Original Article

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FL118, a novel camptothecin analogue, suppressed migration and invasion of human breast cancer cells by inhibiting epithelial-mesenchymal transition *via* the Wnt/β-catenin signaling pathway

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Summary **The aim of the current study was to investigate the effects of FL118, a novel camptothecin analogue, on migration and invasion of human breast cancer cells and the underlying mechanisms of those effects. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay and a plate clone formation assay were used to examine inhibition of the proliferation of MDA-MB-231 cells by FL118. Cell cycle distribution was detected using flow cytometry. A wound healing assay and a transwell assay were performed to detect the effects of FL118 on migration and invasion of MDA-MB-231 cells, respectively. qRT-PCR, Western blotting, and immunocytochemistry were used to study the effects of FL118 on expression of epithelial-mesenchymal transition (EMT)-related molecules and Wnt/β-catenin signaling components in MDA-MB-231 cells. The current results indicated that FL118 inhibited the proliferation, migration and invasion of MDA-MB-231 cells in a dose- and time-dependent manner. FL118 caused MDA-MB-231 cells to accumulate in the S phase. FL118 significantly suppressed the expression of vimentin while enhancing the expression of E-cadherin. Moreover, decreased expression of β-catenin and its targets survivin and cyclin Dl was detected in the nucleus of MDA-MB-231 cells. Taken together, the current results suggest that FL118 inhibited Wnt/β-catenin signaling, leading to suppressed EMT and decreased migration and invasion of breast cancer cells.**

Keywords: FL118, invasion, metastasis, EMT, Wnt/β-catenin, breast cancer

1. Introduction

Breast cancer is currently the leading cause of cancer-related deaths in women (*1*). Worldwide, more than 1.7 million women were diagnosed with breast cancer and 521,900 women died from breast cancer in 2012 (*1*). According to data from the National Central Cancer Registry of China, breast cancer alone is expected to account for 15% of all new cancers in women in 2015 (*2*). Invasion and metastasis, which are estimated to be responsible for approximately 90% of all cancer deaths, are the primary factors that result in the failure of breast cancer treatment (*3,4*). Medications that can suppress invasion and metastasis of breast cancer are greatly needed in clinical settings (*5,6*).

Studies revealed that epithelial-mesenchymal transition (EMT) is one of the major molecular mechanisms promoting cancer invasion and metastasis (*7-9*). EMT is a biological process that allows a polarized epithelial cell, which normally interacts with the basement membrane *via* its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory and invasive capacities (*10*). Studies have reported that Wnt/β-catenin is among the main pathways that regulate EMT, which may be the key mechanism that mediates breast cancer progression (*11,12*). Agents that target Wnt/β-catenin and downstream molecules such as survivin and cyclin D1 have a potent inhibitory effect on cell proliferation, invasion, and metastasis in

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breast cancer (*13-15*).

FL118, a novel camptothecin analogue, was first discovered in high-throughput screening for survivin inhibitors in a previous study by the current authors (*16*). FL118 was found to display potent anticancer activity against several different types of cancer both *in vitro* and *in vivo* (*17-20*). Moreover, its antitumor activity was superior to that of several camptothecin analogues, such as irinotecan and topotecan, that have been approved by the FDA for cancer treatment (*21,22*). FL118 is rapidly cleared from circulation and it effectively accumulates in tumors with a long half-life of elimination, suggesting its potential for use in cancer therapy (*18,20*). However, the effects of FL118 on breast cancer invasion and metastasis have not been reported.

The current study used an aggressive breast cancer cell line, MDA-MB-231, to examine the effects of FL118 on migration and invasion of human breast cancer and the mechanisms for those effects. The aim of this study was to determine whether FL118 would suppress cell migration and invasion and to identify the molecular mechanisms for that anticancer activity.

2. Materials and Methods

2.1. *Reagents and antibodies*

FL118, 11-methylenedioxy-camptothecin (Figure 1), was obtained from a cooperating lab, the American Roswell Park Cancer Institute (RPCI), and was dissolved with dimethyl sulphoxide (DMSO). Antibodies against survivin, E-cadherin, vimentin, and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against β-catenin, lamin B1, and cyclin D1 were purchased from Abcam, Inc. (Cambridge, MA, USA).

