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Molecular Effects of The Calcium Channel Blocker Amlodipine on the Sensitivity of Breast Cancer Cells to Doxorubicin

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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. Methods: We examined the effects of DOX alone or in combination with Amlodipine (AMLO) on the viability of the MCF-7 cells using MTT assay, programmed cell death, and the expression of the anti-apoptotic gene (Bcl-2) and the pro-apoptotic gene (Bax) by quantitative reverse transcription polymerase chain reaction. Combination index (CI) values were calculated using CompuSyn. Results: we found that adding AMLO to DOX potentiated its antiproliferation effect. The value of the combination index (CI) of DOX/AMLO was less than 1 indicating a synergistic effect. Combined DOX/AMLO treatment also caused potentiated apoptosis more than DOX-single treatment. At the molecular levels, DOX/AMLO treatment downregulated the mRNA of Bcl-2; while upregulated the Bax gene compared with DOX alone. Conclusion: the results confirmed the potential of AMLO in sensitizing Breast cancer to DOX by targeting suppressing the Bcl-2 gene while upregulating the Bax gene. Additionally, AMLO could be repurposed to reduce the therapeutic doses of DOX as indicated by the dose reduction index (DRI) and subsequently decrease its side effects (especially cardiotoxicity), along with chemosensitization of breast cancer cells to DOX treatment.

Keywords: Apoptosis, calcium channel blocker, chemoresistance, doxorubicin, Amlodipine.

Introduction

Cancer is a disease related to growth abnormalities with the possibility of conquering other parts of the body other than the place of its origin. It happens after long-term complicated process that starts in a single somatic cell by the accumulation of several DNA alterations to finally come out with a group of cells with unchecked, unorganized, and uncontrolled growth [1]. Breast cancer is the most common cause of cancer in women and the second most common cause of cancer death in women in the U.S. Breast cancer refers to cancers originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk. Worldwide, breast cancer comprises 10.4% of all cancer incidences among women, making it the second most common type of non-skin cancer (after lung cancer) and the fifth most common cause of cancer death. In 2004, breast cancer caused 519,000 deaths worldwide (7% of cancer deaths; almost 1% of all deaths) [2]. Doxorubicin is an antibiotic derived from the Streptomyces peucetius bacterium. It has widespread use as a chemotherapeutic agent since the 1960s. Doxorubicin is part of the anthracycline group of chemotherapeutic agents. Doxorubicin may be used to treat soft tissue and bone sarcomas and cancers of the breast, ovary, bladder, and thyroid. It is also used to treat acute lymphoblastic leukemia, acute myeloblastic leukemia, Hodgkin lymphoma, and small cell lung cancer [3]. DOX is one of the most used chemotherapeutic agents, particularly in advanced or metastasis cancer patients. Mechanically, DOX represses topoisomerase II (Top II) and intercalates directly to DNA double-strand, finally, resulting in the intervention of gene transcription [4]. Cardiotoxicity is the most important side effect of doxorubicin, which is one of the most dangerous dose-limiting toxicities of this drug [5]. Several studies have been conducted to find new strategies to maximize clinical efficacy while limiting side effects of doxorubicin [6]. The second evident problem using DOX is the acquired tumor resistance against it [7]. The development of chemotherapy resistance in Breast Cancer is mediated by multiple signaling pathways involved in the induction of proliferation, cell cycle progression and prevention of apoptosis [8]. Intracellular calcium ions (Ca^{+2}), the most abundant second messenger in the human body, have a substantial diversity of roles

