14 increased, cell viability decreased in a dose-dependent manner. Compound B14 exerted the best antitumor activity and selectivity for MCF-7 and MDA-M-231 cells (IC_{50} = 8.84 μmol/L and 8.33 μmol/L, respectively), while its IC_{50} value for MCF-10A breast epithelial cells was 34.96 μmol/L. B14 has been shown to be a multi-targeted drug that alters the expression of cyclin D1, cyclin E1, and cyclin-dependent kinase 2 (CDK2), and ultimately induces G1 phase cell cycle arrest. At the same time, B14 activates the mitochondrial apoptosis pathway in breast cancer cells. Furthermore, B14 was more effective than curcumin in inhibiting cell migration, invasion, and colony formation. In tumor-bearing mice, analog B14 significantly reduced tumor growth and inhibited cell proliferation and angiogenesis. The pharmacokinetic test found that B14 was more stable than curcumin in vivo. Our data reveal the therapeutic potential of the curcumin analog B14 and the underlying mechanisms to fight breast cancer cells.

KEYWORDS

antitumor, apoptosis, breast cancer, cell cycle arrest, curcumin analog

Hui Shen and Jianfen Shen contributed equally to this work.

1 | INTRODUCTION

Breast cancer is the most common malignancy in women. In 2012, nearly 17 million breast cancer patients were diagnosed worldwide, ~500 000 people died of this disease, 1 in 8 to 1 in 10 women will develop breast cancer during their lifetime.[1,2] Breast cancer is a heterogeneous disease that exhibits distinctly different biological characteristics and clinical manifestations. Breast cancer subtypes are distinguished by 3 receptors: the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2). Triple-negative breast cancer is negative for all 3 receptors. Compared with other subtypes, triple-negative breast cancer is an invasive histological subtype with poor prognosis and high recurrence rate after chemotherapy.[3,4]

Many anticancer therapies currently in use are inadequate not only in terms of their therapeutic efficacy but also because they have undesirable side effects. Certain phytochemicals found in dietary ingredients have shown interesting anticancer properties with no serious side effects. Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a bright yellow hydrophobic polyphenol present in the rhizome of turmeric, and is one of the most widely characterized phytochemicals.[5] Extensive research has shown that the complex chemistry of curcumin allows it to influence a variety of cellular signaling pathways, giving it anti-inflammatory, antioxidant, chemopreventive, and chemotherapeutic properties in addition to many others.[6] The anticancer properties of curcumin have always been a subject of great interest and there is evidence that it inhibits the initiation, progression, and continued survival of cancer cells. Despite these encouraging findings, the clinical application of curcumin in the treatment of cancer is still limited by its low water solubility and instability, which results in poor bioavailability and limited therapeutic effects in vivo.[7,8] Therefore, to improve the flaws and increase the anticancer activity of curcumin, extensive effort has been continuously devoted to the synthesis of new curcumin analogs.[9,10] These engineered analogs show enhanced solubility and biology compared to curcumin. Our research team has been screening curcumin analogs and found through cell experiments that an analog B14 has a highly active anti-breast cancer effect. The aim of this study was to evaluate the anticancer properties of the curcumin analogs synthesized and to elucidate their underlying molecular mechanisms.

2 | MATERIALS AND METHODS

2.1 | Reagents and chemicals

Curcumin was purchased from Sigma (St. Louis, MO). B14, a curcumin analog (Figure 1), was provided by Dr. Ge RS (Department of Anesthesiology, the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University).

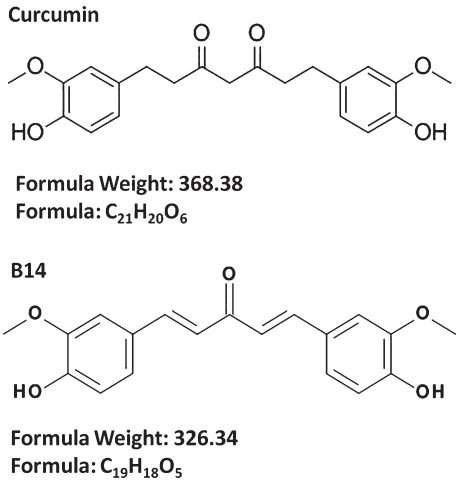

FIGURE 1 Chemical structures of curcumin and B14

In all in vitro experiments, curcumin and B14 compounds were dissolved in medium containing 0.1% DMSO. Human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from Shanghai Institute of Life Sciences Cell Resource Center (Shanghai, China). DMEM and FBS were obtained from Gibco BRL (Grand Island, NY, USA). MTT reagent was from Sigma (St. Louis, MO). The FITC Annexin V Apoptosis Detection Kit and Cell Cycle Staining Kit were purchased from BD Pharmingen (Franklin Lakes, NJ). GAPDH, Cyclin E1, Cyclin D1, Bax, Bcl-2, Cyto-C, TIMP1, Caspase3, cleaved Caspase3, and cleaved Caspase7 antibodies were purchased from Abcam company. Modified RIPA lysis buffer, protease inhibitor cocktail and chemiluminescent immunoblotting reagent were obtained from Thermo Fisher Scientific (Rockford, IL, USA).

2.2 | Cell culture

MCF-7 and MDA-MB-231, as well as normal breast epithelial cells (MCF-10A), were obtained from the Cell Bank of the Chinese Academy of Sciences and cultured in MEM, L-15 medium and MEGM kit medium (Lonza/Clonetics, CC-3150) containing 10% FBS respectively. Cells in exponential growth phase were used for experiments.

2.3 | Animals

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals for China Jiaxing University (Animal use protocol no: SCXK [HU] 2017-0001) and approved by the Ethics Committee of Animal Experimentation of Jiaxing University (JUMC2019-092). Here, 5-wk-old to 6-wk-old female BALB/c-nu/nu mice and 6-wk-old to 8-wk-old female BALB/c mice were purchased from the Qinglongshan Animal Breeding Center (Nanjing, China). Animals were housed at constant room temperature under a 12 h : 12 h, light : dark cyclic, and fed a standard rodent diet and water.

2.4 | Methyl thiazolyl tetrazolium assay

Cell lines (MCF-7, MDA-MB-231, and MCF-10A) were seeded in 96-well plates at a density of 1000 cells per well. Medium containing different concentrations of curcumin (2.5-40 μmol/L) or B14 (2.5-40 μmol/L) was added to the plate with 6 repeat wells in each group. The cells were incubated at 37°C for 12, 24, 48, or 72 h. Then 25 μL MTT (Sigma) was added to each sample. After 3.5 h, 100 μL DMSO (Sigma) was added to each well. The absorbance was read at 490 nm. The viability of the untreated cells was arbitrarily set at 100% and compared with the viability of curcumin, B14-treated cells. IC_{50} was determined using SPSS 16.0 software.

2.5 | Cell apoptosis analysis

Apoptosis analysis was performed with double labeling (Annexin V/Propidium iodide), using an FITC Annexin V Apoptosis Detection Kit (BD Biosciences), in accordance with the manufacturer's recommendations. MCF-7 and MDA-MB-231 cells were grown for 12 h in 6-well plate and then treated with the 5 μmol/L of curcumin or B14 for 48 h; control wells were left untreated. Cells were harvested by trypsinization and resuspended in the binding buffer. Annexin V-FITC and propidium iodide (PI) were added to the binding buffer and incubated for 30 min at 37°C in the dark. Analyses were performed on a BD LSR Fortessa flow cytometer as soon as possible.

