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Research

F-α-DDB-derivative, a novel synthetic of bifendate, plus epirubicin improves antitumor efficacy against triple negative breast cancer without additional cardiotoxicity

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Abstract
Triple negative breast cancer (TNBC) refers to a molecular subtype of breast cancers (BC) with high rate of distant metastases and poor prognosis. Epirubicin (EPI) is widely used for the therapy of TNBC but it's limited in clinical use due to cardiotoxicity and chemotherapy resistance. We previously identified F-α-DDB-derivative, a novel synthetic of bifendate, as a potential agent to improve the therapeutic efficacy of TNBC in combination with EPI since F-α-DDB-derivative inhibited MDA-MB-468, but not as much as EPI. In this study, we investigated the antitumor activity of F-α-DDB-derivative in combination with EPI against TNBC in vitro and in vivo and whether co-treatment would induce additional cardiotoxicity. In human TNBC MDA-MB-468 cells, application of F-α-DDB-derivative (11.5, 23.0, 46.0 μg/ml) in combination with EPI (1.5, 3.0, 6.0 μg/ml) exhibited great inhibition on cell viability and cell proliferation. Additionally, F-α-DDB-derivative (5.75, 11.5, 23.0, 46.0 μg/ml) interacted with EPI (0.75, 1.5, 3.0, 6.0 μg/ml) synergistically to induce apoptosis in MDA-MB-468 cells. This suggests that F-α-DDB-derivative may be more sensitive to apoptotic pathways. Furthermore, we revealed that co-administration of EPI and F-α-DDB-derivative (ip, once every other day for 14 days) significantly increased the therapeutic efficacy of EPI (2.0 mg/kg) or F-α-DDB-derivative (20.0 mg/kg) in mice harboring MDA-MB-468 cell xenografts without additional cardiotoxicity compared to that in EPI monotherapy group. These results implicate that co-treatment of EPI and F-α-DDB-derivative may be a potential therapeutic approach for the treatment of TNBC.

Keywords F-α-DDB-derivative · Bifendate · Epirubicin · Triple negative breast cancer

1 Introduction

According to the latest statistics from the GLOBOCAN 2022 database, breast cancer (BC) is the second most common cancer worldwide. In addition, BC is the fourth leading cause of cancer-related deaths globally and the most common malignant tumor among Chinese women [1]. Triple negative breast cancer (TNBC) refers to BC with no expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), accounting for 10–20% of BC. Compared to other forms of BC, TNBC exhibits a lower overall survival (OS), a higher rate of distant metastases, and a higher rate of recurrence [2, 3]. With the development of contemporary medical technology, cancer treatment methods have advanced significantly. Chemotherapeutic agents have now been clinically proven to be effective

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for TNBC. Epirubicin (EPI) is a type of anthracycline with broad-spectrum anti-tumor activity, and has been widely used in the treatment of TNBC. However, its cardiotoxic side effect has limited its clinical use to some extent, not to mention chemotherapy resistance [4, 5]. Despite the use of lower doses of EPI, serious cardiotoxicity has been observed. Heart failure risk could be a persistent issue throughout life, particularly for young cancer survivors [6, 7].

To address the EPI-induced cardiotoxicity, many efforts have been made. It has been demonstrated that dexrazoxane is effective in preventing or lessening cardiotoxicity caused by anthracyclines. Additionally, there is no proof that tumor response rate, OS, or progression-free survival (PFS) are negatively impacted. Nevertheless, it might be linked to an increased risk of second malignant neoplasm (SMN) [8]. Although derivatives of anthracycline such as liposomal EPI and non-anthracycline alternative therapies have been explored [9, 10], it's still a tough issue to balance the toxicity and anti-tumor efficacy in clinical application. Hence, an effective treatment to reduce the toxicity and enhance the anticancer response of EPI is crucial.

Combination therapy, which involves several medications or regimens, has become popular in recent years as a means of treating cancer since it improves therapeutic efficacy and reduces side effects [11–13]. Bifendate, a synthetic intermediate of *Schizandrin C*, which is widely used to treat chronic hepatitis in China, has been modified to be a potential therapeutic candidate for hepatocellular carcinoma [14]. However, we did not find the antitumor effect of bifendate on TNBC cell lines in our previous experiments.