2.2. *Cell line and cell culture*

MDA-MB-231 cells were donated by Prof. Luo Bing (Department of Microbiology, Qingdao University, Qingdao, Shandong, China). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Hyclone Laboratories, Inc., Logan UT) at 37°C in a 5% CO_2 atmosphere.

2.3. *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay*

Cell proliferation was assessed with an MTT assay. Cells were incubated in 96-well plates (5×10^3 cells/well) 24 h prior to treatment. The cells were then treated with FL118 (0, 1, 10, 100, and 200 nM) for 24, 48, 72, and 96 h and incubated with MTT (5 mg/mL) for 4 h. The culture medium was then replaced with 100 μL DMSO. The optical density (OD) value for each well at 490 nm was measured with a Microplate Reader (BioTek, Winooski, VT, USA) to assess the degree of cell proliferation (*23*).

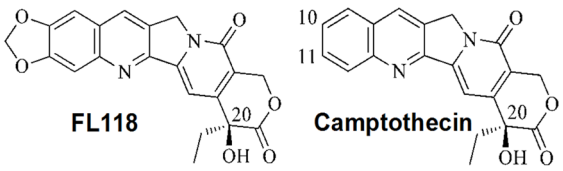

Figure 1. Chemical structures of FL118 and camptothecin.

2.4. *Plate clone formation assay*

Cells (100/well) were seeded in 6-well plates and treated after 24 h. After 12 days, the cells were stained with methylrosanilinium chloride. The number of colonies containing 50 cells was counted under a microscope. Plate clone formation efficiency was calculated using the formula: plate clone formation efficiency = (number of colonies/number of cells inoculated) × 100%.

2.5. *Cell cycle analysis*

Cells were incubated in 6-well plates (3×10^4 cells/well) overnight and then treated with FL118 (100 nM) for 12, 24, 36, and 48 h. The cells were harvested and gently resuspended in a single cell suspension in fluorescence-activated cell sorting (FACS) buffer (PBS containing 2% FBS), followed by drop-wise fixation with 70% ethanol at 4°C overnight. Fixed cells were washed with cold PBS and incubated with RNaseA (10 mg/mL) and propidium iodide (PI, 1 mg/mL) at 37°C in the dark for 30 min, and then analyzed with FACS flow cytometer (Backman Coulter) (*24*). Each assay was repeated in three independent experiments.

2.6. *Transwell assay*

Cell invasions were analyzed in 24-well transwell chambers (8-μm pore size, Corning). About 10 μg/mL Matrigel Matrix (Corning Incorporated, New York, USA) was used to cover the upper side of the chamber. Cells at a concentration of 1×10^5/mL were suspended in 200 μL of serum-free medium and then seeded into the upper chamber, and 650 μL of medium with 15% fetal bovine serum was added to the lower chamber. After incubation for 48 h, cells that had not invaded were removed from the upper surface using cotton swabs. The cells that had invaded were fixed in methanol for 20 min, stained with 0.1% crystal violet, and counted under a phase contrast microscope (five different fields per chamber were examined).

2.7. *Wound healing assay*

Cells were seeded in 6-well plates at a density of 10^6

cells/well and allowed to reach 100% confluence. After treatment with mitomycin C (10 μg/mL, 12 h), a scratch wound was created on the cell surface using a 200-μL pipette tip. The detached cells were washed away with PBS. The medium was changed to serum-free RPMI-1640 with FL118 (10 or 100 nM), and the cells were continuously cultured for 48 h. The wound area was photographed with an inverted phase contrast microscope (Olympus; magnification, 40×) at 0, 12, 24, and 48 h. The migration distance was calculated using Image-Pro Plus.