2.6 | Cell cycle analysis

MCF-7 and MDA-MB-231 cells were seeded in 6-well plates at a density of 3×10^5 per well, grown in plates for 12 h, and then treated with 5 μmol/L of curcumin or B14 for 48 h, and control wells were set up. Cells were collected by centrifugation at 1000 *g* 5 min, washed 3 times with PBS, and resuspended in 70% ethanol overnight at 4°C. After cells were resuspended in PBS containing 40 g/mL RNase A and 50 g/mL PI at 37°C for 30 min, nuclear DNA content was analyzed using a BD LSR Fortessa flow cytometer. Data analysis was carried out using EXPO32/CytoSoft software.

2.7 | Wound healing assay

The breast cancer cells were seeded at a density of 3×10^5 in a 6-well plate. The cells were 100% confluent at 24 h, at which time the monolayer was scraped with a 200 μL pipette tip and washed once to remove non-adherent cells. Complete medium containing curcumin, B14 or DMSO was added and, after 24 h of treatment, cells were observed under a microscope. Inhibition of migration was assessed using ImageJ software, available from the National Institutes of Health website (http://rsb.info.nih.gov/ij). The percentage wound healing was calculated using the following formula: 100 − [(final area/initial area) × 100%].

2.8 | Transwell assay

For measurement of migration ability, 2×10^5 cells were resuspended in 200 μL of serum-free medium and inoculated into the upper chamber of a 24-well Transwell plate with a pore size of 8 μm (Corning, NY, USA), 750 μL of complete medium (containing 10% serum) containing 5 μmol/L of curcumin or B14 was added to the lower chamber; a blank control was set up. After 24 h of treatment, the upper cell layer was removed with a cotton swab, and the migrating cells attached to the lower layer of the Transwell membrane were fixed with methanol, stained with 0.1% crystal violet, and the attached cells were photographed and counted using a microscope (Olympus, Japan). To assay invasion, Matrigel was diluted with serum-free medium to a final concentration of 50 mg/L in accordance with the manufacturer's instructions, added to the bottom of the Transwell chamber, and incubated at 4°C overnight, and then the same step detailed for the migration assay was performed.

2.9 | Colony formation assay

The effects of curcumin and B14 on the ability of MCF-7 cells and MDA-MB-231 cells to form colonies were determined using the colony formation assay. Cells were seeded at 300 cells per well in 6-well plates. Cells were cultured for 24 h in a medium containing DMSO or 5 μmol/L of the compound, and then the medium was replaced with complete medium containing no drug for culture. At 2 wk later, the cells were washed with PBS for 3 times, fixed with methanol for 20 min, and stained with crystal violet for 15 min. The cells were next washed with ddH_2O to eliminate residual crystal violet, and colony number was calculated using Image J software.

2.10 | Reverse transcription-polymerase chain reaction

Total RNA was extracted from MCF-7 and MDA-MB-231 cells using TRIzol reagent (Invitrogen, USA) in accordance with the manufacturer's protocol. Complementary DNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China), and real-time PCR was performed using a StepOnePlus instrument (ABI, USA) with SYBR Green (Bio-Rad, USA) in accordance with the manufacturer's protocol. For cDNA amplification, thermal cycling was performed using the following parameters: 95°C for 10 min, 45 cycles of denaturation at 95°C for 10 s and extension at 60°C for 30 s; a melting curve was obtained from 62°C to 95°C.

2.11 | Western blot analyses

In cell experiments, cells were incubated for 24 h with 5 μmol/L curcumin or B14 before preparation of cell lysates. In animal experiments, a small amount of subcutaneous tumor tissue of mice was taken, cut, ground, and then lysed with lysate to extract protein. Cell lysis buffer, RIPA (20 mmol/L Tris–HCl, pH 7.5, 120 mmol/L NaCl, 1.0% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 10% glycerol, 1 mmol/L EDTA and 1% protease inhibitor cocktail (Roche)), and proteinase inhibitor, PMSF, were added to wells. Cells were incubated for 15 min on ice. The cell lysate was centrifuged at 12 000 *g* for 5 min at 4°C, and then the concentration of the protein in the supernatant was measured using a BCA protein assay kit (Beyotime, China). The supernatant was mixed with protein loading buffer and denatured at 100°C for 10 min. Equivalent amounts of protein extracts from whole-cell or tissues were separated by SDS-PAGE followed by electrophoretic transfer onto PVDF membranes in Tris-glycine buffer. The membrane was blocked with 5% non-fat dried milk in a shaker at room temperature for 1.5 h followed by incubation with the indicated primary antibodies: Cyclin E1 (1:1000; ab33911; Abcam), Cyclin D1 (1:1000; ab134175; Abcam), Bax (1:1000; ab32503; Abcam), Bcl-2 (1:1000; ab182858; Abcam), Cyto-C (1:1000; ab133504; Abcam), Caspase3 (1:500; ab13847; Abcam), active-Caspase3 (1:500; ab32042; Abcam), active-Caspase7 (1:1000; ab256469; Abcam), TIMP1 (1:1000; ab211926; Abcam) and GAPDH (1:2000; ab9485; Abcam) antibodies, at 4°C overnight. Blots were rinsed thoroughly and then incubated with an HRP-labeled species-matched secondary antibody for 1 h. Finally, membranes were washed with 1× TBST for 3 × 10 min and analyzed using an enhanced chemiluminescence (ECL) detection system (GE Healthcare Bio-sciences).

2.12 | In vivo antitumor study

In vivo antitumor activity of analog B14 compared with curcumin was examined using 5-wk-old female BALB/c-nu/nu mice. In total, 2×10^6 MDA-MB-231 cells/200 μL in logarithmic growth phase were subcutaneously (sc) injected into the right flank of nude mice. Once the tumor mass was established (a volume of ~100 mm^3), the mice were randomly assigned to various treatment groups (6 mice per group). Mice in each group were injected ip with B14 (30 mg/kg), curcumin (30 mg/kg) or vehicle control once every 3 d for 3 wk. For drug preparation, 6 mg curcumin (or B14 analog) were dissolved in 100 μL DMSO, and then 1900 μL olive oil (Guangzhou Peiyu Biotechnology Co., Ltd.) was added to make the suspension. At this time, the concentration was 3 mg/mL; the vehicle control was a suspension of 100 μL DMSO and 1900 μL olive oil. Based on a 20 g body weight for each mouse, each mouse was injected ip with 200 μL. Tumor volume was measured by vernier caliper and calculated according to the formula: 0.52 × (axis1 × axis2 × axis3). At the end of experimental week 3, mice were sacrificed and the primary tumor was collected and fixed in 10% buffered formalin.

