Amlodipine inhibits proliferation, invasion, and colony formation of breast cancer cells

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Abstract. Calcium channel upregulation has been implicated in cancer cell proliferation and progression including in breast cancer. Fortunately, the function of calcium channels can be manipulated pharmacologically using calcium channel blockers (CCBs). Amlodipine, a dihydropyridine CCB, has been demonstrated to exert cytotoxic effects in several types of cancers. The present study evaluated the effects of amlodipine on proliferation, caspase activation, colony formation, and invasion of human breast cancer cells. Cell viability was assessed using a colorimetric MTT assay. An Apo-ONE® caspase-3/7 assay was used to measure caspase-3/7 levels. Cell invasion was evaluated using Matrigel invasion chambers. The expression of phospho-(p-)ERK1/2, Bcl-2, and integrin β1 proteins were analyzed using western blotting. A one-way ANOVA with a post-hoc Tukey's multiple comparison tests was used for statistical analysis. Amlodipine significantly inhibited the growth of both MDA-MB-231 and MCF-7 human breast cancer cells in a dose-dependent manner and inhibited colony formation of MCF-7 cells, and this was accompanied by the downregulation of p-ERK1/2 in MDA-MB-231 cells. In addition, treatment with amlodipine resulted in increased caspase-3/7 levels in MDA-MB-231 cells, which was accompanied by the downregulation of the anti-apoptotic protein, Bcl-2. Moreover, amlodipine impaired the invasive abilities of MDA-MB-231 cells, and integrin β1 expression was concurrently downregulated. The present study illustrates the anticancer effects of amlodipine on breast cancer proliferation, colony formation, and invasion *in vitro* and highlights the potential value of amlodipine as an anticancer agent.

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Introduction

Globally, breast cancer is the most prevalent cancer, accounting for 11.7% of all cancer cases and 6.9% of cancer deaths (1). It has been estimated that 284,200 new breast cancer cases were diagnosed in 2021 in the US (2). In Jordan, 2,403 new breast cancer cases were diagnosed last year accounting for 38.5% of all cases of cancer in women (3). Tumor metastasis is a major cause of breast cancer mortality (4). This multi-step process encompasses local tumor invasion, migration of primary cells, and colonization at distal sites (5). Metastatic breast cancer (MBC) represents 6% of newly diagnosed breast cancer cases. However, 20-30% of early-stage breast cancer cases eventually develop into MBC (6). Systemic therapies are the primary treatment options for MBC including chemotherapy, endocrine therapy, and targeted therapy (7). Although combination chemotherapy is commonly used in MBC, it has been associated with increased toxicity (8,9). Furthermore, the emergence of chemotherapeutic resistance limits the effectiveness of breast cancer treatments, thereby increasing disease relapse and death (10). Therefore, the identification of novel strategies targeting the primary tumor with enhanced efficacy and reduced toxicity are needed to improve patient outcomes.

Calcium channel blockers (CCB) have been implicated as anti-cancer molecules in several types of human cancers. For example, amlodipine, a dihydropyridine CCB, has been shown to induce apoptosis, resulting in cell cycle arrest, and suppress the proliferation of cancerous cells in several studies (11-13). In addition, Ji *et al* (14) showed that p-glycoprotein-mediated multidrug resistance could be ameliorated in leukemic cells when an amlodipine derivative was used, which in turn prevented doxorubicin efflux and thus enhanced its efficacy. Moreover, *in vitro* and *in vivo* studies on human epidermoid cancerous cells have shown that several CCBs can inhibit cancer cell growth including amlodipine, nicardipine, and nimodipine (15). However, the exact cellular and molecular anticancer mechanisms of amlodipine have not been studied

in breast cancer cells. In the present study, the effects of amlodipine treatment on breast cancer cell proliferation, apoptosis, colony formation, and invasion were evaluated, and the protein expression levels of the downstream targets were determined as well.

Materials and methods

Cell culture and drug treatment. Triple-negative MDA-MB-231 and luminal MCF-7 breast cell lines were purchased from the American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 media supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified incubator with 5% CO_2 at 37˚C. DMSO was used as a solvent to prepare amlodipine stocks (Tocris Bioscience), with a final DMSO concentration of <0.1% in all experiments.

