Original Article

Co-Delivery of Micellar Berberine and Doxorubicin: A Promising Cardioprotective Strategy and Prospective Therapeutic Anticancer Approach Using Cardiomyocyte and Ovarian Adenocarcinoma Cell Lines as Models

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Abstract

Background: Berberine (Ber) has free radical scavenging abilities and potential chemosensitizing effects. Doxorubicin (Dox) is a highly effective chemotherapeutic agent that is associated with significant cardiotoxicity. We hypothesize that Ber could be loaded into Pluronic F127® micelles (mBer), leading to an improvement in Ber activity. **Objectives:** The objective of this study was to evaluate the potential of Ber in protecting cultured cardiomyocytes against doxorubicin-induced cardiotoxicity (DIC) while concurrently examining its chemosensitizing influence when administered in conjunction with Dox to cultured ovarian adenocarcinoma cells. **Materials and Methods:** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to detect cell viability in cardiomyocytes (H9c2) and ovarian cancer cells (SKOV-3) with either Ber or mBer alone or in combination with Dox. Combination index (CI) analyses were also conducted in order to ascertain the effects of the combinations. **Results:** In H9c2 cells, co-delivery of mBer and Dox resulted in a significantly higher IC50 than Dox alone, and they showed an antagonistic effect in combination (CI > 1). When Ber is incorporated into the F127 micelle, its cardioprotective activity is enhanced. Conversely, the reduction in cell viability and the CI values (CI < 1) showed that this combination was synergistic in SKOV-3, indicating the chemosensitizing effect of mBer. **Conclusions:** The co-administration of mBer and Dox has demonstrated cardioprotective effects against DIC and may hold promise as a prospective therapeutic approach for cancer.

Keywords: Berberine, cardioprotective, cardiotoxicity, chemosensetizing, doxorubicin

Introduction

Doxorubicin (Dox) is a chemotherapeutic drug that belongs to the anthracycline class of antibiotics that has been extensively and effectively used in clinical practice for the treatment of many malignancies, including lymphoma, leukemia, breast and ovarian cancer, and other various adult and pediatric cancers.[1,2] Unfortunately, its clinical use has been hampered by the risk of serious life-threatening cardiotoxicity.[3] Owing to their relatively low ability to regenerate, cardiac muscle is highly susceptible to the long-term toxic effects of Dox.[3] Doxorubicin-induced cardiotoxicity (DIC) has been considered a major health issue associated with Dox therapy and an important challenge in the successful treatment of cancer patients.[4,5] Although extensive investigations have been done to develop an effective treatment for DIC, there is no clinically approved effective treatment currently available.[6,7] Therefore, it is critical to find a way

Access this article online

Quick Response Code:

Website: https://journals.lww.com/mjby

DOI: 10.4103/MJBL.MJBL_814_23

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Submission: 21-Jun-2023 **Accepted:** 10-Aug-2023 **Published:** 30-Apr-2026

How to cite this article: Alyasari NK, Almzaiel AJ. Co-delivery of micellar berberine and doxorubicin: A promising cardioprotective strategy and prospective therapeutic anticancer approach using cardiomyocyte and ovarian adenocarcinoma cell lines as models. Med J Babylon 2026;23:112-8.

to minimize Dox's cardiotoxicity while simultaneously boosting the drug's anticancer efficacy.

Berberine (Ber), a plant alkaloid, is extracted from the Berberis species' roots and bark.[8] Ber is considered to have many biological and pharmacological activities, including anti-inflammatory, anti-diabetic, anti-hypercholesterolemic, cardiovascular, central nervous system, and digestive tract protective actions, as well as antimicrobial and anticancer effects.[9] Many *in vitro* and animal investigations suggest that Ber can protect Dox from damaging cardiac tissues. Ber suppresses Dox metabolism in the cardiomyocytes cytoplasm and reduces the buildup of doxorubicinol, a secondary alcohol metabolite of the drug, in cardiac tissue.[10] Ber protected rat and cultured cardiomyocytes from Dox-induced cardiac tissue-free radical injury by suppressing intracellular Ca^{2+} rise and reducing mitochondrial dysfunction.[11] Ber inhibits p66Shc, a pro-apoptotic protein that controls oxidative stress and promotes the mitochondrial signaling pathway, via activation of Sirtuin 1 expression, revealing a unique method for Ber's cardiomyopathy attenuation.[12] The therapeutic use of Ber for myocardial ischemia/reperfusion damage appears to be promising.[11] On the other hand, Ber can suppress a wide variety of tumors.[13] Ber has the potential to work as an antitumor agent because it can affect many stages of tumor growth, such as cell proliferation, invasiveness, triggering apoptosis, and cell signaling pathways.[13]