2.13 | Immunohistochemistry

Paraffin-embedded samples were sectioned and stained with rabbit monoclonal anti-Ki67 antibodies (Abcam, Cambridge, UK) and rabbit monoclonal anti-CD31 antibodies (Abcam, Cambridge, UK) and stained sequentially with anti-rabbit secondary antibody and avidin-biotin complex. Binding complexes were then visualized with diaminobenzidine. Sections were then counterstained lightly with hematoxylin for microscope examination. Quantitative assay of the immunochemistry data was performed using Image-Pro Plus 6.0 (Media Cybernetics, Inc, Bethesda, MD).

2.14 | In vivo pharmacokinetic study

For this study, we used 50 female BALB/c mice, 6-8 wk old, weighing 22-24 g. Curcumin and B14 were dissolved in DMSO, then diluted to 2.5 mg/mL with normal saline, and ip injections were performed. The dose used was 2 μL per g body weight, so the final dose was calculated to be 5 mg per kg body weight. Approximately 500 μL of whole blood were collected at selected time points in heparinized syringes by cardiac puncture and blood was transferred into microcentrifuge vials (Eppendorf) containing 10 μL heparin. Plasma was separated by centrifugation at 12 000 ×*g* for 8 min and then stored at −80°C until extraction and LC-MS analysis, as described below. The trial design was described previously.[11]

2.15 | Metabolite identifications by LC-MS/MS

HPLC was performed on a Phenomenex Gemini C18 column, the column temperature was 40°C, mobile phase A was a water phase (0.1% formic acid), mobile phase B was acetonitrile, 10-90% B gradient elution, the flow rate was 0.20 mL/min. The results were analyzed by LC-MS/MS, the ion source was a turbo spray source, and the positive ion method was used. Voltage of the ion source was 5.5 kV; temperature of heated capillary was 550°C; CAD was 6 psi; curtain gas was 15 psi; GS1 (N2) was 40 psi; GS2 (N2) was 40 psi; declustering voltage was 10 V; collision energy was 23 eV. Scanning mode was multi-stage reaction monitoring. The ion reactions for quantitative analysis were m/z 369.1→284.9 (curcumin detection), 326.8→202.9 (B14 detection) and 181.2→98.8 (internal standard).

2.16 | Quantification of curcumin and B14 in mouse plasma

For determination of the standard curve, compounds curcumin and B14 were prepared as 10, 50, 100, 200, 500, 1000, 2000, and 5000 ng/mL working solutions; 5 μL of each working solution was added to 45 μL blank plasma. After mixing, 200 μL acetonitrile containing an internal standard was added, mixed for 3 min and then

centrifuged at 21 000 *g* for 10 min. Next, 50 μL were placed into the injection bottle and 5 μL were injected. For pharmacokinetic determination, the stored samples were taken out from the refrigerator, 50 μL plasma was aspirated, and then 200 μL acetonitrile containing an internal standard was added, mixed for 3 min, and then centrifuged at 21 000 g for 10 min. Next, 50 μL were placed into the injection bottle and 5 μL were injected.

2.17 | Statistical analysis

The data are presented as means plus standard deviation (SD) obtained from at least 3 independent experiments. Statistical analysis was carried out using GraphPad Pro. Prism version 6.0 software for *t* test and one-way ANOVA. Significance was determined at values $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

3 | RESULTS

3.1 | Analog B14 inhibited the proliferation of breast cancer cells

Analog B14 inhibited the proliferation of MCF-7 and MDA-MB-231 cells in a time- and dose-dependent manner, at concentrations ranging from 2.5 to 40 μmol/L (Figure 2A; $P < .01$). In addition, we compared the inhibitory effects of the analogs B14 and curcumin on the growth of breast cancer cells. At the same concentration and time,

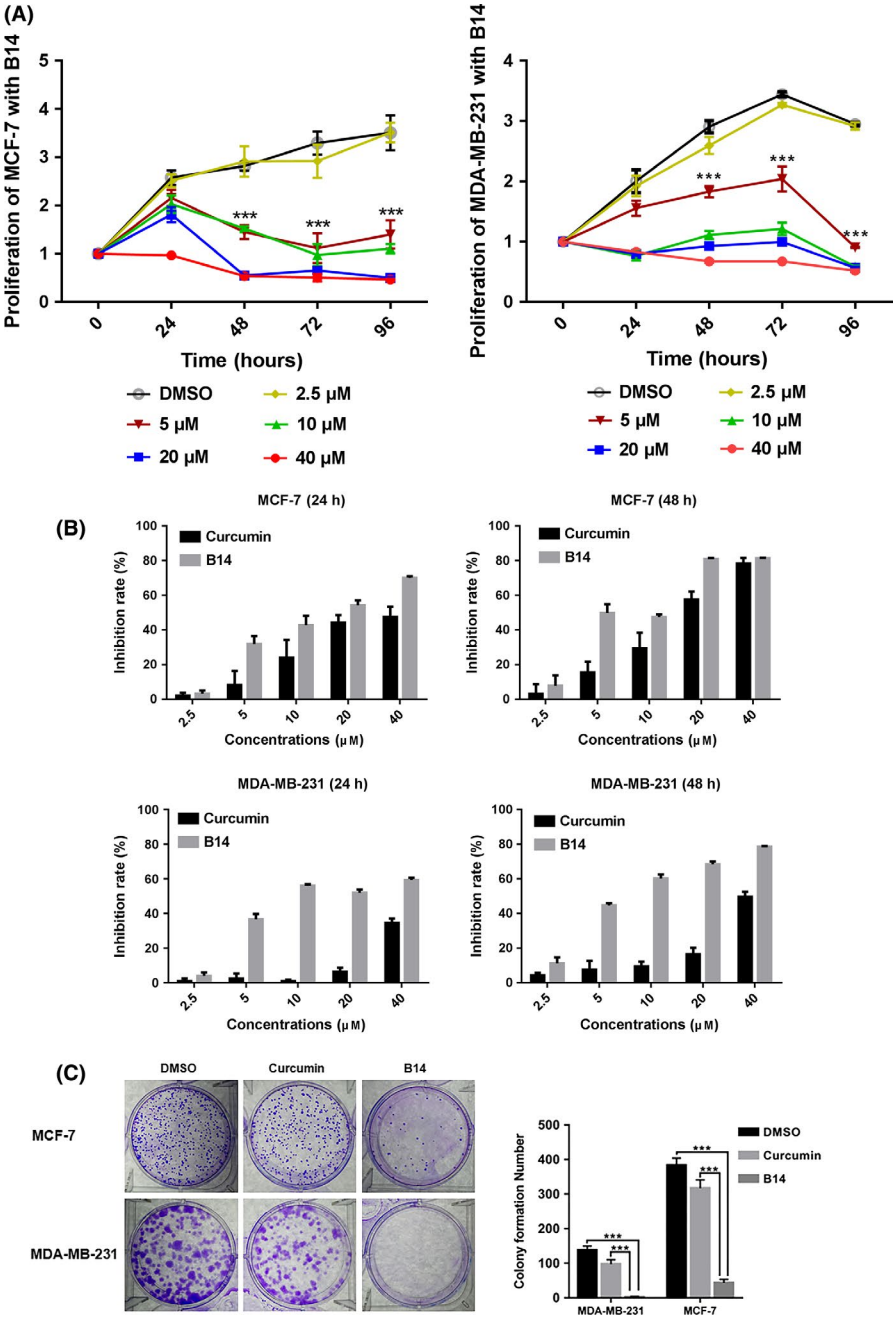

FIGURE 2 Cellular viability of MCF-7 and MDA-MB-231 cells treated with B14. A, Time-dependent and dose-dependent responses of cell viability after B14 treatment. MCF-7 or MDA-MB-231 cells were treated with DMSO (0.1%) or with 0, 2.5, 5, 10, 20, or 40 μmol/L of B14, and after 24 h, 48 h, 72 h, or 96 h their viability was measured using an MTT assay. Each experiment was performed 6 times. B, Effect of B14 or curcumin on proliferation of breast cancer cells at different concentrations. C, Effect of B14 on clonal formation of breast cancer cells. In all experiments, the data represent the mean and standard deviation (SD) of 3 experiments. $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$