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in fundamental cellular physiology, including gene expression, cell cycle control, cell motility, autophagy and apoptosis [9]. Ca^{+2} signaling could regulate pathways such as proliferation and apoptosis suggesting that therapies that modulate Ca^{+2} signaling in cancer cells might be a therapeutic option. Ca^{+2} channels or pumps with altered expression in cancer are obvious targets [10]. By silencing calcium channels by calcium channel blockers such as amlodipine to increase apoptosis that can be used to increase the responsiveness of human cancers toward chemotherapy. Amlodipine belongs to the dihydropyridine class of calcium channel blockers. Like other members of its class, amlodipine inhibits calcium influx into cardiac and vascular smooth muscle via L-type calcium channels [11]. The primary pharmacodynamic action of amlodipine is the reduction of calcium ion (Ca^{+2}) influx through the L-type voltage-sensitive Ca^{+2} channels present in a variety of tissues, including cardiac and vascular smooth muscle cells. In common with other calcium antagonists, amlodipine exerts its effect on Ca^{+2} channels by interacting with specific binding sites present on the αI subunit of the channel complex. Fig. 1. Chemical formulae of amlodipine [12].

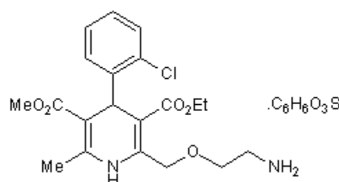

Fig. 1: Chemical structure and formula of Amlopdipine. 2-[(2-Aminoethoxy)methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl 5-methyl ester benzenesulfonate.

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 [13-15]. 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 [16], and nimodipine [17]. However, the exact cellular and molecular anticancer mechanisms of amlodipine have not been studied in breast cancer cells. In this present study, we saw the effects of the combination of doxorubicin/amlodipine treatment on breast cancer cell apoptosis evaluated. Therefore, we sought to investigate the efficacy of AMLO to counteract the drug resistance of the MCF-7 cell line against DOX and to elucidate the underlying mechanism at the molecular level. We report that treatment of Breast Cancer cells with AMLO decreases the expression of the anti-apoptotic Bcl-2 gene and increases the expression of the pro-apoptotic Bax gene.

1. Materials And Methods

1.1. Chemicals and cells

Amlodipine was obtained from Tocris Bioscience™ (Cat. No. 2571). Doxorubicin (MW = 543.5, purity > 98.0%, HPLC) was purchased from Sigma (cat. no. D1515, St. Louis, MO). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO (Invitrogen, CA). MCF-7 breast cancer cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 50 U/ml penicillin/streptomycin, and 2 mM l-glutamine in a humidified incubator at 37 °C and 5% carbon dioxide. Primers were obtained from (Applied Biosystems), RNA extraction kit obtained from (Qiagen, Hilden, Germany) and PCR kit HERA SYBER GREEN/ROX RT-PCR obtained from (Applied Biosystems, Foster City, California, USA). Real-time qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). Analysis of Quantitative DNA content in cultured cells was measured by Ab139418 DNA flow cytometry analysis Kit.

1.2. MTT Cytotoxicity Assay:

The cytotoxic activity of AMLO was measured in vitro against Breast cell line compared to DOX as a reference drug. MCF-7 cells were treated with DOX, AMLO, or DOX combined with AMLO, respectively, for 24 h at different concentrations. Multi-well plates were used in the MTT method, and the final number of cells should not exceed 10^6 cells/cm2 in the log phase of growth for the best results. Also, untreated cells were included for each experiment as control cells. Cells were treated with AMLO or DOX at different concentrations (from 100 to 0.39 uM) for 24 h, and the killing effect of different concentrations was recorded. The half-maximal inhibitory concentration (IC50) values were analyzed and used to determine the concentrations to be used in AMLO/DOX combinations, comprising the ratio of IC50 DOX/IC50 AMLO. The effects of the combination of AMLO on the antitumor activity of DOX on MCF-7 cells were also recorded.

1.3. Drug Combination Analysis.

Drug combination studies were carried out using CompuSyn software version 1.0 (Ting Chao Chou and Nick Martin, Paramus, NJ). The combination index (CI) was measured based on the mass action law of the degree of drug interaction according to

Chou and Talalay. CI calculation is based on the formula CI = (D) 1/ (Dx) 1+ (D) 2/ (Dx) 2, where (Dx) 1 and (Dx) 2 represent the doses of AMLO and DOX in a combination which was required to achieve the same efficacy as that of AMLO (D1) and DOX (D2) when used alone [18]. CI < 1 indicates synergism, where CI = 1 indicates an additive effect and CI > 1 indicates antagonism. Also, the drug reduction index (DRI) values above 1 imply a favorable dose reduction in the drug combination compared to the monotherapy.