Cell viability assay. To evaluate the effects of amlodipine on breast cancer cell viability, the colorimetric MTT cell proliferation assay (ATCC) was performed as described previously (16). Briefly, $1x10^4$ cells/well were cultured in a 96-well plate and incubated overnight. Then, cells were treated with several concentrations of amlodipine or with DMSO as a control. After 48 h of treatment, cells were incubated at 37˚C for 4 h with MTT solution at a final concentration of 500 μg/ml. To solubilize formazan crystals, 100 μl DMSO was added to each well. The optical density was measured at 490 nm on a microplate reader (BioTek Instruments, Inc.). The results are expressed as a percentage of viable cells normalized to vehicle-treated cells using the following equations: % Of viable cells in each well=(Absorbance $_{\text{treatment}}$/Average of Absorbance $_{\text{vehicle in 4 replicates}}$)x100; and % of viable cells for each treatment concentration=Average of normalized % of viable cells in 4 treatment replicates.

Caspase-3/7 assay. An Apo-ONE® homogeneous caspase-3/7 assay (Promega Corporation) was used to assess the effects of amlodipine on the induction of caspase-3/7 activities in MDA-MB-231 cells as described previously (17). Briefly, cells were plated at a density of $1x10^4$ cells/well in a 96-well black plate. After attachment, cells were treated with several concentrations of amlodipine or with DMSO as a control. After 48 h of treatment, the caspase-3/7 reagent was added to each well in a 1:1 ratio with the sample volume at room temperature. After 3 h of incubation, enzyme activity was analyzed using a synergy 2 multi-mode microplate reader (Biotek Instruments, Inc.) at excitation and emission wavelengths of 499 nm and 521 nm, respectively.

Colony formation assay. Assessing anchorage-dependent growth of breast cancer cells was performed using colony formation assays as previously described (18,19). MCF-7 cells were plated at a low density ($2x10^3$ cells/flask) in T25 flasks and incubated for 24 h, then treated with amlodipine or DMSO as a control. Culture media was replaced every 3 days. Following 3 weeks of incubation, PBS was used to wash the cells before fixing them with pre-cooled (1:1) methanol/acetone at -20˚C for 15 min. After staining with 0.1% crystal violet for 5 min at room temperature, the colonies that had formed were visualized using a light microscope (x4 magnification).

Invasion assay. Invasion assays were performed using Corning BioCoat Matrigel Invasion Chambers (Corning Inc.) as described previously (20,21). Cells were resuspended in serum-free media with various concentrations of amlodipine (0, 5 or 10 μM), and a chemotactic serum gradient was generated by placing media supplemented with 10% FBS in the bottom chambers. After 24 h, the invading cells were fixed with ice-cold ethanol at -20˚C for 15 min, stained with 0.1% crystal violet for 5 min at room temperature, visualized using a light microscope (x4 magnification) and counted using ImageJ (version 1.53q; National Institutes of Health). The results are expressed as a percentage of invading cells in treatment groups relative to the control group.

Western blotting. To assess the effects of amlodipine on the protein expression levels of downstream targets, western blotting was performed as previously described (21). Briefly, cells were plated at a density of $5x10^4$ cells/well into a 6-well plate. The following day, the cells were treated with amlodipine (1-25 μM) or DMSO as a control for 48 h. After cell washing with ice-cold PBS, cells were lysed in RIPA buffer (Thermo Fisher Scientific, Inc.) containing protease inhibitor (150 μl/well for 30 min on ice). Cell lysates were transferred into Eppendorf tubes and then centrifuged at 13,000 x g for 5 min at 4˚C. Proteins were quantified in all collected supernatants using a BCA assay. Protein samples were loaded in equal amounts (35 μg per lane) with one lane for 10 μl of the protein ladder into 10% polyacrylamide gels in tris-glycine buffer and run at 200 mv for 40 min at room temperature. After the proteins had been resolved, they were transferred to nitrocellulose membranes, incubated with primary antibodies (all 1:1,000 dilution) for 2 h at room temperature against phospho (p-)ERK1/2 (Cell Signaling Technology, Inc.; cat. no. 5726), ERK1/2 (Cell Signaling Technology, Inc.; cat. no. 9102), Bcl-2 (Cell Signaling Technology, Inc.; cat. no. 3498), and integrin β1 (Cell Signaling Technology, Inc.; cat. no. 4706). GAPDH was used as the loading control (Cell Signaling Technology, Inc.; cat. no. 5174). After washing with TBST buffer, membranes were incubated with the secondary horseradish peroxidase-conjugated antibodies (1:1,000) for 1 h at room temperature [anti-rabbit IgG (cat. no. 7074) or anti-mouse IgG (cat. no. 7076)]. An enhanced chemiluminescent detection kit was used to visualize the immunoreactive protein bands using the Montreal Biotech Fusion Pulse 6 imaging system (Montreal Biotech Inc.). All experiments were repeated three times.

