Amphotericin B potentiates the anticancer activity of doxorubicin on the MCF-7 breast cancer cells

Farzaneh Tavangar¹ · Hamid Sepehri² · Marie Saghaeian Jazi³ · Jahanbakhsh Asadi⁴

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Abstract Despite the improvements in cancer treatment, breast cancer still remains the second most common cause of death from cancer in women. Doxorubicin (DOXO) is widely used for cancer treatment. However, drug resistance limits the treatment outcome. Here, we investigated the toxicity of DOXO in combination with an antifungal agent amphotericin B (AmB) against the MCF-7 breast cancer cell line. The cell viability was measured using MTT assay. The apoptosis was studied by caspase-8 and caspase-9 activity measurements and DNA fragmentation was investigated by TUNEL assay. The combination of two drugs significantly increased the apoptotic index and the caspase-8 and caspase-9 activities in comparison to DOXO-treated cells. Our finding showed that pre-treatment of MCF-7 cells with AmB synergistically exerted the anticancer effect of DOXO through the caspase-dependent apoptosis manner.

Keywords Amphotericin B · Doxorubicin · Apoptosis · Caspase · MCF-7 breast cancer cell line

Introduction

Breast cancer is consistently increasing across the world and it is estimated that breast cancer affects more than one million women every year [1]. Induction of apoptotic cell death is an effective mechanism for cancer treatment [2]. The best advantage of apoptotic induction for cancer therapy is that they can eliminate cells without initiating any inflammatory response [3–9]. Doxorubicin (DOXO) is a chief anticancer agent with anthracycline structure [10]. It is a pivotal chemotherapeutic drug in clinical oncology, which is being used for treatment of breast cancer and other solid tumors [11]. DOXO cytotoxicity is related to different mechanisms including inhibition of topoisomerase II and DNA replication [11], free radical production and oxidative stress [10], increasing the expression of pro-apoptotic Bcl-2 family genes—such as *Bax*, initiating the intrinsic apoptotic pathway, increasing the *Bax/bcl-x_L* ratio, and decreasing the cell viability [12].

However, drug resistant to DOXO limits the therapy outcome in breast cancers [13]. To overcome this problem, the adjuvant chemotherapy in combination with current anticancer drugs may be useful.

Amphotericin B (AmB) is one of the first therapeutic agents that have been used widely for treatment of systematic fungal infections [14, 15]. AmB can form ion-permeable channels in the cell membrane via binding to sterols [16, 17]. It is reported that the membrane permeability disruption mediated by AmB can promote the intracellular drug uptake in treated cells and these pores can transport electrolytes, metabolites, and antitumor agents into cancer cells [18–20].

Here in the current study, we aimed to investigate the effect of AmB combined with the chemotherapeutic agent, DOXO, as a combinational therapy in the viability and apoptosis of MCF-7 breast cancer cells.

✉ Jahanbakhsh Asadi
dr.asadi@goums.ac.ir

¹ Student Research Committee, Golestan University of Medical Sciences, Gorgan, Iran

² Neuroscience Research Center, Golestan University of Medical Sciences, Gorgan, Iran

³ Stem Cell Research Center, Golestan University of Medical Sciences, Gorgan, Iran

⁴ Biochemistry & Metabolic Disorders Research Center, Golestan University of Medical Sciences, Gorgan, Iran

Materials and methods

Drugs and chemicals

DOXO was purchased from TOCRIS bioscience (Cat No. 2252). AmB was also provided from Santa Cruz Biotechnology (Cat No. sc-202462A). APO-BrdUTM TUNEL Assay Kit was bought from Invitrogen (Cat No. A23210). Caspase-8 (Cat No. 4100BF) and caspase-9 (Cat No. 10100BF) Colorimetric Assays were provided from R&D Systems. Protein Assay kit was purchased from Bio-Rad (Cat No. 5000002). MTT powder was provided from Sigma-Aldrich. All the cell culture media and reagents were obtained from Gibco Company.