1.4. Annexin V-FITC (Anx V) Apoptosis Assay.

Apoptosis detection was performed by FITC Annexin-V/PI kit (Becton Dickenson, Franklin Lakes, and NJ) following the manufacturer's protocol. The samples were analyzed by fluorescence-activated cell sorting (FACS) as we previously described [19].

1.5. Reverse transcription and quantitative real-time polymerase chain reaction (PCR)

The MCF-7 cells were treated with DOX alone or in combination with AMLO at concentrations of 0.39–100uM for 48 h were digested with trypsin-EDTA solution, centrifuged, and harvested. The total RNA was extracted from the cells using TRIzol reagent (Life Technologies, Inc.) as described by the manufacturer and reverse-transcribed into cDNA. The primer sequences for Bcl-2, Bax and the housekeeping gene GAPDH were designed using the software Primer version 5.0 (Premier Corporation) (Table 1). Real-time PCR analysis of gene expression was done using the Rotor-Gene Q software package according to the manufacturer's guidelines (Qiagen). The relative level of RNA expression was normalized to GAPDH, and the difference in RNA expression was estimated using the $2^{-\Delta\Delta Ct}$ method [20] which was expressed as the ratio between the expression of each gene. Triplicate measurements were done, and the average of all was analyzed in our results.

Table 1: Primers Sequences of the Target Genes (Bcl-2), (Bax) and the Housekeeping Gene (GAPDH)

Gene	Primer Sequence
Bcl-2	F 5′- TCGCCCTGTGGATGACTGA-3′R R 5′-CAGAGACAGCCAGGAGAAATCA-3′R
Bax	F 5′-TGGCAGCTGACATGTTTTCTGAC-3′R R 5′-TCACCCAACCACCCTGGTCTT-3′R
GAPDH	F 5′- GAAGGTGAAGGTCGGAGTCA-3′R R 5′- TTGAGGTCAATGAAGGGGTC-3′R

1.6. Statistical Analysis.

The experimental results are expressed as mean ± standard error of the mean (SEM). Data analysis was performed using the one-way ANOVA, and a p-value <0.05 was considered significantly different.

2. Results

2.1. Drugs Cytotoxicity and Drugs synergism

By analyzing the MTT cytotoxicity assay records, the cytotoxic order of our tested compounds on the MCF-7 cell line was as follows: DOX combined with AMLO > DOX > AMLO.

The IC50 values of DOX combined with AMLO and of DOX alone on the MCF-7 cell line were very close (24.84 and 1.66, respectively), To further study whether AMLO is affecting the cytotoxic potency of DOX, we carried out combination index analyses using the Chou Talalay equation and CompuSyn software (version 1.0; CompuSyn, Paramus, NJ, USA). Combined treatment of AMLO and DOX yielded significantly greater growth inhibition in a dose-dependent manner. The combination index (CI) was computed for the combination of AMLO /DOX according to the method developed by Chou [21] to confirm and quantify the synergism observed with DOX and AMLO. The two drugs were combined in a constant ratio to calculate CI value using CompuSyn software. Shows a CI less than 1 corresponding to fraction affected (Fa) values from 0.5 to 0.95 which indicates a synergism between the two drugs in inhibiting the proliferation of MCF-7 (Table 2).

Table 2: IC50 of Amlodipine and Doxorubicin as monotherapy or combination therapy in MCF-7 cells

Drug/Combo	CI	DOX	AMLO
DOX		1.61±0.17	
AMLO			24.79±1.33
Combination	0.906	0.55±0.07	13.92±0.45

We further calculated the DRI which represents the actual fold-change of dose attenuation in a synergistic combination at a given effect level compared with the drug alone. The Fa-DRI plot and Fa−log (DRI) plot demonstrate whether the influence of synergistic treatments may ameliorate side effects caused by cytotoxicity to normal cells. Figure 2 demonstrates that the DRI of DOX values were higher than 1, which indicates favorable dose reduction when combined with AMLO. Moreover, the mean DRI of DOX in the combination therapy was 3.01± 0.03, which suggests a three-fold dosage reduction compared to monotherapy.