Statistical analysis. Data were analyzed using GraphPad Prism version 9 (GraphPad Software, Inc.). A one-way ANOVA followed by a Tukey's multiple comparison test was used to compare the difference between multiple groups. The half-maximal inhibitory concentration (IC_{50}) values were obtained by applying a nonlinear regression curve fit analysis. $P<0.05$ was considered to indicate a statistically significant difference. Data are presented as the mean ± SEM.

Results

Cytotoxic effects of amlodipine on breast cancer cells. The *in vitro* biological effects of amlodipine treatment on

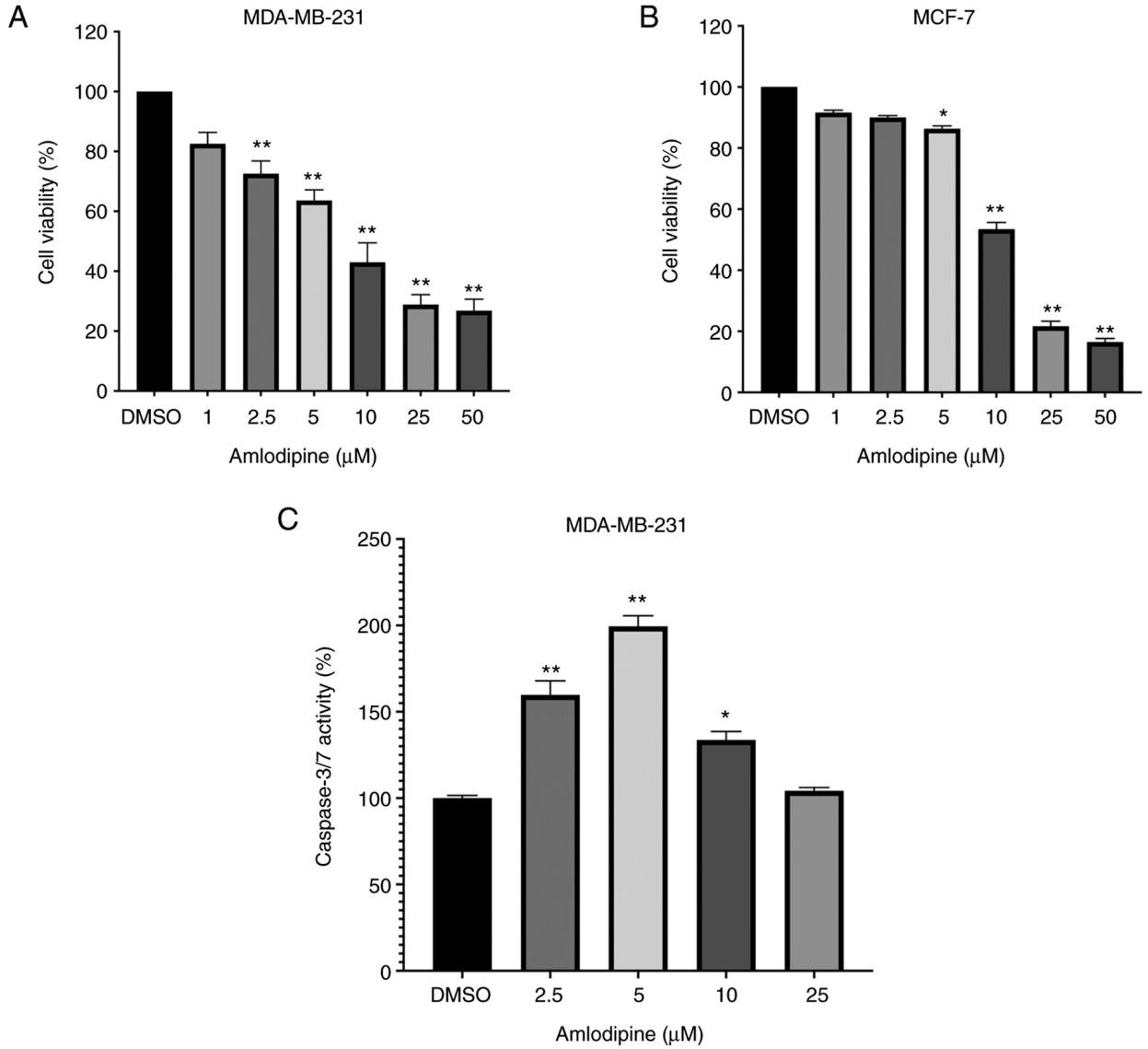

Figure 1. Effect of amlodipine on cell proliferation and caspase-3/7 activity in breast cancer cells. Percentage cell viability of (A) MDA-MB-231 and (B) MCF-7 cells after 48 h of amlodipine treatment. (C) Percentage of caspase-3/7 activity in in MDA-MB-231 cells after 48 h of amlodipine treatment. Data are presented as the mean ± SEM of three independent experiments. *P<0.05, **P<0.01.

MDA-MB-231 and MCF-7 cell proliferation are shown in Fig. 1. Amlodipine treatment reduced MDA-MB-231 and MCF-7 cell viability in a dose-dependent manner. In MDA-MB-231 cells, treatment with 2.5-50 μM amlodipine significantly reduced cell viability compared with the control-treated cells (P<0.05, Fig. 1A). In MCF-7 cells, 5-50 μM amlodipine significantly reduced cell viability compared with the control-treated cells (P<0.05, Fig. 1B). The IC_{50} values for amlodipine in MDA-MB-231 and MCF-7 cells were 8.66 and 12.60 μM, respectively. These findings suggest a cytotoxic effect of amlodipine on breast cancer cells.

