

## Effects of berberine on proliferation, cell cycle distribution and apoptosis of human breast cancer T47D and MCF7 cell lines

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### ABSTRACT

**Objective(s):** Berberine, a naturally occurring isoquinoline alkaloid, has shown antitumor properties in some *in vitro* systems. But the effect of berberine on breast cancer has not yet been completely studied. In this study, we evaluated anticancer properties of berberine in comparison to doxorubicin.

**Materials and Methods:** The antiproliferative effects of berberine and doxorubicin alone and in combination were evaluated in T47D and MCF7 cell lines using MTT cytotoxicity assay. In addition, flow cytometry analysis was performed to evaluate the cell cycle alteration and apoptosis induction in these cell lines following exposure to berberine and doxorubicin alone and in combination.

**Results:** The IC<sub>50</sub> of berberine was determined to be 25  $\mu$ M after 48 hr of treatment in both cell lines but for doxorubicin it was 250 nM and 500 nM in T47D and MCF-7 cell lines, respectively. Co-treatment with berberine and doxorubicin increased cytotoxicity in T47D cells more significantly than in MCF-7 cells. Flow cytometry results demonstrated that berberine alone or in combination with doxorubicin induced G2/M arrest in the T47D cells, but G0/G1 arrest in the MCF-7 cells. Doxorubicin alone induced G2/M arrest in both cell lines. Furthermore, berberine and doxorubicin alone or in combination significantly induced apoptosis in both cell lines.

**Conclusion:** Berberine alone and in combination with doxorubicin inhibited cell proliferation, induced apoptosis and altered cell cycle distribution of breast cancer cells. Therefore, berberine showed to be a good candidate for further studies as a new anticancer drug in the treatment of human breast cancer.

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## Introduction

Breast cancer is one of the most common malignancies with high cancer-related mortality among women worldwide (1, 2). Studies in Iran suggest that breast cancer affects Iranian women at least one decade earlier than the women in developed countries (3). For many years cancer research around the world has been focused on finding better therapeutic strategies and new molecular approaches to reduce the mortality (4). Although advances in the novel targeted therapies in the past decades have improved survival rate in breast cancer, there are still significant numbers of mortalities. This is mainly due to development of drug resistance in cancer cells to available anticancer compounds. A wide variety of mechanisms have been implicated in drug resistance of cancer cells including increased or decreased expression of target proteins, reduced apoptosis via alterations in the expression of Bcl2 family members and increased efflux pump activities. Thus, it is very important to

search for new therapeutic agents with less resistance potential to treat breast cancer (5).

Anthracyclines are a class of antitumor agents widely used for cancer treatment (6, 7). Doxorubicin (Dox) is a widely used anthracycline that has been associated with efficient arrest of cell division and induced apoptotic cell death by intercalation with DNA, generation of free radicals, interaction with cellular membranes, and inhibition of Topo IIA (8). Several multi-drug resistance proteins (MRPs) have been implicated in the elimination of Dox from the cell, including MRP1, MRP2, MRP7, multi-drug resistance 1 (MDR1), and ATP-binding cassette sub-family G member 2 (ABCG2). Therefore, drug resistance along with side effects such as cardiotoxicity seriously limited clinical success of anthracyclines in cancer therapy (9). Cancer cell resistance to anthracyclines, intrinsic or acquired, is induced by multiple factors, such as multidrug resistant protein expression, apoptotic pathway alterations, and drug-detoxifying enzyme induction (10, 11).

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There has been a considerable interest in the use of phytochemicals for cancer treatment. Phytochemicals show promise as potential chemopreventive or chemotherapeutic agents against various cancers (12). Berberine (Brb) is an isoquinoline alkaloid present in the root, rhizome and stem bark of a number of important medicinal plant species such as *Berberis aquifolium*, *Berberis vulgaris*, *Berberis aristata*, *Tinospora cordifolia*, *Rhizoma coptidis* (13). Brb is currently known to have a wide range of pharmacologic effects, including anti-cancer effects, in a variety of human cancer cells (14). Brb has been reported to be able to decrease TPA-induced angiogenesis and migration factors including VEGF and FN in breast cancer cells (15). Brb also showed a decrease in side population (SP) cells in breast cancer cells that was associated with a decrease in ABCG2 expression (16). Brb showed inhibition in cell proliferation and induced apoptosis in prostate cancer cells but not in normal prostate epithelial cells (13). Brb has been reported to decrease cell proliferation in breast cancer cells that was mediated by a mitochondria and caspase-dependent apoptotic pathway (17).