2.8. *Western blot analysis*

The cells were washed twice with ice-cold PBS before being lysed in 200 μL of RIPA buffer containing protease and phosphatase inhibitors. A BCA Protein Kit (Beyotime biotechnology, Shanghai, China) was used to quantify protein concentrations. Equal amounts of protein were separated using 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skim milk in TBST for 2 h and incubated with a primary antibody overnight. The primary antibodies used in this study were as follows: anti-β-actin antibody 1:2,000; anti-survivin antibody 1:500; anti-E-cadherin antibody 1:1,000; anti-vimentin antibody 1:1,000; anti-lamin B1 antibody 1:2,000; anti-β-catenin antibody 1:5,000; and anti-cyclin Dl antibody 1:10,000. The membranes were then incubated with the appropriate HRP-conjugated secondary antibodies. Protein bands were detected with an eECL Western blot kit (CWBIO, China) and visualized using autoradiography on x-ray films (CWBIO, China).

2.9. *Quantitative real-time PCR*

Total RNA from treated cells was isolated using TRIzol reagent (CWBIO, China) in accordance with the manufacturer's instructions. The quality and quantity of total RNA were determined with a Nano-Drop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was reverse transcribed into cDNA using the FAST Quant RT kit (TIANscript, Beijing, China). Quantitative real-time PCR reactions were carried out on a CFX96 Touch™ Deep Well Real-time PCR Detection System (BIO- RAD, California, USA). The primers used in this study were: Survivin: sense 5'-ATACCAGCACTTTGGGAGG-3' and antisense 5'-AGAAAGGAAAGCGCAACC-3'; Vimentin: sense 5'-GGAAGAGAACTTTGCCGTTG-3' and antisense 5'-TGGTATT CACGAAGGTGACG-3'; E-Cadherin: sense 5'-CTGAGAACGAGGCTAACG-3' and antisense 5'-GTCCACCATCATCATTCAATAT-3'; β-catenin: sense 5'-TGGTGA CAGGGAAGACATCA-3' and antisense 5'-CCATAGTGAAGGCGAACTGC-3'; cyclin D1: sense 5'-GCGAGGAACAGAAGTGCG-3' and antisense 5'-GGAGTTGT CGGTGTAGATGC-3'; and β-actin: sense 5'-ACTCTTCCAGCCTTCCTTC-3' and anti-sense 5'-ATCTCCTTCTGCATCCTGTC-3'. Following normalization to β-actin, levels of target gene expression were calculated using the $2^{-\Delta\Delta CT}$ method.

2.10. *Immunocytochemistry*

Cells (10^5/well) were cultured with or without 10 nM FL118 on chamber slides for 48 h and then rinsed three times with PBS. The cells were subsequently fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.3% Triton X-100 for 10 min, and then incubated with primary antibodies against survivin, E-cadherin, vimentin (Santa Cruz, CA, USA), β-catenin, and cyclin Dl (ABCAM, Cambridge, MA, USA) overnight at 4°C. The cells were rewashed and incubated with the appropriate secondary antibodies (ABGENT, San Diego, USA) for 1 h in room temperature. Finally, the cells were washed and restained with DAB for 3-5 min, followed by nuclear staining with hematoxylin. The cover slips were observed under a microscope (Eclipse E-800, Nikon, Japan).

2.11. *Statistical analysis*

Assay results were the average of at least 3 replicates from three independent experiments. The data were analyzed with the Student's *t* test and are presented as mean ± SD. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. *FL118 inhibited the proliferation of MDA-MB-231 cells*

The effect of FL118 on proliferation of MDA-MB-231 cells was examined first. An MTT assay was used to detect the survival rate of MDA-MB-231 cells after treatment with FL118. Results indicated that FL118 inhibited the growth of MDA-MB-231 cells in a dose- and time-dependent manner (Figure 2A). In MDA-MB-231 cells, the IC_{50} of FL118 was 414.3 nM at 48, 102.5 nM at 72 h, and 41.1 nM at 96 h. In a plate clone formation assay, FL118 suppressed colony formation by MDA-MB-231 cells in a dose-dependent manner (Figure 2B). The number of clones that formed decreased significantly after FL118 treatment compared to that in the control. To further investigate the mechanism of FL118-mediated inhibition of the proliferation of MDA-MB-231 cells, cell cycle distribution was analyzed using flow cytometry at 12, 24, 36, and 48 h. Results indicated that FL118 treatment resulted in a reduction in the percentage of cells in the G_0/G_1 and G_2/M phase, while the percentage of S