Since amlodipine reduced breast cancer cell viability, the potential underlying mechanisms of the growth suppression were assessed by analyzing caspase-3/7 activity, which is a well-established marker of apoptosis (22). The results revealed that amlodipine treatment (2.5-10 μM) significantly increased caspase-3/7 activity compared with the control treatment in MDA-MB-231 cells (P<0.05, Fig. 1C) and thus highlighted caspase activation as a potential mechanism underlying the cytotoxic effects of amlodipine. However, caspase-3/7 activity was not assessed in the MCF-7 cells since they do not express caspase-3, and thus caspase activation may be underestimated (23,24).

To further determine the anticancer effects of amlodipine in cell proliferation, whether amlodipine could modulate anchorage-dependent growth was assessed using colony formation assays. Although it may be considered a limitation of the present study for MCF-7 cells to have a high capacity to form colonies compared to MDA-MB-231 cells (25), the MCF-7 clonogenic ability was still assessed following amlodipine treatment. The effect of amlodipine on colony formation of MCF-7 cells is shown in Fig. 2A and B. Amlodipine markedly inhibited colony formation in a dose-dependent manner in MCF-7 cells. Treatment with 2.5-10 μM amlodipine significantly decreased the colony size compared with the control (P<0.05). These findings indicate the ability of amlodipine to suppress the clonogenic proliferation of breast cancer cells over a prolonged period of time.