Therefore, we investigated the effect of Brb and Dox alone and in combination on proliferation, apoptosis induction and cell cycle distribution of breast cancer T47D and MCF7 cell lines.

## Materials and Methods

### Materials

RPMI 1640 and FBS were purchased from Biosera (UK). Pen-strep and trypsin- EDTA were purchased from Gibco (UK). MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide), propidium iodide (PI), and Annexin V-FITC (Anv) were purchased from sigma (Germany). DAPI (4, 6-diamidino-2-phenylindole) and Nonidet P40 were purchased from Roche (Germany). Doxorubicin was purchased from Ebewe (Austria). Berberine was purchased from Sigma (UK).

### Cell culture

MCF7 and T47D cell lines were purchased from Pasteur Institute (Iran). T47D and MCF7 cells were cultured in RPMI1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 mg/ml streptomycin, and incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator.

### Drug preparation

Brb was initially dissolved in DMSO and diluted to different concentrations with complete cell culture medium freshly before adding to the cultured cells. Dox was diluted in complete cell culture medium freshly before adding to the cultured cells. The sub-confluent cells were treated with different concentrations of Brb and Dox alone or in combination and compared to control RPMI (culture

medium containing below 1% DMSO).

### MTT cytotoxicity assay

Proliferation of T47D and MCF7 cells under different conditions was determined using the MTT assay. Briefly, 5000 cells per well were seeded in 96-well plates. After 48 hr, culture media was removed and the cells were treated with Brb and Dox alone or in combination at varying concentrations and time points. Then MTT solution (4 mg/ml in PBS) was added to each well. After 3 hr incubation at 37 °C at 5% CO<sub>2</sub>, DMSO was added to each well to dissolve the formazan crystals. The absorbance of each well was read at 540 nm against 620 nm using a microplate reader (Sunrise, Tecan, Switzerland). The results were presented as a percentage to the control RPMI. Drug concentration that inhibited cell proliferation to 50% of the control RPMI (IC<sub>50</sub>) was determined from at least three independent experiments in quadruplicate format for each treatment.