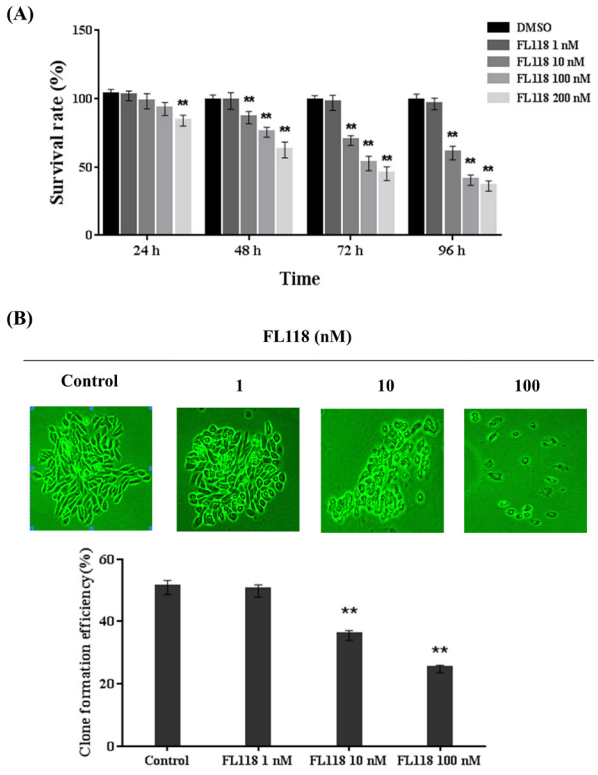

Figure 2. FL118 inhibited the proliferation of MDA-MB-231 cells. (A) The cells were exposed to various concentrations of FL118 for 24, 48, 72, and 96 hours and then subjected to an MTT assay. **(B)** The cells were exposed to various concentrations of FL118 for 12 days and their colony formation activity was evaluated using a plate clone formation assay. $^{**}p < 0.01$ vs. control.

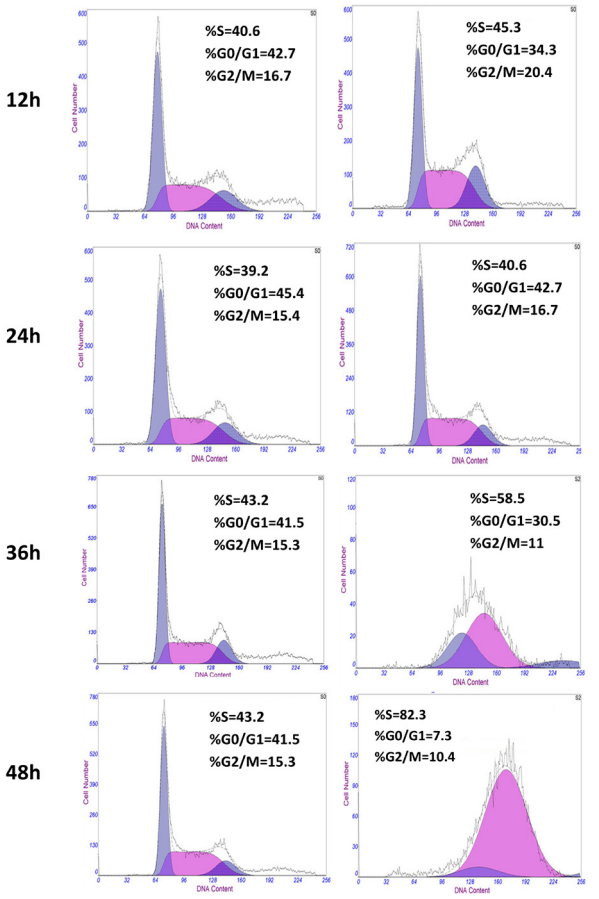

Figure 3. Cell cycle distribution of MDA-MB-231 cells after FL118 treatment. The cells were exposed to 100 nM of FL118 for 12, 24, 36, and 48 h. Cell cycle distribution in the control group (left) and the treatment group (right) was examined using flow cytometry.

phase increased significantly (Figure 3). These results suggested that FL118 suppressed the proliferation of breast cancer cells by halting the cell cycle in the S phase.