Cell line and culture conditions

Human breast cancer cell line (MCF-7) was purchased from cell bank of Pasteur Institute of Tehran, Iran. Cell culture was maintained in the DMEM (Dulbecco's minimal essential medium) supplemented with 10% of the fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified incubator containing 5% CO₂.

Cell treatment

For cell treatment, different concentrations of DOXO and AmB were selected. The primary concentrations for the cytotoxicity assay were selected according to the literature and then the cell viability was checked using MTT assay to calculate the IC₅₀ value.

For DOXO exposure, it has been reported that MCF-7 cell possesses about 40% viability in 1.5 µM while 3 µM was almost resulting to <20% surveillance [21]. Accordingly, in current study, the MCF-7 cells were treated with different concentrations of DOXO (1, 2, 3, and 4 µM) for 24 or 48 h.

However, there was no reporting for AmB toxicity in MCF-7; alternatively, Judith Medoff et al. reported that AmB 30 µg/mL (32 µM) can enhance the actinomycin D toxicity in Hela cell [22]. Then we checked different concentrations of AmB starting from max 29.21 µM to the fewer concentrations of 7.57 and 18.39 µM for 24 h to assess the AmB toxicity in MCF-7 cell line.

Furthermore, MCF-7 cells were treated with both drugs in combination together with concentration of 0.5 µM DOXO for 21 h and different concentrations of AmB for 24 h.

Cell viability assay

Cell viability was measured using MTT assay. The cells (7×10^3) from exponential growth phase were seeded in a 96-well plate with the final volume of 100 µL. Twenty-four hours later, the cells were treated with various concentrations

of DOXO, AmB individually, and in combination together for different time points. As the manufacturers' protocol, the supernatant was removed and DMEM medium without phenol red supplemented by MTT solution was added to treated cells. Finally, the optical density which represents the cell viability was measured by a spectrophotometric micro plate reader at 570 nm. The percent of growth inhibition was calculated as $[1 - (\text{OD treated cell} / \text{OD non-treated cell})] \times 100$. Indeed, for comparing the attached cell number, the treated cells were imaged with light microscopy and at least three fields were counted with ImageJ software (v 2).

TUNEL assay

For the implementation of Transferase dUTP Nick End Labeling (TUNEL assay), the number of 4×10^6 cells was seeded in a 75-cm² flask. After 24 h of incubation, cells were treated with DOXO (0.5 µM) alone and in combination together with different concentrations of AmB (as mentioned in the cell treatment method). TUNEL assay was performed according to the manufacturer's recommendation (APO-BrdUTM TUNEL Assay Kit, Invitrogen) then the cells were imaged with inverted fluorescent microscope (Nikon, Ti-U model). The results were reported as apoptotic index that was calculated as the percentage of the fluorescence-labeled cells per 700 total cells which was counted in five-seven areas.

Caspase-8 and caspase-9 assays

To measure the caspase activity, a colorimetric assay was used to measure the caspase-8 or caspase-9 enzymatic activity in treated cell lysates using caspase-8 colorimetric assay (Cat No. 4100BF, R&D system) and caspase-9 colorimetric assay (Cat No. 10100BF, R&D system) kits. For normalization, the protein content of cell lysate was measured with the protein assay kit, and then the similar amounts of the cell lysate were used for caspase activity measurements. According to the manufacturer's recommendation, 200 µg of total protein (cell lysate) of each sample was added to a 96-well plate containing the complete reaction buffer 1X (with DTT), then 5 µL of caspase-8 substrate (IETD-pNA) or caspase-9 substrate (LEHD-pNA) was added to each well. Following 2-h incubation in 37 °C, the optical density (OD) was acquired with a micro plate reader using a wavelength of 405 nm. The non-treated cell lysate was regarded as negative control. The mean OD of blank wells (including all factors without substrate) was subtracted from test wells, and then the resulted OD is directly proportional to the level of cellular caspase.

Statistics analysis

The GraphPad Prism 6 was used for statistical analysis and data visualization. The two-way analysis of variance (ANOVA) test followed by Tukey's or Dunnett's post hoc test was performed for comparing results. Differences were considered significant when the *P* value was <0.05.