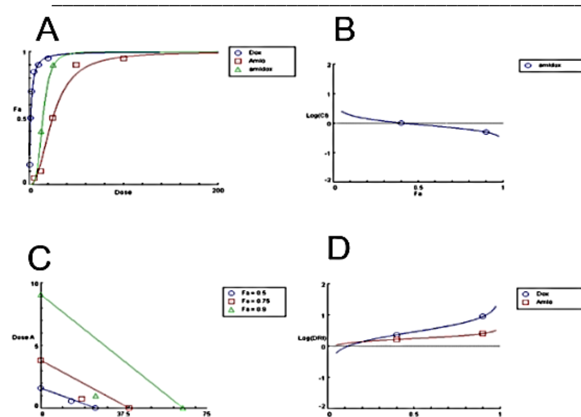

Fig. 2: The graphic representations obtained from the CompuSyn Report for AMLO and DOX combinations (A) The combined inhibition effects of Amlodipine and Doxorubicin against the MCF-7 cell line as analyzed with the CompuSyn system. (A) Dose-Effect Curve (B) Logarithmic Combination Index Plot (Log (CI)-Fa) (C) Isobologram for Combination, and (D) Logarithmic Dose Reduction Index Plot (Log (DRI)-Fa). Data are average of three independent experiments ± SD.

2.2. Annexin V-FITC (Anx V) Apoptosis Assay

Annexin-V/PI-based flow cytometry apoptosis assay is a helpful tool that can clarify whether cell death is due to programmed apoptosis or uncontrolled necrosis. It enables researchers to identify the early apoptotic cells within a cell population. AMLO is also tested for its apoptotic effect on the MCF-7 cell line, compared to DOX and DOX/AMLO combination. Since the combination of AMLO with DOX achieved the highest antitumor activity against the MCF-7 cell line, it was further evaluated for its effect on the cell cycle of the cell line compared to both DOX and AMLO alone.

Treatment of MCF-7 cells with IC50 concentration of DOX, AMLO, and DOX/AMLO combination showed a marked increase in the AnxV-FITC apoptotic cells percentage in both early in both concentrations the first concentration of DOX 0.4uM and the second concentration of DOX 0.8uM (Figures 3, 4)

These findings confirmed that the cytotoxic activity of either DOX, AMLO, or DOX/AMLO combination is due to physiological apoptosis, not nonspecific necrosis. Furthermore, as reported in all parts of our research, DOX/AMLO combination achieved the best increase in the percent of apoptotic cells confirming the concept of chemo-sensitization of the combination compared to DOX alone.

2.3. Apoptosis Genes expression analysis.

To unravel the underlying molecular mechanisms of the found synergistic antitumor effects of AMLO and DOX on breast cancer, we studied the expression of apoptosis-related genes. Bcl-2, an antiapoptotic gene, Bcl-2 expression was significantly decreased in AMLO/DOX combination therapy compared with either treatment alone. Which we used two concentrations of DOX the first concentration DOX 0.4 (0.665 AMLO + 0.465 DOX) given 0.410 and the second concentration of DOX 0.8 (0.665 AMLO + 0.335 DOX) given 0.240 Figure (5A).