### Apoptosis assay

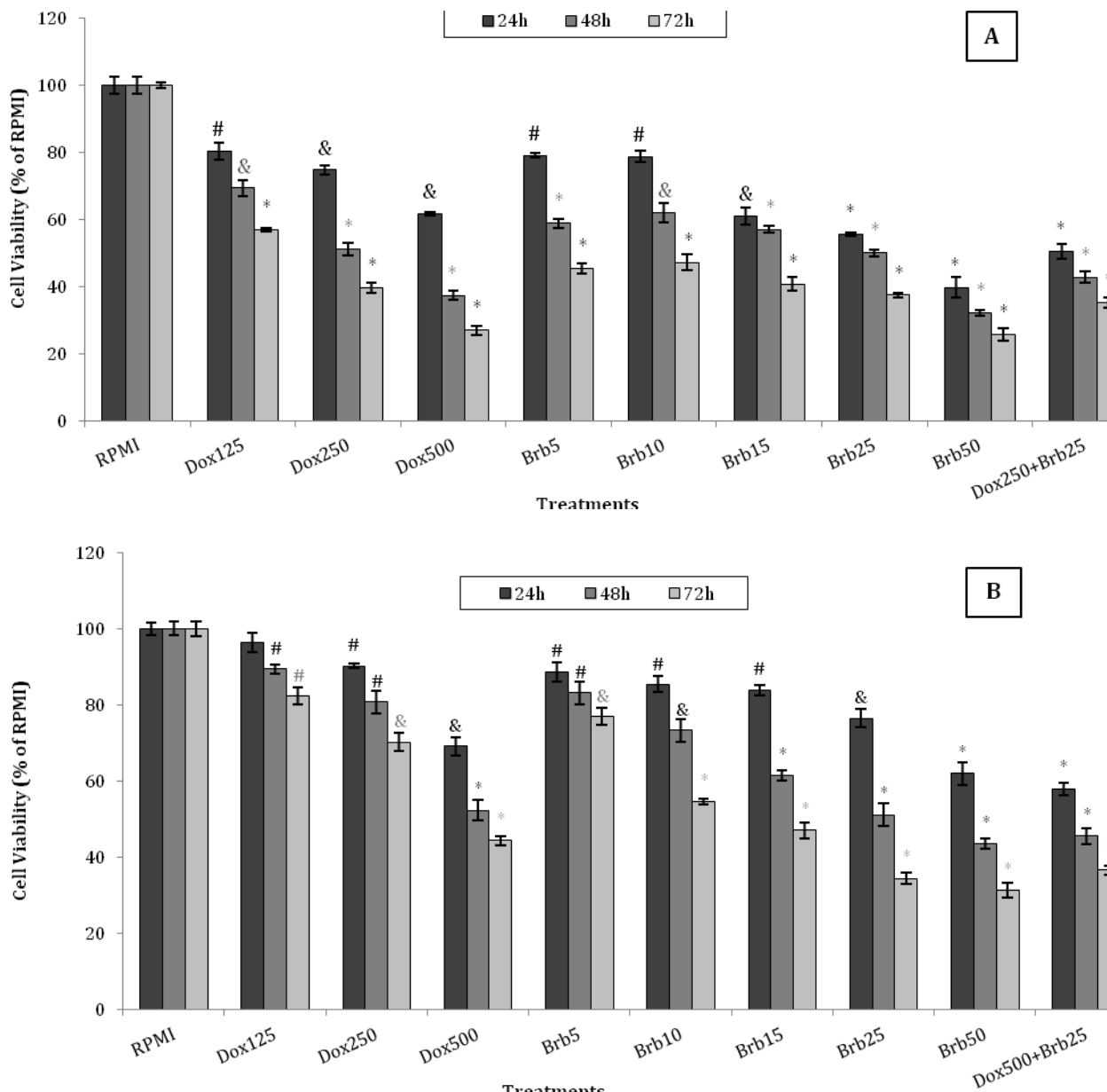
T47D and MCF7 cells were seeded into 6-well plates at a density of 2.5×10<sup>5</sup> cells/well. The cells were exposed to IC<sub>50</sub> of Brb and Dox alone or in combination for 48 hr and then cells were harvested, washed twice with PBS, resuspended in binding buffer, and stained with Annexin V-FITC (Anv) plus PI for 15 min at 4 °C in dark. Then stained cells were resuspended in binding buffer and assessed for apoptosis by Partec-PAS (Germany) flow cytometer and data was processed using FloMax software. In addition, stained cells were examined under the fluorescent microscope (Olympus IX81, Japan) using FITC (Green) and PI (Red) filters. Green (Annexin V-FITC+) cells are apoptotic and red (PI+) cells are necrotic.

### Cell cycle distribution analysis

T47D and MCF7 cells were seeded into 6-well plates at a density of 2.5×10<sup>5</sup> cells/well. The cells were exposed to IC<sub>50</sub> of Brb and Dox alone or in combination for 48 hr and then cells were harvested, washed twice with PBS and stained with DAPI cocktail containing NDP40 for 30 min at 4 °C in dark. Cell cycle distribution, based on DNA content of cells, was then determined by Partec-PAS flow cytometer, and data was processed using FloMax software.

### Statistical analysis

All experiments were repeated at least three times and data presented as mean±SD. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego CA). One-way ANOVA was used with Tukey test to compare the data groups with control, and differences were considered significant if *P* values were <0.05.



**Figure 1.** Cytotoxic effects of Berberine and Doxorubicin on T47D and MCF7 cells. The T47D (A) and MCF7 (B) cells were treated with different concentrations of Brb (5-50  $\mu$ M) and Dox (125-500 nM). After 24 hr, 48 hr and 72 hr of treatment, cell proliferation was determined using MTT assay. Each experiment was repeated at least three times in quadruplicates for each concentration and the results are presented as mean $\pm$ SD. The IC<sub>50</sub> values were then determined and used in the subsequent experiments. # denotes  $P < 0.05$ , &  $P < 0.01$ , and \*  $P < 0.001$  for significant difference between treatments in comparison to control RPMI.