3.2. *FL118 suppressed the invasive and migratory capabilities of MDA-MB-231 cells*

Tumor cell invasion and migration are critical steps in the metastatic process (*25*). To assess the ability of breast cancer cells to invade Matrigel, a transwell insert system was used to monitor the process of invasion. In this assay, a low concentration of FL118 that had little effect on cell proliferation was used. After treatment with 10 nM of FL118 for 48 h, the number of invading MDA-MB-231 cells decreased markedly in comparison to the control group, as shown in Figure 4A. The effect of FL118 on cancer cell migration was examined using a wound healing assay. Results indicated that cells treated with FL118 at 10 nM or 100 nM had a markedly reduced migratory capacity compared to that of the control group 24 and 48 h after wound creation (Figure 4B). Taken together, these results suggest that FL118 inhibits the invasion and migration of MDA-MB-231 cells at low concentrations.

3.3. *FL118 increased E-cadherin and decreased vimentin expression in MDA-MB-231 cells*

The effect of FL118 on the expression of EMT markers, including E-cadherin and vimentin, was examined in MDA-MB-231 cells using Western blotting, qRT-PCR, and an immunocytochemistry assay. As shown in Figure 5A, the results of Western blotting indicated that 10 or 100 nM of FL118 significantly decreased vimentin expression while markedly increasing E-cadherin expression. Expression of vimentin and E-cadherin mRNA in MDA-MB-231 cells was examined using qRT-PCR. Results indicated that FL118 reduced the level of vimentin mRNA while increasing the level of E-cadherin, which is consistent with the results of Western blotting (Figure 5B). After treatment with FL118, the expression profiles of EMT-related markers were verified using an immunocytochemistry assay. Consistent with the above results, the immunocytochemistry assay indicated that the expression of vimentin decreased significantly while expression of E-cadherin increased when MDA-MB-231 cells were treated with 10 or 100 nM FL118 for 48 h (Figure 5C). All of these results suggest that