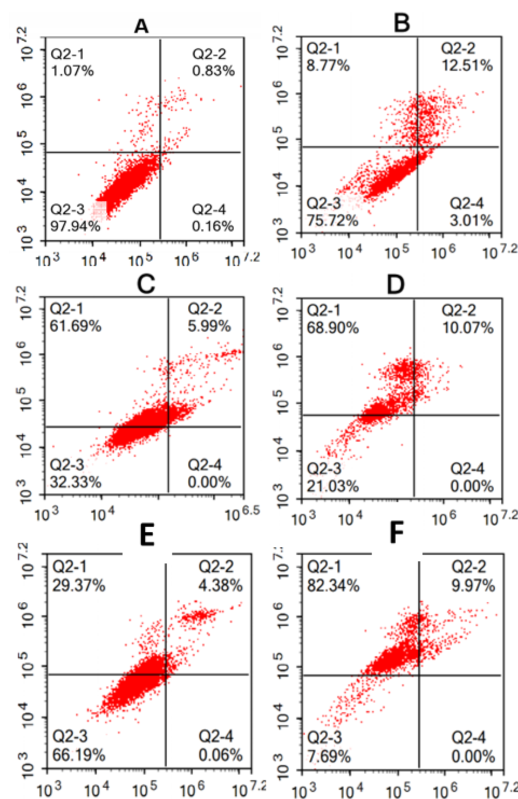

Fig. 3: The Graphical representation of the flow cytometry analysis of cell apoptosis induced in MCF-7 cell line by variable treatments as detected by the annexin V-FITC-positive assay. (A) Untreated control cells. (B) Cells treated with AMLO. (C) Cells treated with DOX (0.4 uM). (D) Cells treated with DOX (0.4 uM)/AMLO combination. (E) Cells treated with DOX (0.8 uM). (F) Cells treated with DOX (0.8 uM)/AMLO combination. (Lower right: early apoptotic; upper right: late apoptotic; lower left: viable; upper left: necrotic)

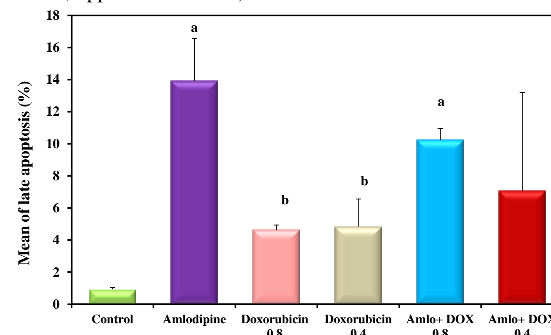

Fig. (4): Comparison between the different studied groups according to total (early+late) apoptosis of MCF-7 cells after different treatments. Different letters indicate significant differences between treatments ($p < 0.05$).

Moreover, the BAX gene is responsible for many roles in promoting apoptosis, BAX expression was significantly increased in AMLO/DOX combination

therapy compared with either treatment alone, and also we used two concentrations of DOX the first concentration of DOX 0.4 (1.960 AMLO + 3.150 DOX) given 4.450 and the second concentration of DOX 0.8 (1.960 AMLO + 4.360 DOX) given 5.750 Figure (5B).

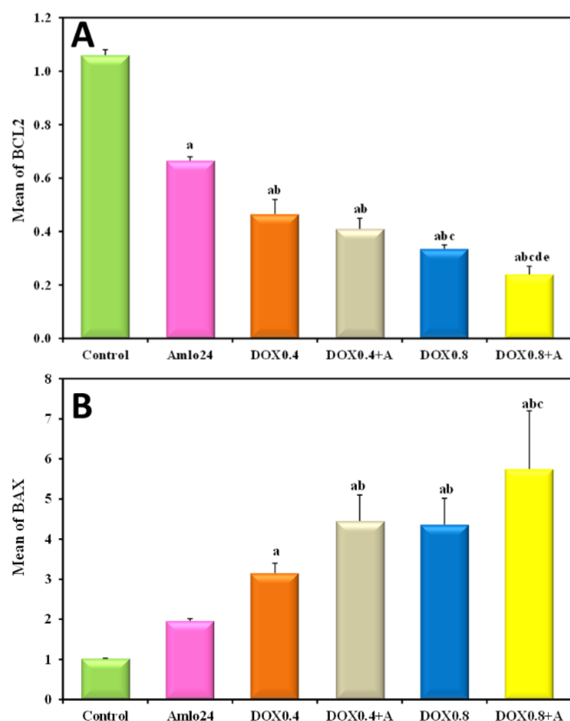

Figure 5: Impact of conjugates DOX, AMLO, and DOX/AMLO combination on the expression of (A) anti-apoptotic gene Bcl2 and (B) proapoptotic gene BAX in MCF-7 cells. Different letters indicate significant differences between treatments ($p < 0.05$).