results for B14 were significantly higher than curcumin for inhibition rate of breast cancer growth (Figure 2B). We assessed the viability of these cells using MTT assays following 48 h of treatment with different concentrations of either curcumin or B14. Calculation of IC_{50} values from these data suggested that B14 is several times more effective than curcumin with regards its impact on general viability (Table 1). IC_{50} values of curcumin analog B14 in these cancer cell lines were determined to be 8.84 μmol/L and 8.33 μmol/L, in MCF-7 and MDA-MB-231 cells, respectively. Interestingly, B14 had little effect on the viability of non-tumor normal breast epithelial cells MCF-10A, with an IC_{50} value of 34.96 μmol/L. This showed that the influence of B14 on cell viability was selective for cancer cells.

Next, we performed a cell clone formation experiment in which breast cancer cells were cultured for 24 h in medium containing 5 μmol/L curcumin or B14, and then culture was continued for 2 wk by replacing with new complete medium. The results showed that, compared with the curcumin group and the control group, (DMSO), B14 significantly inhibited clonal proliferation of the 2 breast cancer cell lines ($P < .01$) (Figure 2C).

3.2 | Analog B14 blocked breast cancer cell cycle G1/S phase transition

To explore the effects of B14 on the cell cycle of breast cancer, we used flow cytometry to detect cell cycle changes in 2 breast cancer cells (MCF-7 and MDA-MB-231) after B14 treatment. The 2 breast cancer cell lines were cultured for 48 h in medium containing 5 μmol/L B14, and then subjected to flow cytometry; the curcumin group and the blank group were set up in the experiment. As shown in Figure 3A, B14 can cause breast cancer cell cycle arrest in the G1/S phase. In MCF-7 cells, the percentages of cells in G1 phase in the blank control group and the curcumin group were 40.88% and 44.42%, respectively; the percentage of cells in G1 phase in the B14 group increased to 52.38%. In MDA-MB-231 cells, cells in G1 phase in the control group and the curcumin group were 34.85% and 38.50%, respectively, and percentage in G1 phase for the B14 group increased to 47.92%. These data suggested that B14 can block the G1/S transition in the breast cancer cell cycle; B14 had a better cell cycle arresting ability in estrogen-negative (ER−) breast cancer cells MDA-MB-231 than estrogen-positive (ER+) breast cancer cells MCF-7.

TABLE 1 IC_{50} values for cell viability in human breast cancer

	MCF-7	MDA-MB-231	MCF-10A
Curcumin	16.85	42.01	>80
B14	8.84	8.33	34.96

Note: Cells were treated for 48 h and cell viability was analyzed by MTT assays. IC_{50} values (μmol/L) were subsequently determined for each cell line.

3.3 | Analog B14 induced apoptosis in breast cancer cells

To confirm apoptosis induction in breast cancer cells by B14, cells were cultured in a medium containing 5 μmol/L B14 or curcumin; apoptosis was detected by flow cytometry 48 h later using an apoptosis kit. We found that B14 significantly induced apoptosis in breast cancer cells compared with the blank group with the same concentration of curcumin (Figure 3B).

3.4 | Analog B14 decreased the migration capacity of breast cancer cells

To test whether B14 affected the migration of breast cancer cells, we performed scratch and Transwell migration experiments to calculate the wound healing rate and the number of cells passing through the Transwell chamber (Figure 4A,B). Both experiments demonstrated that B14 significantly inhibited the migration of breast cancer cells compared with the curcumin group and the control group, and the inhibitory ability for ER− breast cancer cells (MDA-MB-231) was significantly more compared with that of ER+ breast cancer cells (MCF-7).

3.5 | Analog B14 inhibited the invasive ability of ER−breast cancer cells

We used ER+ (MCF-7) and ER− (MDA-MB-231) breast cancer cells to detect the effect of B14 on invasion ability. The curcumin group and the control group were set up in the experiment. The experimental method was Transwell invasion. B14 inhibited the invasiveness of ER− breast cancer cells at the same concentration as curcumin (Figure 4C), but had no effect on ER+ breast cells (negative result not shown).

3.6 | The analog B14 altered the expression of molecules involved in cycle, apoptosis, migration, and invasion in breast cancer cells

To explore the underlying mechanism by which B14 affected breast cancer cells, we performed real-time PCR to detect the expression of related molecules. Cells were cultured for 24 h in medium containing 5 μmol/L of B14 or curcumin, and then cells were collected for detection. We found that the expression levels of a gene (*p21*) that blocks G1/S phase transition were upregulated, and expression levels of genes (*CDK2*, *Cyclin D1*) that promote G1/S phase transition were downregulated. The expression levels of genes (*Bax*, *Cyto-C*) that promote apoptosis were increased, and the expression levels of a gene (*Bcl-2*) that inhibits apoptosis were decreased. The expression levels of a gene (*VEGF*) that promotes cell migration were decreased, and the expression