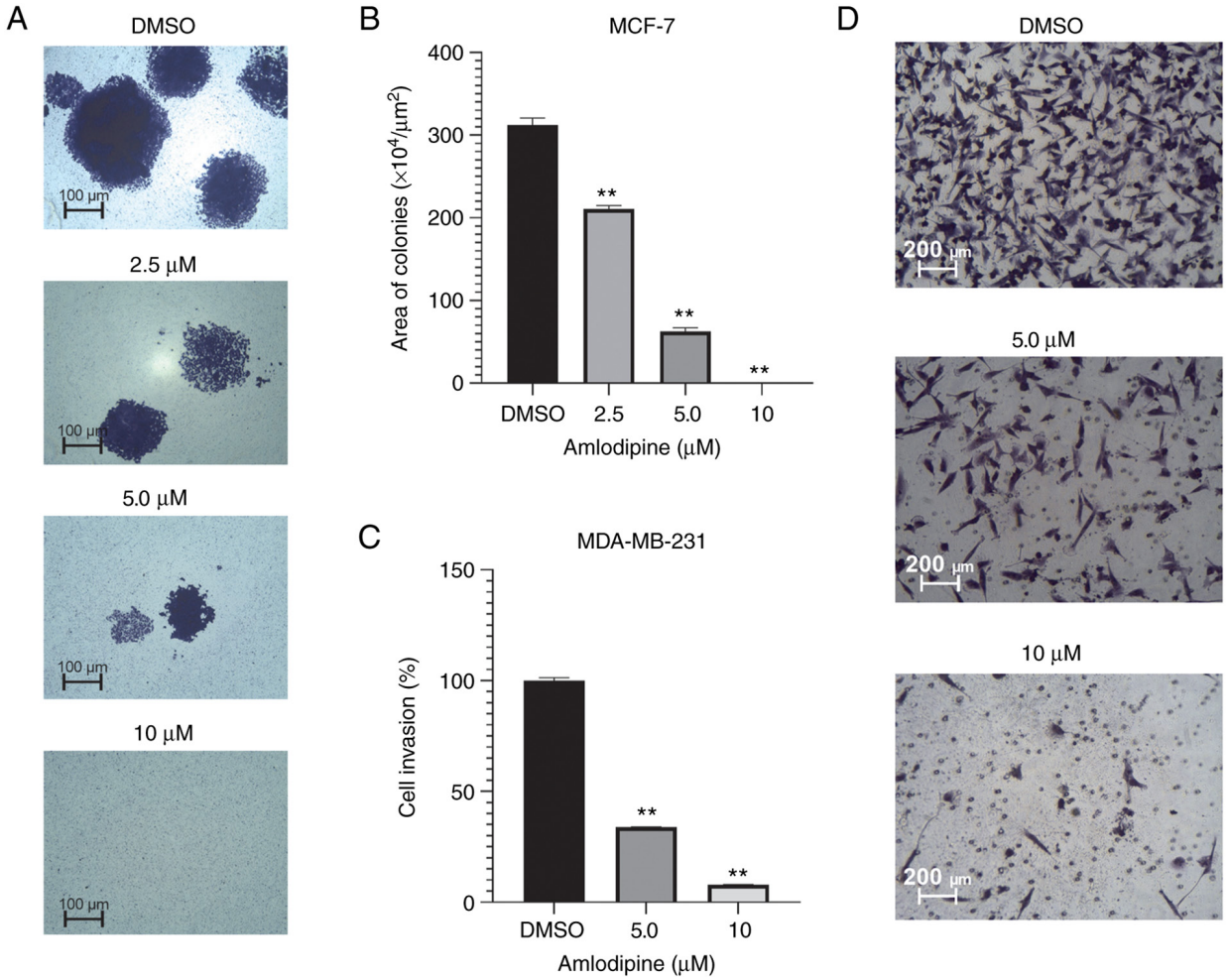

Figure 2. Effect of amlodipine on colony formation and invasion of breast cancer cells. (A) Representative images of colony formation and (B) quantitative analysis of the area of the colonies formed by MCF-7 cells following treatment with amlodipine (2.5, 5 or 10 μM) compared with the control treated cells. Scale bar, 100 μm. (C) Quantitative analysis of cell invasion percentage and (D) representative microscopic images for cell invasion by MDA-MB-231 cells treated with amlodipine (5 or 10 μM) compared with the control treated cells. Scale bar, 200 μm. **P<0.01.

Effect of amlodipine on the invasion of breast cancer cells. To mimic the *in vivo* process of cell invasion through the extracellular matrix, the effects of amlodipine on the invasive abilities of MDA-MB-231 cells were evaluated using Matrigel invasion chambers. As shown in Fig. 2C and D, amlodipine significantly suppressed MDA-MB-231 cell invasion in a dose-dependent manner. Treatment with 5 and 10 μM amlodipine significantly reduced MDA-MB-231 invasiveness by >60 and 90% compared with the control-treated cells, respectively. Since MCF-7 cells are not highly invasive cells (25), invasion assays were not performed using these cells, which is considered a limitation of our study. These results provide robust evidence of the anti-invasive effects of amlodipine on breast cancer cells *in vitro*.

Anticancer effects of amlodipine may be mediated via ERK1/2, integrin β1, and Bcl-2 inhibition. To further shed light on the potential signaling molecules driving the anticancer effects of amlodipine on breast cancer cells, the expression levels of key proteins involved in cell proliferation, apoptosis, and invasion were evaluated using western blotting. The results indicated that amlodipine reduced the protein expression levels of the anti-apoptotic protein Bcl-2, in both MDA-MB-231 and MCF-7 cells compared with the control (Fig. 3). As MCF-7 cells are not highly invasive cells, p-ERK1/2 and integrin β1 protein expression levels were only assessed in MDA-MB-231 cells. Amlodipine treatment reduced ERK1/2 phosphorylation in MDA-MB-231 cells compared with the control treatment. Moreover, amlodipine reduced the protein expression levels of integrin-β1 in MDA-MB-231 cells compared with the control treatment. These findings may partially explain the possible molecular drivers of the anti-cancer effects of amlodipine.