Ber's low water solubility, low bioavailability, and low permeability across biological membranes have hampered its use in therapeutic settings thus far.[14] Ber is a substrate for P. glycoprotein, which results in a rapid outflow of Ber from cells.[14,15] In view of this, attaining the necessary Ber concentration in the organ of interest constitutes a considerable challenge. Because of Ber's low efficacy and bioavailability, a new approach is needed to improve these issues. Micelles are an amphiphilic polymeric nanocarrier that can be used to overcome problems.[16]

Taking into account these findings, we hypothesized that combining Dox and Ber as a promising cardioprotective strategy will not only minimize the cardiotoxicity caused by Dox, but also serve as a novel approach for tumor therapy. Despite Ber's poor water solubility, incorporating it into Pluronic F127® polymeric micelles may improve the compound's stability and cellular permeability. In order to test this hypothesis, the current study was conducted with two objectives in mind. Initially, utilizing an embryonic cardiomyoblast cell line, we examined how the cardiotoxicity of Dox changed when Ber was incorporated into Pluronics F127® micelles (mBer) and delivered in combination with Dox. Secondly, the effect of mBer on Dox's *in vitro* anticancer activity was evaluated using an ovarian adenocarcinoma-derived cell line.

Materials and Methods

Chemicals

Dox, berberine hydrochloride (Ber), pluronic F127, dimethyl sulfoxide (DMSO), and cell culture supplies including Dulbecco's Modified Eagle's Medium (DMEM), trypsin ethylenediaminetetraacetic acid, penicillin/streptomycin, amphotericin B, sodium pyruvate, glutamine, and fetal bovine serum (FBS) were all purchased from Sigma Aldrich (Burlington, MA, USA). Chemicals of analytical grade were used in this research. Ber loaded into pluronic F127 nanomicelles (mBer) was created based on the thin film hydration method.[16]

Tissue culture

In flat bottom 96-well cell tissue culture plates, H9c2 rat embryonic cardiomyoblast cell line (ATCC® CRL-1446™), and SKOV-3 Human Caucasian Ovarian Adenocarcinoma cell line (ATCC® HTB-77™) were cultured separately in DMEM medium which was supplemented with 10% FBS, 1% of penicillin 10,000 international unit/10 mg streptomycin, and 25 µg amphotericin B. For maintenance, 1 mM sodium pyruvate and 2 mM glutamine were also added to culture media. The cultured cells were incubated at 37°C in an air atmosphere with 5% CO_2 for 24 h after being seeded at a density of 5.0×10^3 cells per well and the cellular attachment to the well bottom was achieved.

Cell culture viability assay, and IC50 calculation

Experimental groups

Both types of cell lines were subdivided into seven treated groups according to the received treatment. Experimental groups were treated separately with 1 µL of DMSO and contained various serial concentration dilutions of either: free Ber, Berberine-loaded Pluronic F127 Micelles (mBer), Dox, Bre-Dox, or mBer-Dox combination, at a constant molar ratio of 1:1, and empty F127 micelles. Another set of cells that serve as the solvent control were treated only with phosphate buffered saline (PBS) containing 0.5% DMSO. All the wells had a 0.5% final concentration of DMSO. Concentrations of Ber or mBer used were varied from 25 to 1600 µM and 2.5 to 40 µM in H9c2 cells and SKOV-3 cells respectively.