3. Discussion

Although alterations in Ca^{+2} signaling may not be a requirement for the initiation of cancer, the consequences of altered calcium transport in cancer cells may be significant and contribute to tumor progression. Cancer cells use the same calcium channels, pumps, and exchangers as non-malignant cells. However, there are often key alterations in calcium channels and pumps in cancer cells.

Such changes in cancer cells may include the expression of calcium channels or pumps (or their specific isoforms) not normally present in non-malignant cells of the same cell type, pronounced changes in the level of expression, altered cellular localization, altered activity through changes in post-translational modification, gene mutations, and changes in activity or expression associated with specific cancer-relevant processes (e.g. migration) [22].

These changes are often reflected in alterations in Ca^{+2} flux across the plasma membrane or intracellular organelles. The influx of calcium across the plasma membrane into the cell is a key trigger or regulator of cellular processes relevant to tumor progression, including proliferation, migration, and apoptosis. Ca^{+2} permeable ion channels of almost every class have now been associated with aspects of tumor progression.

The free calcium ion concentration is tightly and precisely controlled in cellular compartments, to generate intracellular calcium signals with various amplitudes, as well as different temporal and spatial properties. [23] This precise control is essential for differential modulation, in an individual cell, of various signaling pathways and intracellular Ca^{+2} regulated proteins involved in specific cellular processes. These include regulation of the cell cycle, proliferation, apoptosis, gene transcription, and cell migration [24]. Since all these functions are relevant to tumorigenesis, the remodeling of intracellular Ca^{+2} homeostasis and of Ca^{+2} signals is thought to be a crucial event in leading to, or maintaining, malignant phenotypes. Indeed, tumor transformation is associated with a major rearrangement of Ca^{+2} transporting molecules (changes in expression and/or function), which participate with other signaling pathways. This may result in enhanced survival (evasion of apoptosis), excessive proliferation, malignant angiogenesis, cell migration, and metastasis [25-26].

Recent studies have also shown that calcium channel expression is upregulated in breast cancer cells [27]. In the present study, treatment of breast cancer cells with the CCB, amlodipine, resulted in a dose-dependent reduction in breast cancer cell viability.

Similarly, recent studies have shown that silencing calcium channel expression inhibited breast cancer cell growth both in vitro and in vivo [28]. To ascertain the underlying mechanism(s) of amlodipine-induced growth suppression, breast cancer cellular apoptosis was assessed by measuring BAX activity. The results showed that amlodipine induced BAX activity in MCF-7 cells, which may contribute to apoptosis. Which was the activation of BAX was accompanied by downregulation of the anti-apoptotic protein Bcl-2, which strongly indicated that BAX-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 [29-30]. In agreement with the findings of the present study, a previous study demonstrated that amlodipine treatment induced apoptosis in MCF-7 cells via downregulation of Bcl-2 protein expression [31].

In addition, activation of BAX-dependent apoptosis has been reported with other dihydropyridine CCBs [32]. Moreover, Wong et al [32] reported that treating cancer cells with calcium channel inhibitors may also lead to BAX-independent apoptosis. In the present study,

amlodipine treatment of breast cancer cells resulted in BAX-dependent apoptosis as shown by the activation of BAX.

The results of the present study are consistent with a recent study that showed the ability of several CCBs, including amlodipine were accompanied by downregulation in Bcl-2 expression and activation of BAX.

In conclusion

The results of the present study showed that amlodipine exerted anticancer effects on apoptosis at least in part, achieved by the inhibition of Bcl-2 expression and activation of BAX indicating the induction of BAX-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.

4. Conflicts of interest

"There are no conflicts to declare".

5. Funding sources

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