Discussion

Voltage-activated calcium channels are widely distributed in all types of human cells (26). Several studies have shown that calcium channel expression is altered as an adaptive mechanism in human cancers such as in breast, prostate, and colorectal cancer (26-28). Interestingly, recent studies have implicated calcium channels in cancer cell proliferation, invasion, and metastasis (29-31). Recent studies have also shown that calcium channel expression is upregulated in breast cancer cells (31,32). In the present study, treatment of breast cancer cells with the CCB, amlodipine, resulted in a dose-dependent reduction in breast cancer cell viability.

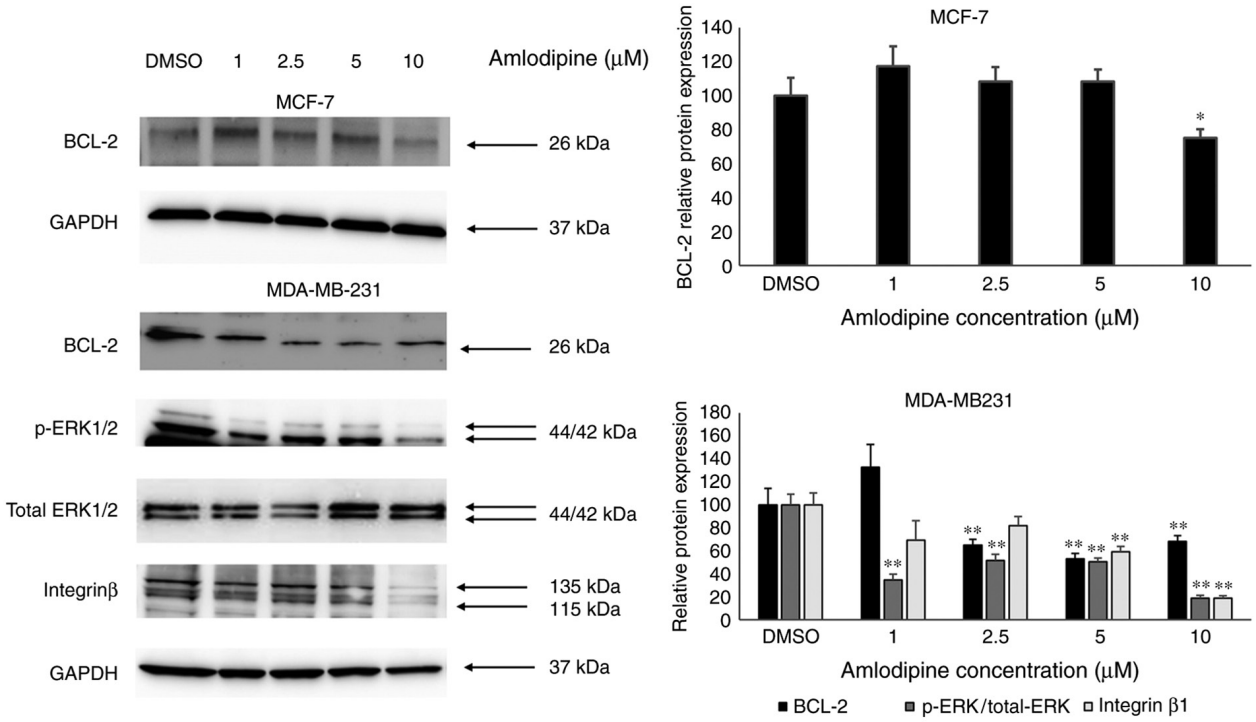

Figure 3. Effects of amlodipine treatment on the protein expression levels of Bcl-2, p-ERK1/2 and integrin β1 in breast cancer cells. Western blotting was used to examine the changes in the expression levels of the relevant proteins in MCF-7 and MDA-MB-231 cells following 48-h treatment with 1-10 μM amlodipine. Western blot analyses and quantifications with statistical analysis are shown. *P<0.05, **P<0.01. p-, phospho.