Dox concentration varied from 0. 125 to 8 µM and 0.0125 to 0.6 µM in H9C2 cells and SKOV-3 cells, respectively. Ber-Dox and mBer-Dox concentrations were ranged from 0.125 to 8 µM and 0.0125 to 0.6 µM in H9c2 cells and SKOV-3 cells, respectively, and for void micelles were ranged from 2.5 to 1600 µM in both cell lines. After the treatment, the plates were incubated for another 48 h, and the cell viability assay was performed to determine the level of cell viability.

MTT assay

The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test was used

to evaluate cells viability based on their metabolic activity. Normal viable cells have NAD(P)H-dependent cellular oxidoreductase enzymes, mainly located in the mitochondria and other organelles such as endoplasmic reticulum, which can reduce a yellow tetrazolium salt into insoluble purple crystal compound called formazan. The resultant formazan crystals can be solubilized by adding DMSO, resulting in a colored soluble solution that can be quantified by measuring the absorbance using a microplate reader. The number of viable cells is proportional to the rate of tetrazolium reduction.[17]

The assay was performed by adding 10 µL of the MTT reagent of 5 mg/mL to each well containing cultured cells and the cells were incubated further under the same conditions for 4 h. Next, the supernatant will be removed using multichannel pipette followed by adding 150 µL of DMSO and 25 µL of glycine to each well with well mixing to lyse the cells and ensure complete solubilization of the formazan crystals. Using a microplate (ELISA) reader, the 492 nm absorbance of tested cells and control cells was used to assess the relative proportion of inhibited cells, assuming 100% survival from the vehicle-only control. The percentage inhibition of treated cells was estimated using the following formula:

$$\text{Percentage of inhibited cells} = 100 - \left(\frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \right) \times 100$$

The half-maximal inhibitory concentration (IC_{50}), which shows the dose required to produce a 50% inhibition of cellular viability, was calculated for each drug alone or in combination. The results of the experiment were produced by plotting the measured absorbance after various treatments against the different concentrations of the test substances using the linear regression analysis. The triplicate was performed for all concentrations in all treated groups.

Combination index calculation

At the same concentrations used in the experimental groups for the calculation of the inhibitory concentrations and IC_{50} for Ber, Dox, Ber-Dox, and mBer-Dox utilizing the MTT test, fractions of affected cells (Fa) versus combination index (CI) graphs for Ber and Dox in combination in two different cell lines, H9c2 and SKOV-3, were also constructed utilizing CI analysis.[18,19] CalcuSyn software (Paramus, New Jersey) was used to analyze the CI equation algorithms. The CI can be estimated for different Fa. The benefit of showing CI versus Fa is that it displays the interaction between DH and Ber in combination at all affected cell levels (1%–99%).

We got the CI equation for the two drugs as follows:

$$CI = \left(\frac{Ber1}{BerX} \right) + \left(\frac{Dox1}{DoxX} \right)$$

where Ber1 and Dox1 stand for concentrations of Ber and Dox alone, respectively, to exert a certain effect. BerX and DoxX display the concentrations of Ber and Dox, respectively, in the combination that has the same effect.

When the CI is 1, there is additive interaction; when it is <1, there is synergy; and when it is >1, there is antagonism.[18]

We used the same principle to figure out how Dox and mBer interact in the two different cell lines by calculating the CI value and comparing it to how Dox and free Ber interact.

For each H9C2 and SKOV-3 cell line, we generated a plot of mean CI versus Fa data that is representative of three replicates.

Statistical analysis

In this investigation, we utilized a one-way analysis of variance (ANOVA) in conjunction with the least significant differences as a post hoc test to examine normally distributed data and identify statistically significant distinctions. GraphPad Prism, version 9.5.0, was employed for this purpose, with a significance level of $P < 0.05$ being deemed statistically significant.

Ethical approval

The research was conducted in accordance with the ethical principles articulated in the Declaration of Helsinki. The local ethics committee of the College of Medicine/ University of Al-Qadisiyah in Iraq reviewed and approved the study protocol procedure (document number 208) on October 28, 2022.