Results

Cytotoxicity of AmB or DOXO in MCF-7 cancer cells

To evaluate the cytotoxicity of different concentrations of AmB and DOXO, first of all, the MCF-7 cells were treated with each drug alone in different time points and the growth inhibition was measured by MTT assay as described above. As it was expected, both drugs exhibited dose-dependent toxicity (Fig. 1a). Then, the final concentration of IC_{50} equal to 2.41 μ M was obtained for DOXO in 24-h treatment. For AmB, the 24-h exposure of MCF-7 cells resulted in IC_{50} equal to 41.6 μ M for 24 h (Fig. 1b). Because the 48-h drug exposure led to high growth inhibition, therefore, the 24-h time point was selected as an incubation time for the next experiments. According to the toxicity level, for the following

experiments, all the concentrations were considered below the IC_{50} values.

Combination of DOXO and AmB is more toxic for MCF-7

The synergistic toxicity of AmB with DOXO chemotherapeutic agent was measured with the MTT assay of MCF-7 cells that were treated with both AmB (24 h, increasing doses) and DOXO (21 h, 0.5 μ M). Then, the total growth inhibition of the wells containing the co-treated cells were compared to DOXO (0.5 μ M)-treated cells statistically. The result illustrated that all the tested AmB concentrations can significantly increase the growth inhibitory effect of DOXO (0.5 μ M) up to 60% in a dose-dependent manner (Fig. 2). Interestingly, 3 h AmB pre-treatment following with 21 h co-exposure to DOXO (0.5 μ M) and AmB resulted more toxicity than 24 h co-treatment (data not shown). This finding suggests that the AmB pre-treatment method is more effective to synergize the anticancer toxicity of DOXO in cancer cells.

The morphological changes of MCF-7 cells during pre-treatment with AmB and DOXO

To monitor cytotoxicity morphologically, treated MCF-7 cells were imaged by light microscopy after 24 and 48 h (Fig. 3a). Results indicated observable morphological changes

Fig. 1 The cytotoxicity of increasing concentrations of DOXO (a) and AmB (b) alone in MCF-7 cells. The growth inhibition was calculated according to MTT optical density. The toxicity of both drugs increase proportionally to the exposure dose. The equation, R^2 value, and the calculated IC_{50} values are shown in both graphs

Fig. 2 The cytotoxicity of DOXO and AmB combination in MCF-7 cells. The growth inhibition was calculated according to MTT optical density. The combinational toxicity was compared to DOXO (0.5 μM) alone treated cells. AmB pre-treatment resulted in more growth inhibition in DOXO-treated cells significantly in a dose-dependent mechanism

including many cells with distorted shapes with cell shrinkage. Then, the number of attached cells was counted to compare the cell number decrease. As shown, DOXO-treated cells had a mild decrease in the cell population, but no considerable morphological changes were observed. The AmB exposure alone resulted in both morphological changes and cell number decrease; however, the combination of both drugs was more toxic leading to more deformed and floated cells with significantly less attached cell number (Fig. 3a, b).

DNA damage induced by different concentrations of AmB and DOXO or both drugs

The DNA integrity was evaluated as apoptotic damage in response to cytotoxicity of both drugs by TUNEL assay.

Figure 4a shows the apoptotic cells stained with Alexa green. The nuclear DNA fragmentation during apoptosis process results in high molecular weight fragments which can be cleaved into the low molecular weight fragments. Briefly, the free ends of fragmented DNA are targeted by terminal deoxytransferase (TdT), which adds labeled UTP into 3-OH of the DNA fragments that is detectable by fluorescent imaging. A considerable note is that doxorubicin can show fluorescent interaction.

The DNA fragmentation was examined in DOXO, AmB, or co-treated MCF-7 cells, and the non-treated cells were considered as negative control. The apoptotic index represents the percentage of DNA-fragmented cells to the total cell count. The results illustrated that AmB pre-treatment increases the apoptotic index in the DOXO-treated MCF-7 cells significantly (Fig. 4b). The magnitude of the apoptotic index in the AmB/DOXO low dose (0.5 μM) rise up approximately similar to DOXO high dose (4 μM).