**Results**

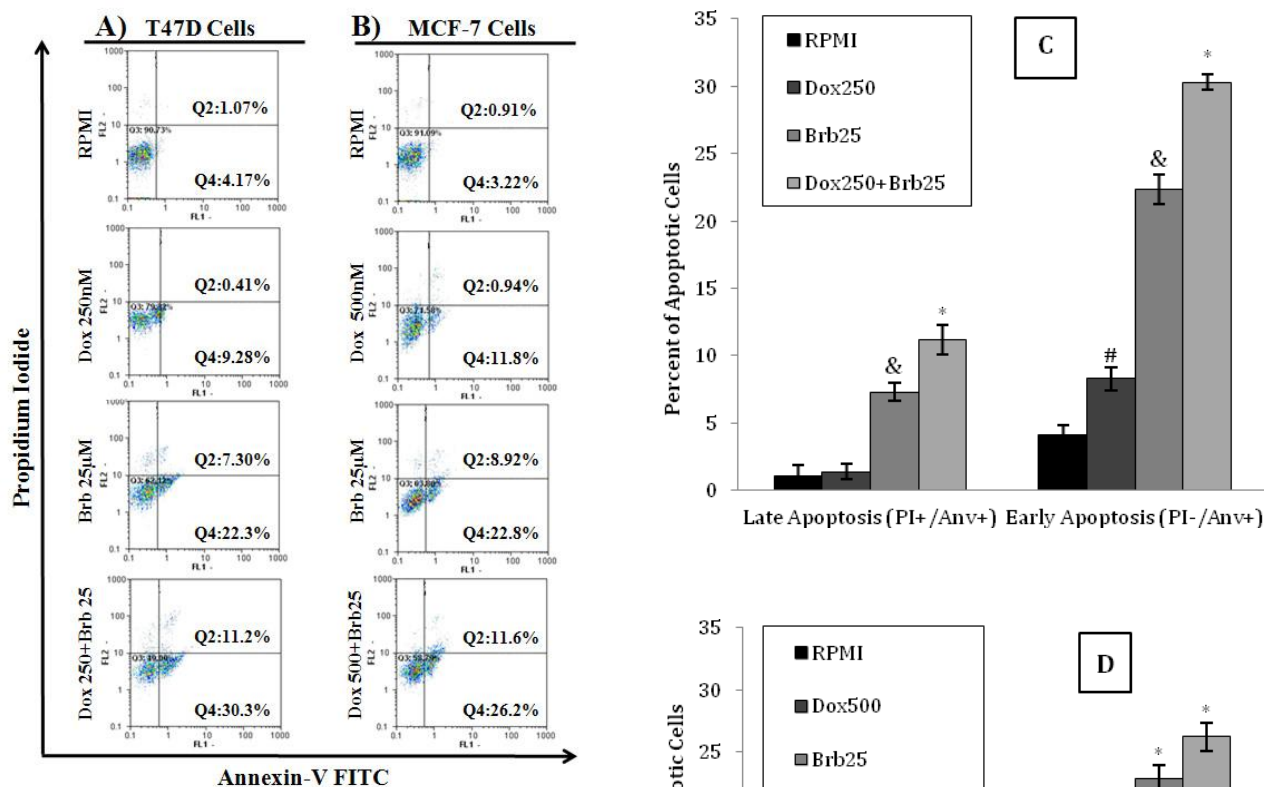
**Cytotoxic effects of treatments on T47D and MCF7 cells**

MTT assay was used to determine the effect of different treatments on proliferation of T47D and MCF7 cells. Cell proliferation was significantly decreased following treatment of T47D and MCF7 cells with Brb and Dox in a concentration- and time-dependent manner ( $P < 0.001$ ), (Figure 1 A and B). The IC<sub>50</sub> was observed at 25  $\mu$ M concentration of Brb after 48 hr of treatment in both cell lines. The IC<sub>50</sub> for Dox was determined to be 250 nM and 500 nM after

48 hr treatment in T47D and MCF-7 cells, respectively. Co-treatment with Brb and Dox significantly increased cytotoxicity in T47D compared with MCF7 cells ( $P < 0.05$ ).