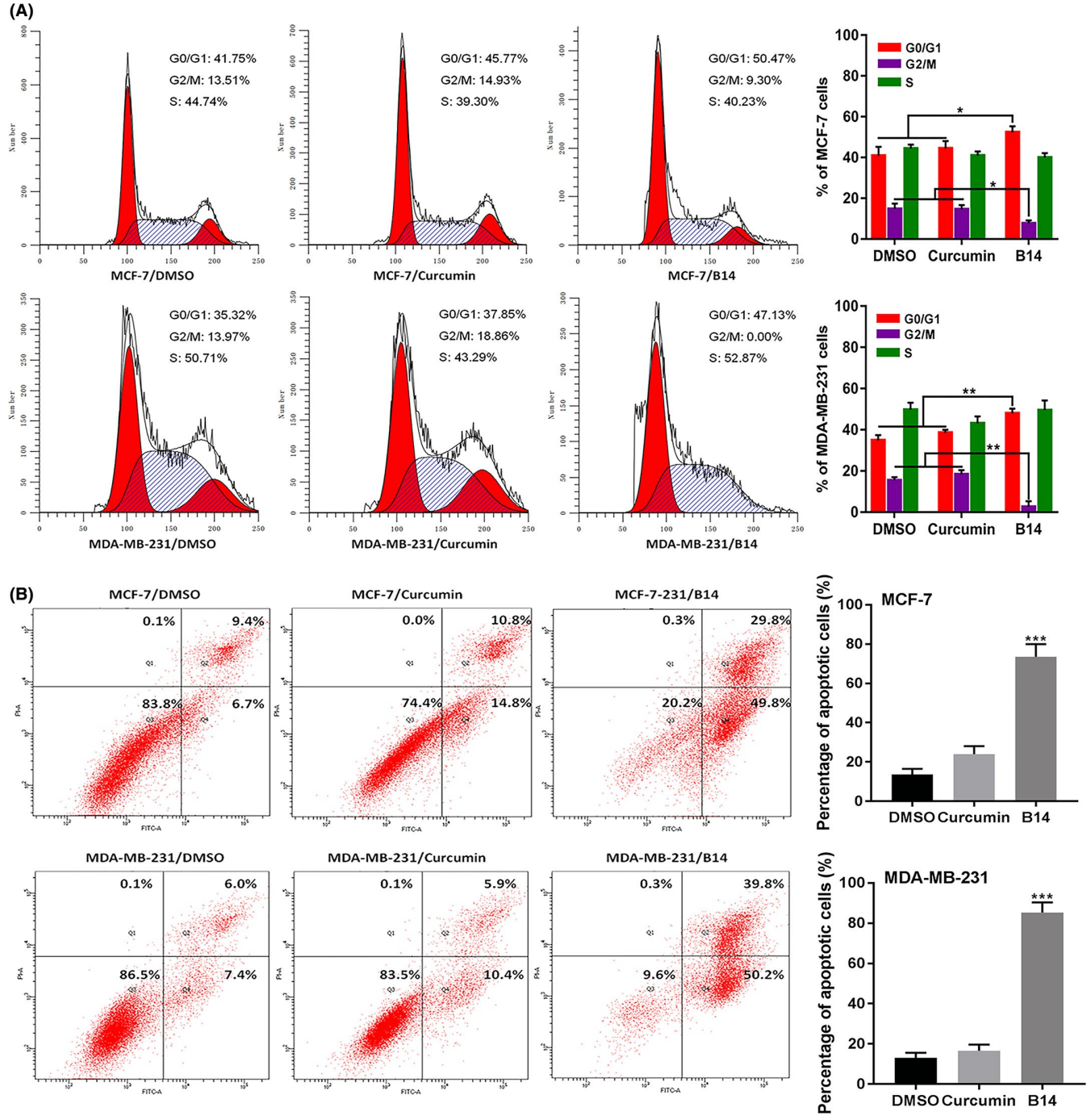

FIGURE 3 Analog B14 treatment significantly induced G1/S phase cell cycle arrest and apoptosis in breast cancer cells. Cells were treated with 5 μmol/L of curcumin or B14 for 48 h. Cells were harvested for analysis of cell cycle distribution (A) and apoptosis (B). Data from a typical experiment are depicted, and similar results were obtained in 3 independent experiments. Data are presented as mean ± SD of 3 independent experiments. $^{*}P < .05$ and $^{**}P < .01$ vs DMSO and curcumin

levels of genes (*TIMP1*, *TIMP2*) that inhibit cell invasion were increased (Figure 5).

At the same time, we examined the expression levels of related proteins using western blot analysis of ER− breast cancer cells (MDA-MB-231). The results confirmed that addition of B14 could change protein levels related to metastasis, cell cycle, and apoptosis in breast cancer cells (Figure 6). Addition of B14 could upregulate the pro-apoptotic proteins Bax, Cyto-C, active-Caspase3 and active-Caspase7, and downregulate the inhibitory protein Bcl-2. Protein levels of Cyclin E1 and Cyclin D1, which promote normal cell cycle progression, were reduced. Furthermore, the expression levels of the protein TIMP1 associated with inhibition of cancer cell invasion were increased. The above results indicated that B14 can inhibit proliferation, promote apoptosis, and inhibit metastasis and invasion by regulating the expression of mRNA and proteins of certain genes.