Interestingly, the number of TUNEL-labeled green cells in AmB/DOXO wells was more than the number of DNA-fragmented cells in the DOXO-treated cell plus the AmB-treated ones (Fig. 4a). For example, the apoptotic index of AmB 18.3 μM with DOXO 0.5 μM ~50% is more than the accumulation of DOXO 0.5 μM apoptotic index ~10% plus the apoptotic index of the AmB 18.3 μM treated cells ~20%. It suggests that the observed synergism probably is not a simple accumulation effect; however, the underlying mechanism needs to be clear.

Caspase-8/9 activity assay

To confirm the apoptosis of treated cells, the activities of two of the main apoptosis regulators: caspase-8 (central hallmark of the extrinsic apoptosis) and caspase-9 (central hallmark of the intrinsic apoptosis), were measured in the treated cells.

Then the caspase activities of the cell lysates were compared with both non-treated and DOXO-treated (0.5 μM) cells. Our findings showed that, however, DOXO (0.5 μM) cannot increase the cellular caspase activity more than non-treated cells, AmB pre-treatment significantly increases both the caspase-8 and caspase-9 activities (~3–3.5-folds). The most caspase-9 activity was recorded in the AmB 29.2 μM + DOXO 0.5 μM that was significantly higher than non-treat or DOXO 0.5 μM alone (*P* value <0.00001) (Fig. 5).

The intrinsic apoptosis (caspase-9) is significantly more activated when increasing the AmB pre-treatment concentrations, but the extrinsic apoptosis (caspase-8) does not change significantly proportional to the AmB dose. The AmB dose-dependent caspase-9 activation suggest that the underlying mechanism of anticancer synergism of AmB with DOXO is probably associated with the intrinsic apoptosis rather than extrinsic pathway; however, both the extrinsic and intrinsic apoptosis pathways crosstalk with each other.

Discussion

Breast cancer still remains the most common type of malignancy among women. Although novel therapeutic methods are evolving, current approaches still rely on surgery, chemotherapy, radiation therapy, and hormonal therapy. However, these conventional therapeutic methods are associated with side effects. To minimize the inevitable side effects and enhance the effectiveness of present approaches, anticancer combination therapy has emerged. Numerous anticancer agents with different molecular mechanisms have been examined in combination to evaluate their effectiveness in a dose- and time-dependent manner [3, 23]. In the present study, we showed that amphotericin B can potentiate the anticancer

Fig. 3 The morphological changes of treated MCF-7 cell line. **a** MCF-7 cells were treated with different doses of DOXO and AmB, and then were imaged to investigate the morphology of the cells. Notice the cells with shrinkage and distorted shapes in drug-treated MCF-7 cells indicated by arrows. **b** The quantitative comparison of the treated MCF-7 cell number. Using ImageJ software, the adherent cells were counted in at least three different fields as shown treatment leads to significant decrease in cell count when comparing with non-treat cells. Data were shown as a mean $\pm$ SE

effects of doxorubicin on the apoptosis induction of MCF-7 breast cancer cells. DOXO and cyclophosphamide are the first-line chemotherapies for breast cancer therapies [3]. Similar to the other anticancer chemicals, high doses of DOXO administration are associated with complicated side effects such as nephrotoxicity [5]. The synergistic effect of DOXO and other factors like lovastatin and lapatinib has been reported for cancer treatment [5, 9]. On the other hand, the non-chemotherapeutic agent, amphotericin B, has been reported to have toxicity promotion function with cisplatin

[24], 1,3-Bis(2-chloroethyl)-1-nitrosourea [25], and actinomycin D [22] drugs in different cancer cells.

Ozol et al. in 1983 reported that AmB can increase the anticancer function of DOXO in the ovarian carcinoma and leukemia cells [26]. However, no finding is available supporting amphotericin B synergism with DOXO in MCF-7 breast cancer cell inhibition.