**Apoptosis**

In order to study the induction of apoptosis, T47D and MCF7 cells were treated with IC<sub>50</sub> of Brb and Dox alone and in combination and analyzed by flow cytometry after double-staining with AnnexinV-FITC (Anv) and PI. Representative data of flow cytometry analysis are shown in Figure 2-A and B. In T47D



**Figure 2.** Apoptosis induction in T47D and MCF7 cells

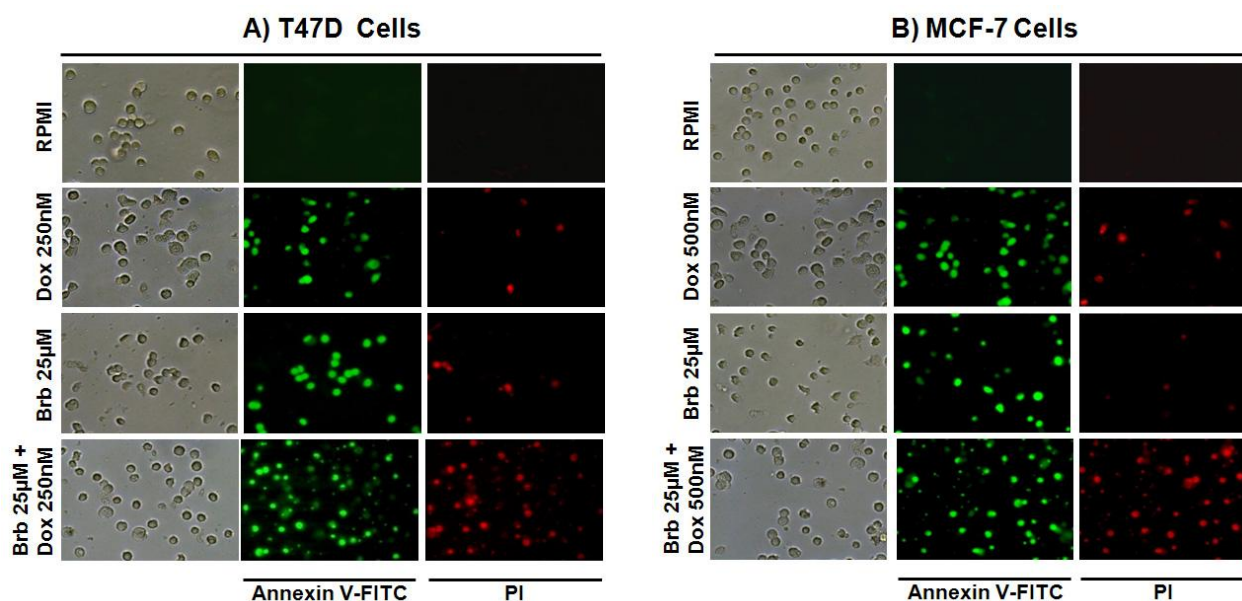
The T47D (A and C) and MCF7 (B and D) cells received different treatments for 48 hr to evaluate induction of apoptosis using Annexin V-FITC (Anv) and PI double-staining by flow cytometry. Representative images of flow cytometry for apoptosis in the T47D (A) and MCF7 (B) cells are shown following treatment with Brb and Dox alone or in combination in comparison to control RPMI. The percentage of early (Q4: PI-/Anv+) and late apoptotic (Q2: PI+/Anv+) cells in T47D (C) and MCF7 (D) cells are presented for each treatment in comparison to control RPMI. Data are presented as mean $\pm$ SD of three independent experiments. # denotes  $P < 0.05$ , &  $P < 0.01$ , and \*  $P < 0.001$  for significant difference between treatments in comparison to control RPMI

cells, the early (Q4: PI-/Anv+) and late apoptosis (Q2: PI+/Anv+) rate together for the control RPMI was 5.24%, while those of the Dox (250 nM) and Brb (25  $\mu$ M) alone were 9.69% ( $P < 0.05$ ) and 29.6% ( $P < 0.01$ ), respectively (Figure 2-C). In MCF7 cells, the early (Q4: PI-/Anv+) and late apoptosis (Q2: PI+/Anv+) rate together for the control RPMI was 4.13%, while those of the Dox (500 nM) and Brb (25  $\mu$ M) alone were 12.74% ( $P < 0.01$ ) and 31.72% ( $P < 0.001$ ), respectively (Figure 2-D). A significant increase in the percentage of apoptotic cells was observed in both T47D (41.5%,  $P < 0.001$ ) and MCF7 (37.8%,  $P < 0.001$ ) cells following co-treatment with IC<sub>50</sub> of Brb and Dox, in comparison to other treatments (Figure 2-C and D). In addition, stained cells with Annexin V-FITC and PI showed increase in apoptotic cells stained green (Annexin V-FITC+) following treatment with IC<sub>50</sub> of Brb alone or in combination with Dox in comparison to Dox alone or RPMI in both T47D and MCF7 cell lines (Figure 3-A

and B). Furthermore, cells stained red (PI+) increased in cells treated with Brb alone or in combination with Dox in comparison to Dox alone or RPMI in both T47D and MCF7 cell lines (Figure 3-A and B).