Results

mBer reduces DOX-mediated cytotoxicity in H9c2 cells

On rat's H9c2 cell culture, the potential inhibitory effect of different serial concentration dilutions of free Ber, mBer, Dox, Bre-Dox, or mBer-Dox combination, and empty F127 micelles was tested and compared with the control group of the cell line treated only with PBS containing 0.5% DMSO, which is considered the vehicle control group. In the MTT experiment, cell viability was expressed as a percentage of inhibition of treated cells relative to untreated control. Treatment of H9c2 cells with free Ber or mBer did not affect the viability of cells except after relatively high concentrations for both Ber forms [Figure 1]. As shown in Figure 2, the inhibitory effect of Dox on the viability of H9c2 cells increases as Dox concentration increases. Starting at 2.85% inhibition at the lowest utilized concentration 0.125 µM and increasing to 89% inhibition at the highest tested concentration 8 µM. Combination of Ber and Dox considerably mitigated the cytotoxicity of

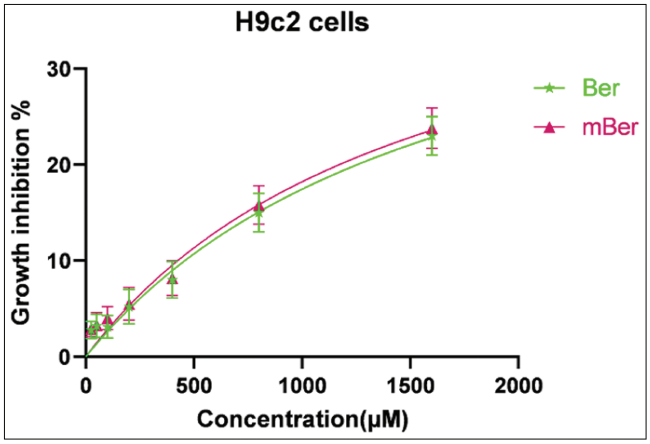

Figure 1: The growth inhibition percentage of free Ber and mBer in H9C2 cell line. Data presented as mean ± SD (μM); $n = 3$

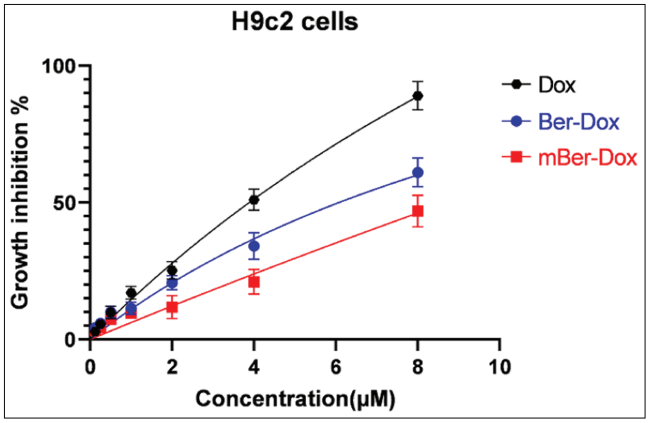

Figure 2: A comparison of growth-inhibition percentages of Dox, Ber-Dox, and mBer-DoX in H9C2 cell line. Data presented as mean ± SD (μM); $n = 3$

Dox on H9c2 cells [Figure 2]. The IC_{50} values of free and micellar-loaded Ber in H9c2 cells after 48 h of incubation were 3,565.08 ± 37 μM and 3439.46 ± 39 μM respectively [Figure 3], indicating that both Ber and mBer are particularly nontoxic to cultured cardiomyocytes (H9c2 cells). Empty polymeric micelles alone exhibited no cytotoxic to H9c2 cultured cells. Following 48 hours of incubation, the 50% growth inhibition concentration (IC_{50}) of Dox in H9c2 cells was 4.42 μM [Figure 3]. The increase in the IC_{50} of Ber-Dox when compared to the IC_{50} of Dox [Figure 3] demonstrated that Ber had cardioprotective properties. When the micellar form of Ber is used with Dox, the cardioprotective effect of Ber against Dox in H9c2 cells is clearly improved. When combined with Dox, mBer is more effective and potent at protecting cultured cardiac cells than free Ber [Figure 2], as evidenced by the fact that the IC50 of the combination of mBer and Dox was significantly ($P < 0.05$) higher than that of Ber-Dox in combination [Figure 3].