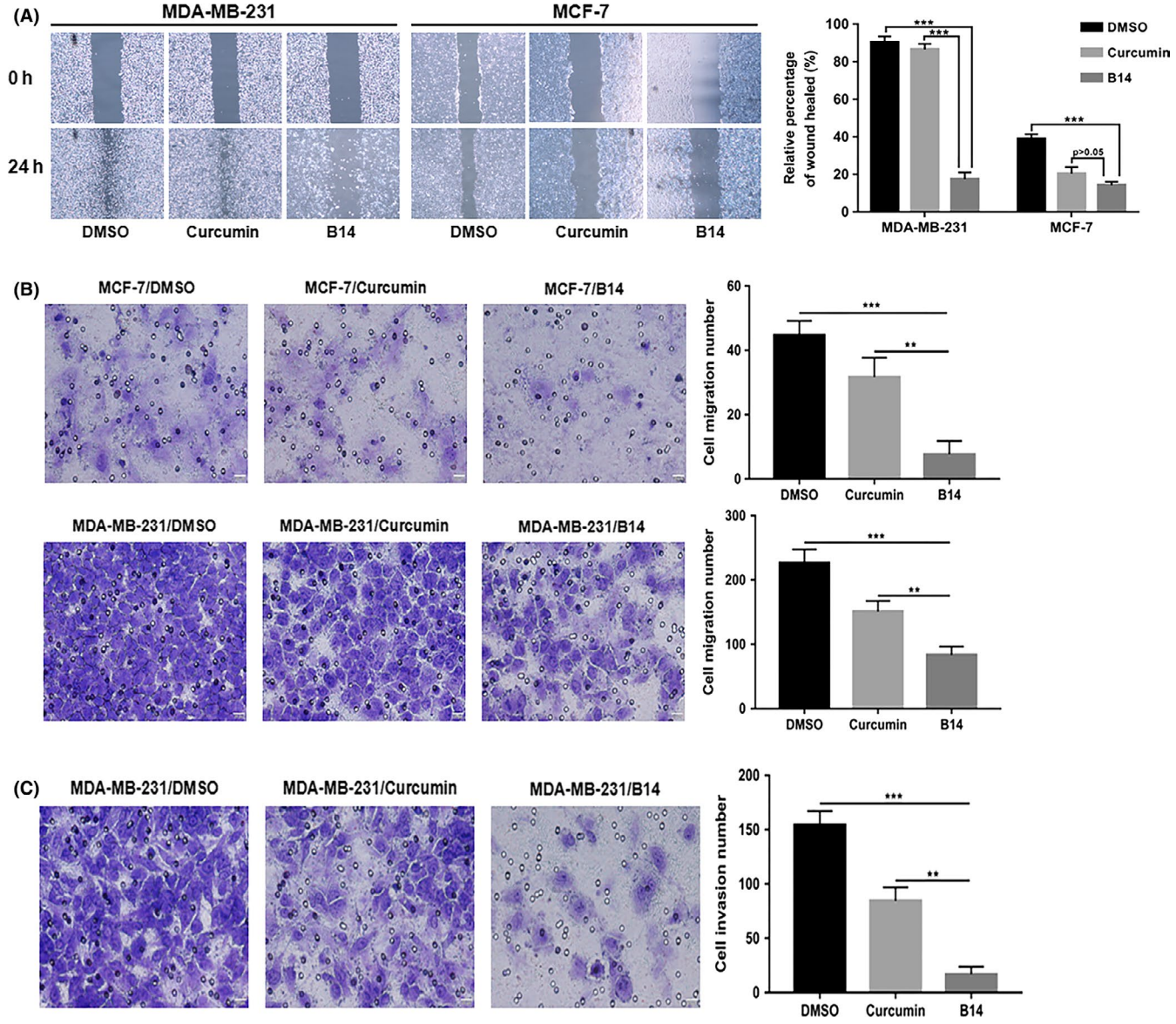

FIGURE 4 B14 inhibited cell migration and invasion in breast cancer cells. A, The effect of B14 on MCF-7 and MDA-MB-231 cell migration was evaluated by a wound healing assay. MCF-7 and MDA-MB-231 cells were scratched and treated with 5 μmol/L of B14 for 24 h. Migration inhibition (%) after treatment with B14 was calculated, and quantitative results are illustrated in the right panel. B, The inhibitory effect of B14 on MCF-7 and MDA-MB-231 cells migration was detected by a Transwell assay. Cells in serum-free medium were plated onto the upper chamber of the Transwell. Complete medium (10% serum) containing B14 at the indicated doses was added to the lower chamber. After 24 h, cells on the bottom side of the Transwell membrane were stained, photographed and counted using a microscope (Olympus, Japan). Migration inhibition (%) was calculated and quantitative results are illustrated in the right panel. C, For the invasion assay, the Transwell membrane was pre-coated with Matrigel, following which MDA-MB-231 cells were plated and treated as described above. Invasion inhibition (%) was calculated and quantitative results are illustrated in the right panel. Data are represented as mean ± SD of each group. $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$

3.7 | In vivo inhibition of tumor growth activity

To determine the safety and effectiveness of curcumin analog B14 in vivo, female BALB/c nude mice were injected sc with breast cancer MDA-MB-231 cells When the tumor mass had formed (a volume of ~100 mm^3), vehicle control, curcumin (30 mg/kg), or B14 (30 mg/kg) were injected ip once every 3 d for 3 wk. The mean tumor volume of the mice in the analog B14-treatment group was significantly reduced compared with that of the control mice; the tumor volume in the B14-treatment group was also significantly smaller than that in the curcumin group at the same drug concentration. Furthermore, the mean tumor weight of the B14-treatment group was also significantly smaller than that of the control group and curcumin group (Figure 7A). B14 was well tolerated with no obvious weight loss over the treatment period, suggesting that it was relatively nontoxic to mice (Table 2).

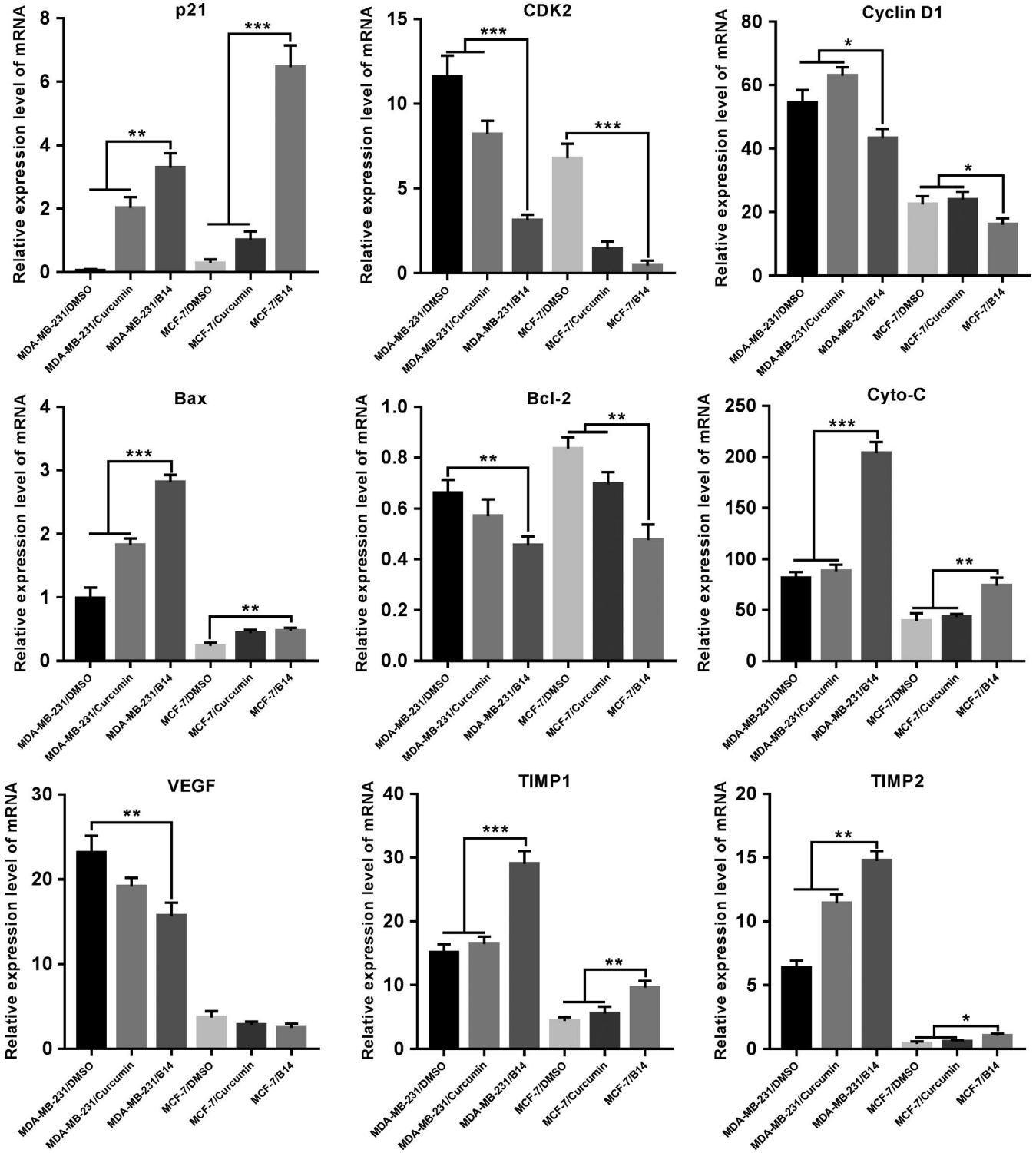

FIGURE 5 Molecular expression related to cycle, apoptosis, migration, and invasion in breast cancer cells

Immunohistochemistry staining of tumor sections showed that proliferation index determined by Ki67 staining and microvessel density determined by CD31 staining was significantly decreased in both the curcumin-treated and analog B14-treated groups; values for the B14-treatment group were significantly smaller than for the curcumin group (Figure 7B). B14-treated tumors showed similar changes in expression of apoptosis-related proteins (Bax, Bcl-2 and Caspase3), cycle-associated protein (Cyclin D1) and invasion-related protein (TIMP1) as those in the B14-treated breast cancer cell lines (Figure 7C). These results indicated that analog B14 had a strong therapeutic effect on breast tumors in vivo.