#### Cell cycle distribution

The effect of Brb and Dox alone and in combination on cell cycle distribution of T47D and MCF7 cells was determined by flow cytometry and showed alterations in G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phases of cell cycle under different treatment conditions (Figure 4-A and B). In T47D cells, 56.54% of control RPMI cells were in G<sub>0</sub>/G<sub>1</sub> phase, 29.56% in S phase and 13.90% in G<sub>2</sub>/M phase (Figure 4-C). In MCF-7 cells, 71.41% of control RPMI cells were in G<sub>0</sub>/G<sub>1</sub> phase, 21.71% in S phase and 6.89% in G<sub>2</sub>/M phase (Figure 4-D). Compared with control RPMI, Brb 25  $\mu$ M induced cell accumulation in the G<sub>2</sub>/M phase ( $P < 0.01$ ) while reducing the number of cells in



**Figure 3.** Microscopic evidence of apoptosis induction in T47D and MCF7 cells

Stained T47D (A) and MCF7 (B) cells with Annexin V-FITC and PI were examined under the microscope, which showed increase in green (Annexin V-FITC+) and to a lesser extent in red (PI+) cells following treatment of cells with Brb alone or in combination with Dox in comparison to Dox alone or control RPMI. Dual stained (PI+/Annexin V-FITC+) cells that indicate late apoptotic event are also seen at different extent in T47D and MCF7 cells.

G0/G1 ( $P < 0.05$ ) and S phase ( $P < 0.001$ ) in T47D cells. Brb 25  $\mu\text{M}$  induced G0/G1 cell accumulation in comparison to control RPMI (80.86% versus 71.41%,  $P < 0.001$ ) in MCF7 cells. In comparison to control, a significant increase in G2/M arrest was observed in both T47D (76.69%,  $P < 0.001$ ) and MCF7 (32.74%,  $P < 0.001$ ) cells treated with Dox at concentrations of 250 nM and 500 nM, respectively (Figure 4-C and D). The percentage of G2/M arrest following treatment of T47D cells with the combination of Brb and Dox and the pattern of cell cycle distribution was almost similar to treatment with Brb 25  $\mu\text{M}$  alone (31.80% versus 33.55%). In the MCF7 cells, G0/G1 arrest was observed with the combination of Brb and Dox in comparison to control RPMI (81.42% versus 71.41%,  $P < 0.001$ ), which was almost similar to Brb 25  $\mu\text{M}$  alone (81.42% versus 80.86%). Brb alone or in combination with Dox differently induced cell cycle arrest. It was G0/G1 in MCF-7 cells but G2/M in T47D cells (Figure 4-C and D).