Antagonistic action of mBer and Dox in H9c2 cells

The CI was used to figure out how Ber and Dox affect H9c2 cells together. The plots of CI versus all fractions

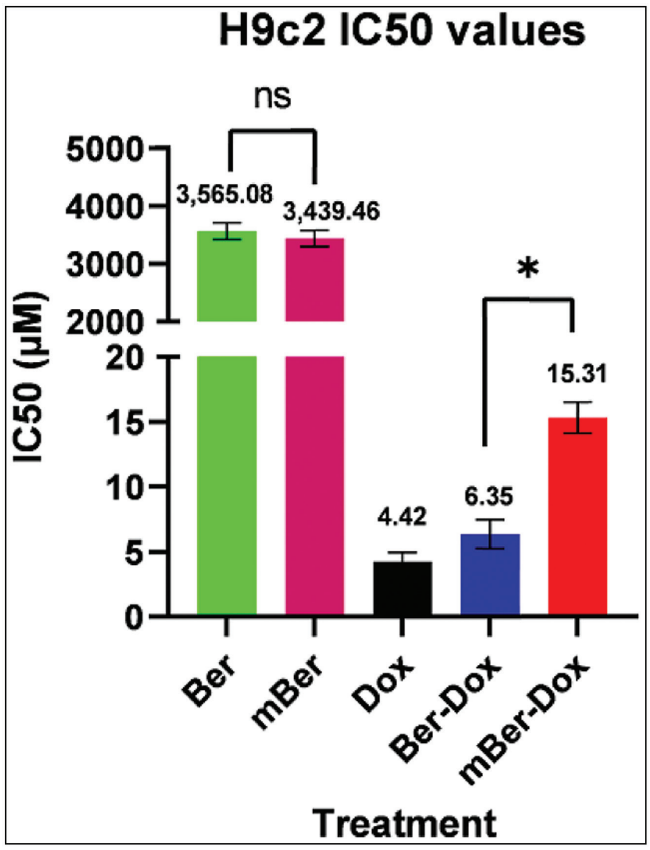

Figure 3: The IC 50 values of Dox, Ber-Dox, and mBer-DoX in H9C2 cell lines. Data presented as mean ± SD (μM); $n = 3$. ns: no significant difference $P > 0.05$. * Denoted to significant differences $P < 0.05$

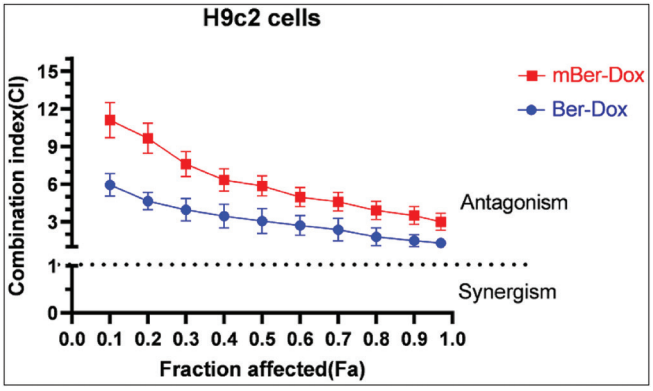

Figure 4: CI versus Fa plots for the Ber-Dox and mBer-Dox combinations in H9c2 cells. CI values of 1, 1, and >1 correspondingly imply synergy, additivity, and antagonism. Data presented as mean CI ± SD; $n = 3$

affected for Ber-Dox and mBer-Dox are shown in Figure 4. Values of CI that are less than 1 indicate synergy, while values of CI that are greater than 1 indicate antagonism. Considering the data, both the Ber-Dox and mBer-Dox combinations were antagonistic (CI > 1) in H9c2 cells [Figure 4], but the mBer-Dox combination was more antagonistic than the Ber-Dox combination. These data further support Ber's antagonistic effect against DIC, and the cardioprotective effect of Ber is enhanced when it is loaded in to the F127 micelle.