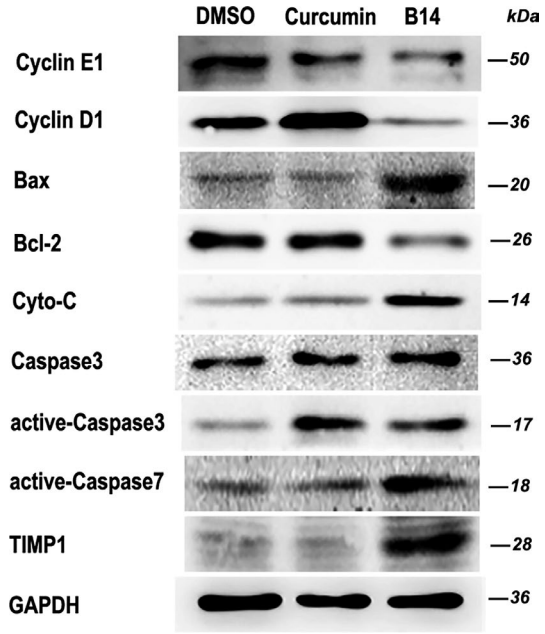

FIGURE 6 Immunoblots for levels of proteins associated with invasion, cycle, and apoptosis of ER− breast cancer cells

3.8 | In vivo administration of curcumin and B14

To compare the stability of curcumin and B14 in mice, both compounds were injected ip. A low dose of 5 mg/kg was chosen for this preliminary assessment to ensure solubility of both compounds. Although clearance for both compounds from the plasma was rapid, B14 had overall higher mean levels compared with curcumin for all time points tested (~2-fold). Concentrations of curcumin and B14 as a function of time following administration in mice are depicted in Figure 8. The average difference between the 2 compounds indicated that B14 was more stable in vivo than curcumin.

4 | DISCUSSION

Despite the great advances in diagnosis and treatment, breast cancer remains a major health problem and a major cause of death among women worldwide.[1,2] Curcumin has been shown to prevent canceration or prevent tumor formation and progression in a variety of cancer types and to inhibit angiogenesis and tumor metastasis in a variety of tumor models.[12-14] Pharmacological activity of curcumin mainly results from impacting multiple molecular targets and cell signaling pathways. It has been reported that curcumin modulates the growth of tumor cells through regulation of multiple cell signaling pathways, including cell proliferation pathways (cyclin D1, c-myc), cell survival pathways (Bcl-2, Bcl-xL, cFLIP, XIAP, c-IAP1), caspase activation pathways (caspase-8, 3, 9), tumor suppressor pathways (p53, p21) death receptor pathways (DR4, DR5), mitochondrial pathways, and protein kinase pathways (JNK, Akt, and AMPK).[15-18] Because curcumin has a variety of properties, the combination of curcumin with a single target or pathway drug is generally more effective compared with a single targeted drug alone.[19,20] Considering that curcumin has significant varieties of biological activities, along with low toxicity, affordability, and easy synthesis, curcumin fulfills the characteristics for an ideal lead compound in the development of new agents for the treatment of a variety of diseases, especially a variety of malignancies.[21-23] Due to the low bioactivity and availability of curcumin, it is limited for the clinical treatment of tumors, therefore there is a need to develop more effective curcumin analogs to inhibit proliferation, metastasis, and invasion of tumor cells at lower doses, as a more effective cancer treatment.

Curcumin and its derivatives have attracted much attention due to their anti-inflammatory, antioxidant, and antitumor effects. These biological effects are mainly attributed to the important elements of the curcumin structure. Some other derivatives have shown improved antitumor and anti-inflammatory actions compared with curcumin because of the high level of methoxylation, nonsaturation of the diketone moiety, and a low level of hydrogenation.[24] Modifying the chemical structure of curcumin can improve its pharmacological activity and the pharmacokinetic properties of a drug molecule. Some other derivatives have shown improved antitumor and anti-inflammatory actions compared with curcumin because of the high levels of methoxylation, the nonsaturation of the diketone moiety, and a low level of hydrogenation.

We demonstrated that a novel analog, B14, was more potent than curcumin in inhibiting cell viability, cell colony formation, cycle, apoptosis, migration, and invasion. These results indicated that the analog B14 was capable of functioning simultaneously in multiple pathways in tumor cells. In terms of cell viability, the analog B14 showed a time-dependent and concentration-dependent inhibition of breast cancer proliferation and, at the same concentration, the analog B14 had a stronger inhibition rate on breast cancer than curcumin. In addition, we found that B14 has an effective and selective antitumor activity on MCF-7 and MDA-MB-231 cells, but not on MCF-10A breast epithelial cells. Cell cycle analysis revealed that the analog B14 reduced the expression of Cyclin D1, Cyclin E1, and CDK2, resulting in cell cycle arrest at the G1 phase, while curcumin at the same concentration did not arrest the cell cycle. Previous studies have demonstrated that curcumin and its synthetic analogs induced G1 arrest in colorectal, colon and prostate cancers,[25-27] and induce G2/M arrest in leukemia and cholangiocarcinoma.[28,29] This suggested that cell cycle arrest induced by curcumin and its analogs was specific for different cancer cell types, and that B14 in this study induced G1 arrest in breast cancer. Apoptosis analysis showed that B14 significantly induced apoptosis in breast cancer cells at a concentration of 5 μmol/L. From changes in apoptotic molecules (Bax, Bcl-2, Cyto-C), B14 induced the activation of mitochondria apoptotic pathways in breast cancer cells. Cell migration and invasion ability were significantly inhibited by B14, and the expression of migration-associated molecules (VEGF) and invasion-related molecules (TIMP1, TIMP2) also changed. In addition, we found that B14 had a stronger effect on ER− breast cancer cells (MDA-MB-231) than on ER+ breast cancer cells (MCF-7) in terms of cycle, migration, and invasion, which may