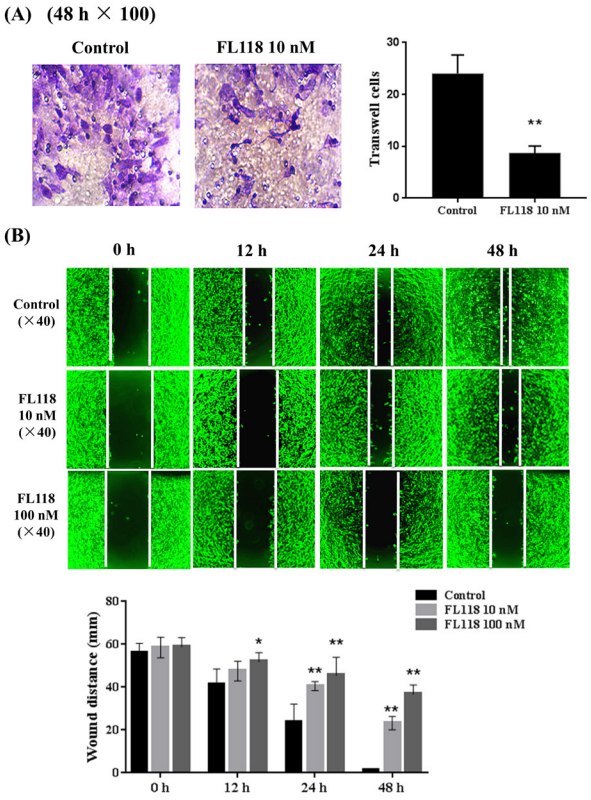

Figure 4. FL118 suppressed invasion and migration of MDA-MB-231 cells. (A) Effect of FL118 on cell invasion was examined using a transwell assay. The cells were exposed to 10 nM of FL118 for 48 h, and images (100×) from a Matrigel-coated Boyden chamber were used to count invading cells. $^{**}p < 0.01$ *vs*. control. **(B)** The effect of FL118 on cell migration was determined using a wound healing assay. The cells were exposed to 10 or 100 nM of FL118 for 12, 24, and 48 h, respectively, and the wound width was measured at each time point. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs*. control.

low concentrations of FL118 that had little effect on cell proliferation markedly suppressed the EMT of MDA-MB-231 cells.

3.5. *FL118 decreased the levels of β-catenin, survivin, and cyclin D1 in MDA-MB-231 cells*

To investigate whether FL118 suppressed EMT *via* the Wnt/β-catenin pathway, Western blotting and qRT-PCR were used to examine changes in protein and mRNA levels of β-catenin and its downstream targets survivin and cyclin D1. After treatment with 10 or 100 nM of FL118 for 48 h, the levels of β-catenin, survivin, and cyclin D1 protein and mRNA were down-regulated in MDA-MB-231 cells (Figure 6A and 6B). Expression of β-catenin, survivin, and cyclin D1 was verified using an immunocytochemistry assay. Results indicated that β-catenin, survivin, and cyclin D1 expression decreased significantly after treatment with FL118 (10 nM) for 48 h. These results suggest that FL118 downregulated the Wnt/β-catenin pathway, which may have contributed to the inhibition of cell migration and invasion by FL118.

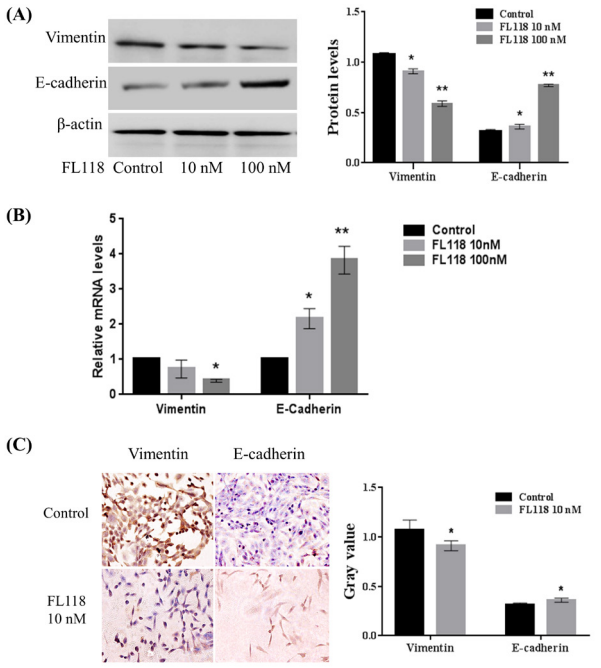

Figure 5. FL118 increased E-cadherin while decreasing vimentin expression in MDA-MB-231 cells. The cells were exposed to 10 or 100 nM of FL118 for 48 h, and E-cadherin and vimentin expression was examined using Western blotting **(A)**, qRT-PCR **(B)**, and an immunocytochemistry assay **(C)**, respectively. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs*. control.