Chemosensetizing effect of mBer in SKOV-3 cells

To assess the possible inhibitory effects of various concentrations of free Ber, mBer, Dox, Bre-Dox, or mBer-Dox combination, and void F127 micelles on human SKOV-3 cells, the MTT assay was performed, and the results were also represented as a percentage of inhibition relative to the untreated control. The results demonstrated that both Dox and Ber reduced cell viability in the SKOV-3 cell line in a concentration-dependent manner [Figures 5 and 6].

Treatment of SKOV-3 cells with free Ber exhibited dose-dependently low cytotoxicity. mBer augments the cytotoxicity of Ber in comparison to its free form [Figure 5]. Micelles devoid of Ber had no effect on SKOV-3 cells. Dox's cytotoxicity against SKOV-3 cells is clearly increased when combined with Ber. Whereas there is a shift in the potency and efficacy of the cytotoxicity against SKOV-3 cells when mBer was used in combination with Dox compared to the situation when Ber and Dox were used in combination [Figure 6]. Also, contrary to what was seen in H9c2 cells, when SKOV-3 cells were treated with both Ber and Dox, the IC50 was significantly ($P < 0.05$) lower than when Dox was used alone. This further suggests that Ber makes cancer cells more sensitive to chemotherapy when it is combined with Dox. Intriguingly, the micelle form of Ber enhances Ber's chemosensitizing effect on SKOV-3 cells, as indicated by a further significant ($P < 0.05$) drop in IC_{50} value [Figure 7].

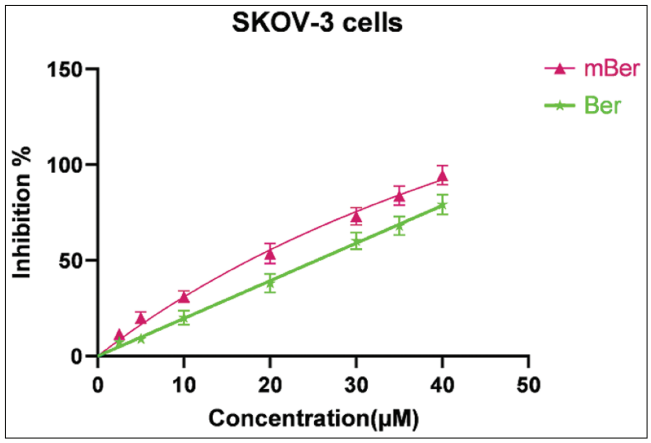

Figure 5: A comparison of the growth inhibition percentages of free Ber and mBer in SKOV-3 cells Data presented as mean ± SD (μM); $n = 3$

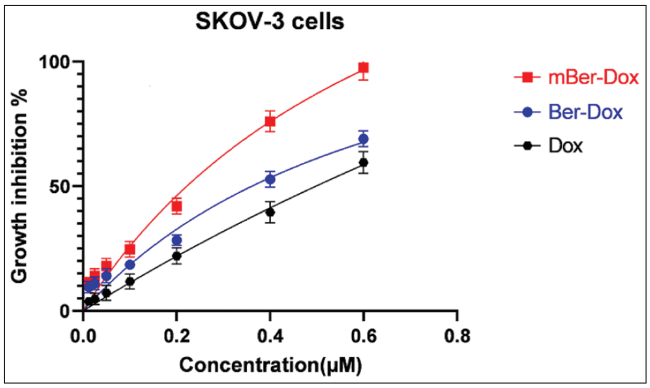

Figure 6: Growth-inhibition percentages of Dox, Ber-Dox, and mBer-DoX in SKOV-3 cell line. Data presented as mean ± SD (μM); $n = 3$