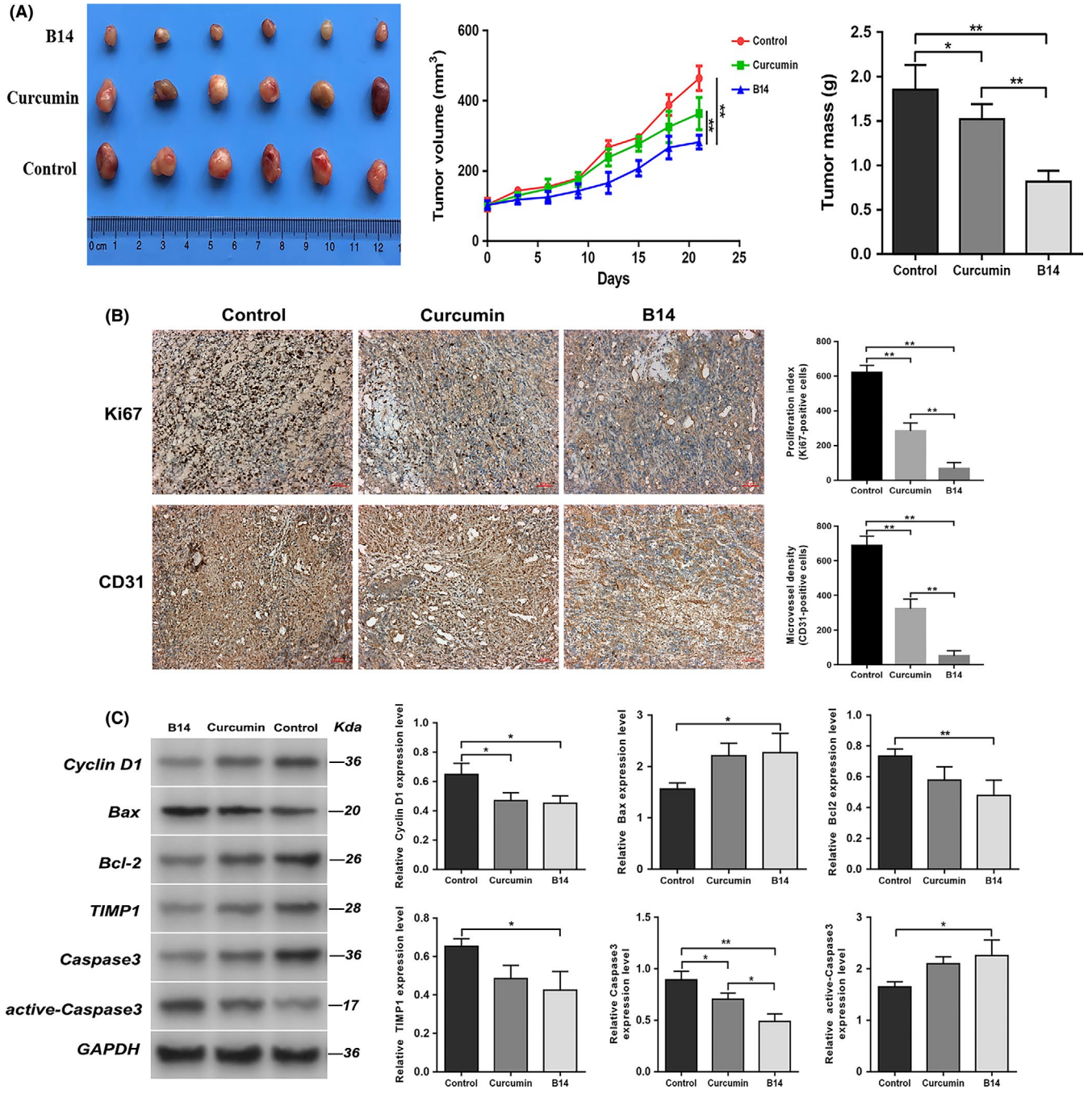

FIGURE 7 B14 inhibited MDA-MB-231 xenograft tumor growth in nude mice. MDA-MB-231 breast cancer cells were injected subcutaneously on the right side of nude mice. When the tumor mass was formed (a volume of ~100 mm^3), vehicle control, curcumin (30 mg/kg) or B14 (30 mg/kg) were injected intraperitoneally once every 3 d for 3 wk. A, Effect of B14 and curcumin on tumor volume and weight (n = 6). B, Curcumin analog B14 inhibited proliferation and angiogenesis in breast cancer tumors. Tumor section (5 μm) was carried out for immunohistochemistry of Ki67 and CD31. C, The tumor tissues from each group (n = 3) were collected, proteins were extracted from tumors, and were subjected to western blot analysis for the determination of Cyclin D1, Bax, Bcl-2, TIMP1, Caspase3 and active-Caspase3 (n = 3). Results are shown as mean ± SD. $^*P < .05$, $^{**}P < .01$

mean that B14 has a more effective therapeutic effect on estrogen receptor-negative breast cancer with a higher degree of malignancy. Compared with the control group in the MDA-MB-231 xenograft model, the treatment with analog B14 significantly reduced tumor volume and mass. Although curcumin treatment also reduced the tumor volume, the therapeutic effect was less than that of B14. Immunohistochemical assay showed that treatment with analog B14 and curcumin reduced the proliferation (Ki67) and angiogenesis (CD31) of cancer cells in the tumor, and the inhibition effect of B14 was stronger at the same dose. Protein analysis showed similar results to that of the in vitro experiments in terms of apoptosis, cycle, and invasion.

TABLE 2 Body weight of mice before and after curcumin or B14 intervention

Treatment	Body weight (g)	
	Start	End
B14	20.83 ± 0.62	22.90 ± 0.28
Curcumin	20.71 ± 0.71	23.62 ± 0.42
Control	20.48 ± 0.58	23.54 ± 0.21

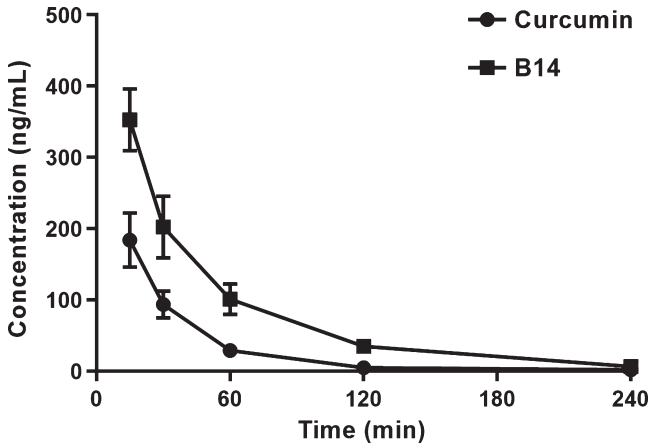

FIGURE 8 Curcumin and B14 stability detected by LC-MS/MS. Curcumin and B14 were injected ip into mice (n = 5 for each time point for curcumin and B14, respectively) at 5 mg/kg of body weight, and compound concentrations (ng/mL) were detected in plasma samples collected at 15, 30, 60, 120, or 240 min

To further characterize the ability of analog B14 as a potential chemotherapeutic agent for breast cancer, we decided to administer B14 to mice and evaluate its stability in vivo. For comparative studies, we included curcumin in the described in vivo experiments, as a compound with known pharmacokinetic properties and metabolic profile. The results showed that B14 and curcumin were rapidly removed from plasma after administration (5 mg/kg). However, compared with curcumin, B14 levels in plasma were generally higher (~2 times), which indicated that the curcumin analog B14 was more stable in vivo. It should be noted that plasma concentrations of both compounds (curcumin and B14) were very low (less than 400 ng/mL at all time points) at a dose of 5 mg/kg, which indicated the need to consider higher doses or different routes of administration to achieve efficacy.

The results of in vitro and in vivo studies indicated that B14 had better bioavailability compared with curcumin and could exert antitumor effects on breast cancer through mitochondrial apoptotic pathways and cell cycle G1 arrest. A series of curcumin analogs, as with curcumin, mostly exhibited multiple targeting mechanisms. Although anticancer effects have been reported in vitro and in vivo, the molecular mechanisms of these compounds remain unclear. The study of the curcumin analog B14 multitargeting antitumor mechanism may provide a new strategy for the design and development of anticancer drugs based on curcumin. At present, the anti-inflammatory activity of curcumin and its analogs and its influence on immune cells are also the entry points for studying treatment of various diseases.[30-32] Therefore, in further studies, we should demonstrate whether the anti-inflammatory effects of B14 and its effects on the immune response include crosstalk and complementation with its antitumor properties.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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