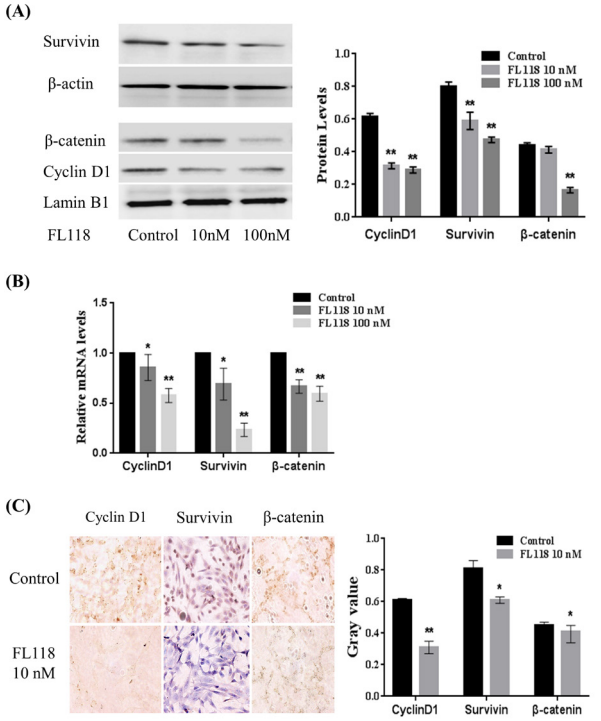

Figure 6. FL118 decreased the levels of β-catenin, survivin, and cyclin D1 in MDA-MB-231 cells. The cells were exposed to 10 or 100 nM of FL118 for 48 h, and β-catenin, survivin, and cyclin D1 expression was examined using Western blotting **(A)**, qRT-PCR **(B)**, and an immunocytochemistry assay **(C)**, respectively. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs*. control.

4. Discussion

Invasion and metastasis remains a challenge in the treatment of breast cancer (*26*). The MDA-MB-231 cell line is a well-established cell line used to examine cancer metastasis (*27*), which is why the current study examined the effects of FL118 on that cell line. FL118 at low concentrations that had little effect on cell proliferation significantly suppressed migration and invasion by MDA-MB-231 cells. FL118 decreased the level of vimentin expression while increasing the level of E-cadherin, suggesting that it reversed the process of EMT. Furthermore, FL118 reduced the level of expression of β-catenin and its downstream targets cyclin D1 and survivin, implying that the Wnt/β-catenin signaling pathway might play a role in the antitumor activity of FL118.

EMT plays a key role in tumor metastasis and progression. In this significant biological process, epithelial cells lose their polarity and cell-cell adhesion, and those cells acquire migratory and invasive properties to become mesenchymal stem cells (*28*). Loss of the epithelial marker E-cadherin and acquisition of the mesenchymal marker vimentin are considered to be important characteristics of EMT (*28*). E-cadherin is an adhesion molecule expressed in the epithelioid cell phenotype and it plays a key role in the process of cancer invasion. Low expression of E-cadherin might significantly enhance the invasion and metastasis of breast cancer (*29*). Vimentin is also an important marker of mesenchymal cells. The up-regulation of vimentin is closely related to invasion by and metastasis and EMT of breast cancer cells (*30*). The current results indicated that FL118 effectively increased the expression of E-cadherin and it decreased the expression of vimentin in MDA-MB-231 cells, supporting the contention that FL118 suppresses cell migration and invasion by inhibiting EMT.

The Wnt/β-catenin pathway is one of the key signaling pathways triggering EMT, and survivin and cyclin D1 are known downstream targets of Wnt/β-catenin signaling (*31*). The current study thus explored whether Wnt/β-catenin signaling was involved in the anti-invasive activity of FL118. Results indicated that levels of β-catenin expression and its downstream targets cyclin D1 and survivin were significantly suppressed by FL118. FL118 may accelerate the degradation of β-catenin in the cytoplasm, thereby suppressing the translocation of β-catenin into the nucleus and thus suppressing the expression of downstream targets. A study has indicated that survivin is an evolutionarily conserved activator of cell migration, invasion, and metastatic dissemination (*32*). Cyclin D1 can activate the downstream gene Snail and mediate the occurrence of EMT (*33,34*). Thus, blocking of the Wnt/β-catenin signaling pathway by FL118 presumably contributed to its suppression of migration and invasion of breast cancer cells.

In conclusion, this study found that FL118 suppressed migration and invasion of breast cancer cells by inhibiting EMT *via* the Wnt/β-catenin signaling pathway. These findings revealed new molecular mechanisms for the anticancer activity of FL118 and provide an experimental basis for the further development of FL118.

Acknowledgements

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