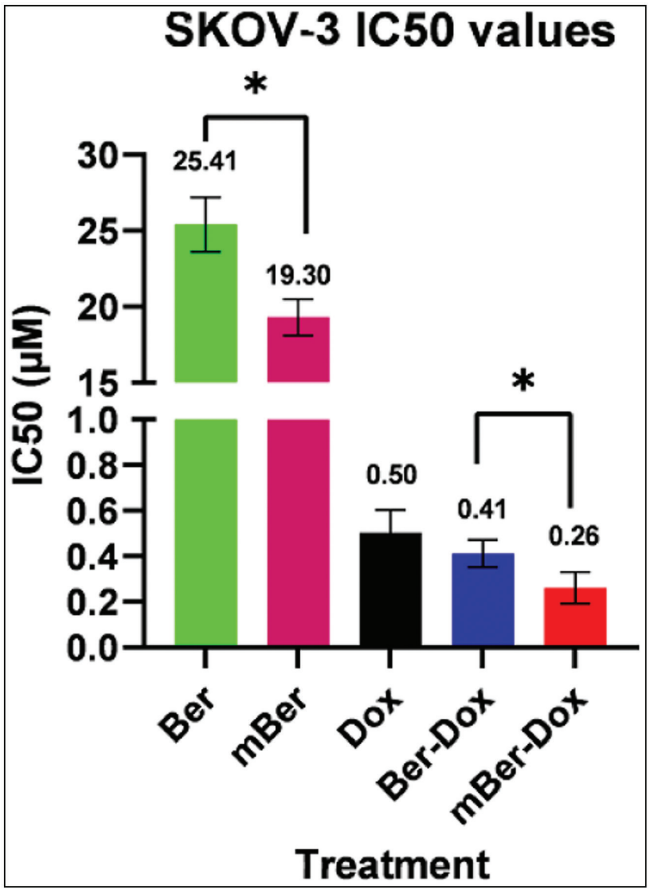

Figure 7: The IC 50 values of Dox, Ber-Dox, and mBer-DoX in SKOV-3 cell lines. Data presented as mean ± SD (μM); $n = 3$. * Denoted to significant differences $P < 0.05$

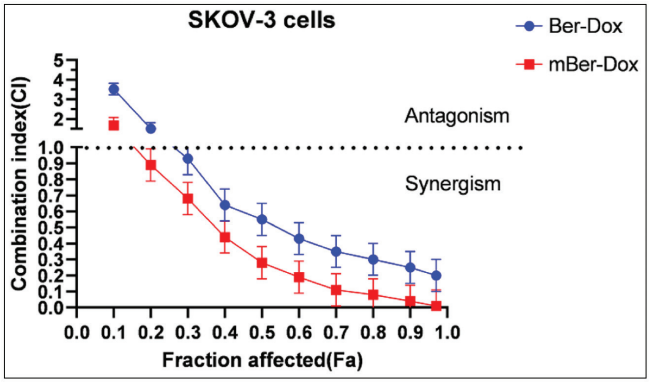

Figure 8: Ber-Dox and mBer-Dox CI values in SKOV-3, where values below 1, at 1, and above 1 denote synergistic, additive, and antagonistic effects, respectively. Data presented as mean CI ± SD; $n = 3$

Synergistic action of Ber and Dox in SKOV-3 cells

In SKOV-3 cells, both combined treatment showed antagonistic effect (CI < 1) only at relatively low concentrations (low fraction effected) which is less relevant to clinical therapy, but substantially showed synergistic effect as their concentrations increase. The effect of Ber-Dox combination was found to be synergistic after a Fa of 0.25, whereas the mBer-Dox co-delivery showed synergistic effect earlier after a Fa of 0.15. Notably, after those points, which are more relevant to clinical therapy, it was revealed that they both have an antagonistic cytotoxic effect. In general, the mBer-Dox combination demonstrates superior cytotoxic synergy in SKOV-3 cells than the Ber-Dox combination [Figure 8].

Discussion

The purpose of this study was to find a strategy to make Dox less harmful to the heart without diminishing its anticancer activity. We show that Ber, a naturally occurring phytochemical molecule, can protect cultured neonatal rat cardiomyocytes from Dox's toxic effects and make a cultured human breast cancer cell line more sensitive to the drug's effects.