Our findings indicated the synergistic action of AmB pre-treatment in combination with DOXO. The DNA fragmentation and the caspase-8/9 activity results suggest that AmB can

Fig. 4 DNA fragmentation in the treated MCF-7 cells. TUNEL staining and subsequent imaging using fluorescent microscopy ($\times 100$ magnification) were carried out for combination chemotherapeutic drugs, DOXO, and antifungal drug AmB in MCF-7 cell line. The green-labeled cells show the DNA-fragmented apoptotic cell (a). At least 3–5 fields were counted to measure the percentage of apoptotic index. Both the high DOXO concentration (4 μ M) and AmB pre-treatment (18.3 and 29.2 μ M) increase the apoptotic index in comparison to the low DOXO concentration (0.5 μ M) (b). Data were shown as a mean $\pm$ SE for repeated experiments

Fig. 5 Spectrophotometric analysis of caspase-8 and caspase-9 activities following pre-treatment with AmB plus DOXO and DOXO alone in MCF-7 cells. Data were shown as a mean $\pm$ SE for three experiments. The results were compared with the non-treated cells (asterisk on the bars) or DOXO 0.5 μ M (asterisk on the lines). The gray- and black-colored bars or stars are related to the caspase-8 and caspase-9 results, respectively

increase the DOXO toxicity with a mechanism dependent to apoptotic induction.

Our finding showed that the AmB pre-treatment is more effective than co-treatment of both drugs and then it seems that the synergistic effect of AmB might be before DOXO uptake process probably through membrane perforation.

Indeed, it is reported that AmB can alter the cell membrane potential by efflux of the K^+ ions through permeable pores [8]. Consequently, cellular potassium loss can activate caspase cascade and cell shrinkage, and can finally induce apoptosis in the living cells [27–29].

Therefore, AmB alone can inhibit cancer cells through apoptosis independent to the chemotherapeutic agent, which is in concordant with our findings of DNA fragmentation in the AmB-treated MCF-7 cells (Fig. 4a).

However, it is reported that the potassium efflux can in turn increase the cytotoxicity and apoptotic effect of the anticancer chemotherapeutics, leading to toxic synergism [8]. In contrast, high level of k^+ concentration prevents the induction of apoptosis [29].

In our study, we found both intrinsic and extrinsic apoptotic responses in treated MCF-7 cells; however, the caspase-9 activation (intrinsic pathway) was found to be directly

dependent to the AmB concentration. Generally, the mitochondrial-initiated pathway or intrinsic apoptosis is activated within cells by various stimuli including treatment with cytotoxic drugs and redox unbalance [30, 31]. However, both the internal and external apoptosis can crosstalk with each other affecting cell viability. For example, internal oxidative stress increases permeability of the lysosome membrane leading to cathepsin D and E release into the cytoplasm that will consequently activate caspase-8 (extrinsic apoptosis) [32–35]. Brajtburg et al. showed that AmB under auto-oxidation condition can produce free radical unbalance, one of the key mediators of the extrinsic and intrinsic apoptosis [36]. Furthermore, it has been reported that AmB can induce phosphorylation of the mitogen-activated protein kinase (MAPK) family [15] and the MAPK signaling members such as *p38*, *JNK*, *ASK1*, and *ERK* activation can regulate the caspase-8 activity [31, 37, 38]. Considering the evidences discussed above, our findings suggest that the AmB pre-treatment that probably increases the toxicity of the DOXO chemotherapeutic agent in MCF-7 cells is dependent to both the intrinsic and extrinsic apoptosis pathways.

Conclusion

In conclusion, we showed that pre-treatment of the AmB can successfully increase the anticancer effect of the DOXO in a dose-dependent manner in the MCF-7 breast cancer cells. The underlying mechanism of the AmB pre-treatment in the MCF-7 sensitization to DOXO is associated with both the internal and external cellular programmed cell death responses.

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Compliance with ethical standards

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