## Discussion

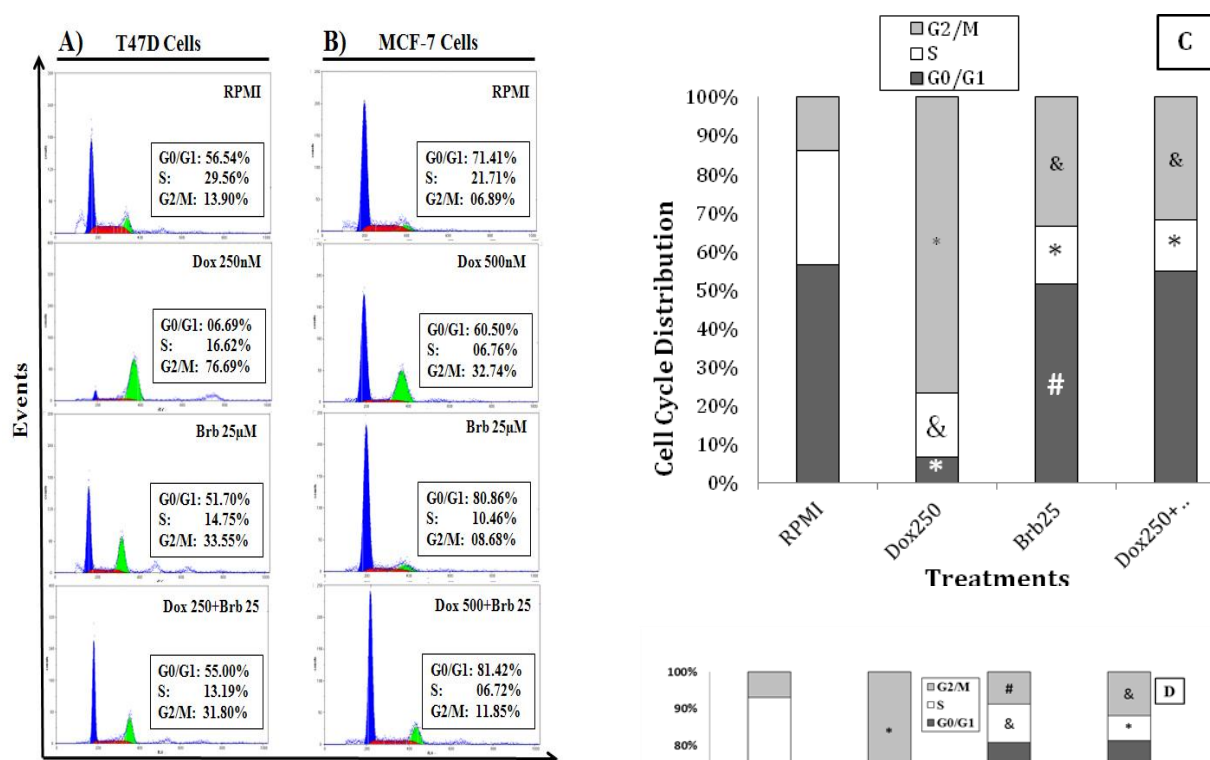
Results of this study showed a concentration- and time- dependent manner in cytotoxic effects of Brb on T47D and MCF7 cancer cells. In addition, Brb alone or in combination with Dox induced cell cycle arrest and apoptosis in both cancer cells.

Apoptosis contributes to cell death in tumors treated with various anticancer agents (18, 19). Despite the fact that many tumors initially respond to therapy, cells can subsequently survive and gain resistance to these treatments (20).

Anthracyclines such as Dox have been shown to be able to upregulate several pro-apoptotic and

downregulate anti-apoptotic proteins in Bcl2 family (20). In addition, anthracyclines seem to achieve their antitumor effects by affecting several intracellular processes, causing DNA intercalation as well as generation of free radicals. A key mechanism, however, seems to be the inhibition of the DNA damage repair enzyme topoisomerase II (TopoII) (21). Changes in Topo IIA expression levels as a major determinant of response to the Topo IIA inhibitors show that suppression of Topo IIA levels induces resistance to Dox *in vitro* and *in vivo* (22).

Brb is an isoquinoline derivative alkaloid that is prevalent throughout Chinese, Unani, and Indian Ayurvedic medicines (23). Brb also has a wide range of pharmacologic effects, including inhibition of protein synthesis, suppressing lipid peroxidation, blocking effect on cell cycle progression, and induction of apoptosis in a variety of cancer cell lines such as breast cancer cells (24-26). Brb is potentially useful for the development of therapeutic regimen in cancer and decrease in the metastatic potential of highly metastatic cancer cells (27). Brb is one of several known DNA intercalators with cytotoxic activity (28, 29). Growth inhibitory effects of Brb on multiple types of human cancer cells have been reported (30). Several studies have shown cytotoxic effects and apoptosis induction with Brb in different human cancer cells including breast (17, 31, 32), colon (33-36), prostate (13, 37, 38), ovarian (39), thyroid (40), bladder (41), liver (42, 43), pancreatic (29), nasopharyngeal (44), oral squamous (45), epidermal (46), and leukemia (47) cancers. Studies have reported that Brb at 50  $\mu\text{M}$  concentration had the most reducing effect on cell viability in T47D