Ber, which is known to have numerous pharmacological effects, has anticancer potential due to its ability to eliminate free radicals, induce apoptosis and cell cycle arrest, reduce angiogenesis and inflammation, and modulate the activity of multiple signaling pathways.[20] Because it has a low water solubility and is difficult to pass through biological membranes, Ber's therapeutic potential is currently limited.[14] We were able to load Ber into amphiphilic polymeric micelles, which could be a way to solve problems with Ber's bioavailability and solubility. In this experiment, the MTT test was used to find out how effective Ber and its micellar form are at protecting the cardiomyocytes in vitro. We found that Ber has a cardioprotective effect. Interestingly, the polymeric nanoformulation of Ber greatly outperformed the activity of the free Ber, as evidenced by the experimental results of this work, which demonstrated that Ber loaded micelles treatment with increasing concentrations provided a significant protection against Dox. This significant improvement in the cardioprotective activity of the polymeric nanoformulation of Ber over the free form of Ber, as measured by the IC_{50} value on H9c2 cells, can be attributed to the advantages of micelles in enhancing the permeability of Ber and the cellular retention effect, which permits the release and availability of Ber nanoparticles readily in the treated cells. Ber's antagonistic effect against Dox in H9c2 cells may be a result of this compound's free radical scavenging activities and/or interference with cell signaling pathways, which leads to the attenuation of Dox-induced damage. It was reported that Ber exhibits cardio-protective properties via multiple mechanisms, such as minimizing free radical injury, increasing catalase and superoxide dismutase activity, and preventing mitochondrial dysregulation. In a similar way, Ber protects cultured cardiomyocytes from Dox toxicity by lowering SIRT1-induced p66shc expression, which reduces the damage caused by Dox.[12]

The current investigation has also employed the CI index to identify compound interactions. The Fa versus CI plots suggest that Ber in H9c2 cells generally acts as an antagonist for Dox, possibly by scavenging free radicals. On the other hand, we found that free Ber has an inhibitory effect on SKOV-3 cells, and this ability was augmented when nanoformulation of Ber was used. Intriguingly, we also confirmed that Ber and Dox work well together in SKOV-3 cell lines. This suggests that Ber makes SKOV-3 cells more sensitive to chemotherapy when it is combined with a highly toxic drug like Dox. The CI index corroborated the IC50 value, demonstrating that the synergistic anticancer activity of mBer and Dox was improved. One possible explanation is that F127 micelles improve Ber's permeability, delivery, and retention inside the cell. Ber, in particular, is rapidly effluxed from cells because it is a substrate for P-glycoprotein drug efflux transporter.[21] Inhibition of the P-glycoprotein by Pluronic F127 micelles has been reported.[22] These findings suggest that F127 micelles contribute to enhanced cellular retention of Ber. One way that both Dox and Ber act as anti-cancer agents is by inducing apoptosis.[23,24] As per the idea of "independent similar action," which is a situation in which the combined effect of two drugs is equivalent to the greater of their individual effects,[25] this seems to be the case. Thus, the combination of Dox and Ber may have a synergistic anti-cancer effect.

In agreement with our findings, it has been reported that Ber significantly reduced the apoptosis in H9c2 myocytes induced by hypoxia.[26] Conversely, a number of studies have indicated that Ber promotes apoptosis in cancer cell lines in a concentration-dependent way.[27] According to the findings of these studies, it can be concluded that the effect of Ber apoptosis differs depending on the type of cell employed and the concentration used.

Conclusions

Ber loaded on F127 polymeric micelles could be a good way to improve Ber activity and protect the heart from DOX toxicity. This confirms that mBer provides a greater cardioprotective effect than free Ber in cultured cardiomyocyte cells through its antagonistic effect against Dox-induced toxicity. We also confirmed that the IC_{50} of Ber loaded on F127 polymeric micelles is enough for it to work as a promising and effective anticancer agent in an ovarian adenocarcinoma cell line *in vitro* through a synergistic effect. The combination of mBer and Dox could be a novel strategy for protecting the heart from Dox-induced toxicity and offers the possibility of a

promising and effective agent for cancer treatment. To confirm the chemosensitizing and cardioprotective effects of Ber, additional *in vivo* investigations on both animals and humans are required. Thus, our findings provide a rational strategy for further devising a potentially effective phytochemical adjunctive therapy with Dox for chemotherapy patients.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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