Similarly, recent studies have shown that silencing calcium channel expression inhibited breast cancer cell growth both *in vitro* and *in vivo* (31,32).

To ascertain the underlying mechanism(s) of amlodipine-induced growth suppression, breast cancer cellular apoptosis was assessed by measuring caspase-3/7 activity. The results showed that amlodipine induced caspase-3/7 activity in MDA-MB-231 cells, which may contribute to caspase-dependent apoptosis. Although this finding was limited by a lack of flow cytometry analysis to confirm the occurrence of apoptosis, activation of caspase-3/7 pathways was accompanied by downregulation of the anti-apoptotic protein Bcl-2, which strongly indicated that caspase-dependent apoptosis occurred in the breast cancer cells following amlodipine treatment. The Bcl-2 gene promotes cell survival and protects cells against apoptosis. High expression of Bcl-2 is associated with lower apoptosis-mediated death and contributes to resistance to chemotherapy. Moreover, Bcl-2 protein expression is typically altered in breast cancer cells (33,34). In agreement with the findings of the present study, a previous study demonstrated that amlodipine treatment induced apoptosis in MDA-MB-231 cells via downregulation of Bcl-2 protein expression (35). In addition, activation of caspase-dependent apoptosis has been reported with other dihydropyridine CCBs (36). Moreover, Wong *et al* (36) reported that treating cancer cells with calcium channel inhibitors may also lead to caspase-independent apoptosis. In the present study, amlodipine treatment of breast cancer cells resulted in caspase-dependent apoptosis as shown by the activation of caspase3/7. However, the increase in caspase3/7 activity appeared to decrease at higher concentrations (10-25 μM), which could be due to the dominance of caspase-independent apoptosis at higher concentrations.

To further illustrate the antiproliferative effect of amlodipine, the tumorigenic ability of breast cancer cells was assessed using colony formation assays whilst being treated with amlodipine. The inhibitory effects of amlodipine on colony formation were notable in MCF-7 cells and in agreement with previous findings in gastric cancer (37). To the best of our knowledge, this is the first study to provide proof of the inhibitory effects of amlodipine on breast cancer colony formation. In the present study, the effects of amlodipine on breast cancer cell proliferation and colony formation were accompanied by a reduction in ERK1/2 phosphorylation. Recent studies have also shown the inhibitory effects of amlodipine and other CCBs on the ERK1/2 pathway in gastric cancer (38), hepatic cancer (39), ovarian cancer (40), and melanoma (41). Taken together, the current and previous studies highlight the suppressive effects of amlodipine on cell proliferation, resistance to apoptosis, and tumorigenic potential via inhibition of major signaling proteins such as ERK1/2 and Bcl-2.

Previous studies have implicated calcium channels in breast cancer cell adhesion and invasion (30,31,42). For example, silencing calcium channel expression in breast cancer cells has been associated with a reduction in cell motility and adhesion (31). In the present study, amlodipine significantly reduced MDA-MB-231 breast cancer cell invasion. Filopodia structures are finger-like cytoplasmic projections that extend beyond the cell's edge and promote cancer cell invasion (42,43). The results of the present study are consistent with a recent study that showed the ability of several CCBs, including amlodipine, in inhibit filopodia formation and thus impairing breast cancer cell invasion (30). In the present study, the anti-invasive effects of amlodipine were accompanied by downregulation in p-ERK1/2 and integrin β1 protein expression. These proteins

are well-established key players in breast cancer cell migration, invasion, and metastasis (44-46). Together, these findings suggest that the anti-invasive effects of amlodipine are mediated via at least the inhibition of p-ERK1/2 and integrin β1 expression.

In conclusion, the results of the present study showed that amlodipine exerted anticancer effects on cell proliferation, colony formation, and invasion, and they were, at least in part, achieved by the inhibition of p-ERK1/2, integrin β1, and Bcl-2 expression and activation of caspase-3/7, indicating the induction of caspase-dependent apoptosis. This study highlights amlodipine as a potential therapeutic agent for the management of breast cancer and may provide novel insights for future research on the effects of amlodipine in the sensitization of breast cancer cells to chemotherapy.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

MAYA was involved in the study conception and design. MAYA, RAS and MA performed the experiments. MAYA, RAS, and KHA conducted the data analysis. MAYA and RAS wrote the first draft of the manuscript. All authors revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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