**Figure 4.** Cell cycle alterations in T47D and MCF7 cells

The T47D (A and C) and MCF7 (B and D) cells received different treatments for 48 hr and were stained with DAPI cocktail and analyzed by flow cytometry to determine cell cycle distribution pattern. Representative images of flow cytometry for cell cycle distribution of the T47D (A) and MCF7 (B) cells are shown following treatment with Brb and Dox alone or in combination in comparison to control RPMI. Alterations in the percentage of T47D (C) and MCF7 (D) cells in G0/G1, S, and G2/M phases of cell cycle are presented as the mean $\pm$ SD of three independent experiments. # denotes  $P < 0.05$ , &  $P < 0.01$ , and \*  $P < 0.001$  for significant difference between treatments in comparison to control RPMI

cells (31). In addition, treatment with Brb inhibited growth in both MDA-MB-231 and MCF7 cells (16, 32), and exhibited a significant cytotoxic effect on MCF7 cells without affecting the breast normal epithelial cells (17). It has been reported that the highest cytotoxic concentration induced intercalation of Brb with DNA (48). Dox, which is a commonly used drug for cancer treatment (49), has been reported to significantly inhibit the melanoma tumor growth following combination with Brb (50). In our study, results of MTT assay also showed that treatment with combination of Brb and Dox increased cytotoxicity on T47D and MCF7 cells to a greater extent than Brb and Dox alone. This suggests that Brb may be an effective chemotherapeutic agent against breast cancer.

Control of cell cycle progression in cancer cells is considered to be a potentially effective strategy for the control of tumor growth. The molecular analyses of human cancers have revealed that cell cycle regulators are frequently mutated in most common malignancies (13). It has been reported that treatment with Brb induced cell cycle arrest at G0/G1 phase in different

cancer cells including breast (32, 49), liver (43, 51), cholangiocarcinoma (52), thyroid (40), bladder (41), neuroblastoma (53), glioblastoma (54), oral squamous (45) and epidermal (46) cancers. In some studies on prostate cancer cells, it was reported that at low concentration, Brb induced G0/G1 arrest, and upon exposure to higher concentration of Brb, cells exhibited G2/M arrest (13, 37, 38). Similar results have been reported in melanoma cells (55). Our *in vitro* data indicated that treatment of MCF-7 cells with Brb resulted in significant G0/G1 phase arrest of cell cycle progression which indicates that one of the mechanisms by which Brb may act to inhibit the proliferation of cancer cells is inhibition of cell cycle progression. Notably, this effect was not seen in T47D cell line. Similar to Dox, treatment of T47D cells with Brb alone or in combination with Dox resulted in G2/M arrest of cell cycle progression. However, in MCF-7 cells G0/G1 arrest was observed following treatment with Brb in combination with Dox. Similarly, in another study, treatment with combination of Brb and Paclitaxel induced G2/M arrest in digestive tract cancer cells (56).

Apoptosis plays a crucial role in eliminating the mutated neoplastic and hyperproliferation neoplastic cells from the system and therefore is considered a protective mechanism against cancer progression (57). Acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer. Apoptosis is tightly regulated by anti-apoptotic and pro-apoptotic effector molecules, including proteins of the Bcl2 family, and can be mediated by several different pathways (58). In this study, the effects of Brb alone and in combination with Dox were determined on the induction of apoptosis in both T47D and MCF7 cells. Our flow cytometry and microscopic data indicate that treatment of T47D and MCF7 cells with Brb alone and in combination with Dox resulted in significant induction of apoptosis. A significant enhancement in the percent of apoptotic cells was observed in both T47D and MCF7 cell lines following treatment with combination of Brb and Dox. Similarly, apoptosis induction was reported after combination treatment with Brb and Paclitaxel in digestive tract cancer cells (56).

## Conclusion

Our findings indicate that Brb and Dox, alone and in combination, exhibit antiproliferative effects against human breast cancer T47D and MCF7 cell lines, and this effect was mediated through interference with normal cell cycle distribution and induction of apoptosis. Therefore, Brb may be used as a good candidate drug for treatment of human breast cancer. More importantly, our findings suggest that Brb combined with Dox can be a novel combination in treatment of breast cancer with greater effects and possibly less side effects. Further investigation and in particular *in vivo* evaluations are required to explore clinical applications of Brb alone or in combination with available anticancer drugs in breast and other cancers.

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