In recent years, the research of fluorine chemistry in the field of medicine has been deepening, and a number of fluorine-containing medicines have been introduced, such as fulvestrant, Enhertu, abemaciclib, etc. [15–17]. Since the atomic radius and volume of the fluorine atom (F) is similar to that of the hydrogen atom (H), when F replaces H in a molecule, it does not lead to a significant change in the molecular spatial site resistance. However, as the most electronegative atom in the periodic table, when fluorine atoms replace hydrogen atoms in a molecule, the electronic properties of the molecule are altered considerably, which causes changes in the lipophilicity of the molecule, changes in the electrostatic interactions with the target structures (e.g., proteins), and inhibitory effects on some metabolic pathways [18]. Therefore, the modification of the molecular structure of drugs by fluorine chemistry is expected to provide more possibilities for drug development and enhancement of drug efficacy. Previously, we have discovered that F-α-DDB-derivative, a novel synthetic of bifendate synthesized by Shanghai Institute of Organic Chemistry has antitumor effects on TNBC. In the present study, we demonstrate the synergistic effect of EPI and F-α-DDB-derivative on TNBC in vitro and in vivo without additional cardiotoxicity. According to our research, treating TNBC with a combination of EPI and F-α-DDB derivative may be a novel treatment approach.

2 Materials and methods

2.1 Cell line and cell culture

The human triple negative breast cancer line MDA-MB-468 was purchased from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China). MDA-MB-468 cell was routinely cultured in at 37 °C in a humidified incubator (ThermoFisher, MA, USA) with 5% CO_2 in Dulbecco's modified eagle medium (DMEM) (CM-0290B) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) (Procell, Wuhan, China). Every 1–2 days, take out the culture bottle to observe the cell growth status and the color of medium. Spray the outer wall of the culture flask with 75% alcohol and transfer it to an ultra-clean bench, pour off the original complete medium, add 1–2 mL of PBS, shake the flask gently to rinse and pour out the PBS, and repeat this process 2–3 times. Add 5–6 mL of medium, spray the outer wall of the culture flask with 75% alcohol, and place it in a cell culture incubator at 37℃ with 5% CO_2 for further incubation. Eppendorf (5810R) centrifuge (Hamburg, Germany) was used for cell pellet preparation and reagent separation. Eppendorf Research Plus pipettes (Hamburg, Germany) were used for precise liquid handling.

2.2 Animals

Female BALB/c mice (4–6 weeks, weighing 17–23 g) were obtained from the Shanghai Model Organisms Center, Inc. The animals were kept in stainless steel cages with a 12-h light/day cycle, 22 ± 2℃, and 55–65% humidity under stringent control. They also had unlimited access to food and drink. All animal experiments were performed according to the guidelines and approval of SRCMO-IACUC (NO.2024-0003).

2.3 Reagents

Epirubicin (56390-09-1) (E408486-1) was purchased from Aladdin (Shanghai, China). F-α-DDB-derivative was obtained from Shanghai Institute of Organic Chemistry (Chinese Academy of Sciences, Shanghai, China).

Phosphate buffered saline (PBS) (KGL2206-500), 4% paraformaldehyde fixative (KGC3121-500), Trypan Blue staining solution (KGL2320-12) and Crystal violet solution (KGE1202-100) were procured from KeyGEN BioTECH (Nanjing, China). Dimethyl sulfoxide (DMSO) (D9170-1 VL) was provided by Sigma-Aldrich (Gillingham, UK). We obtained 0.25% trypsin (C0203-100) from the Beyotime Institute of Biotechnology (Haimen, China). Polyethylene glycol 300 (PEG300) (HY-Y0873) was from MedChemExpress (New Jersey, US). Polysorbate 80 (Tween80) (T485984-10) was from Aladdin (Shanghai, China).

2.4 Cell viability assay

The effect of EPI and F-α-DDB-derivative was evaluated by the cell counting kit-8 (CCK8) assay. Cells were seeded into 96-well plates (Corning, NY, USA) at a density of 2.5×10^4 cells per well. After 24 h, cells were treated with different concentrations of EPI (0.25, 0.5, 1.0, 2.0, 4.0, 8.0 μg/ml) and F-α-DDB-derivative (0.5, 1.0, 5.0, 10.0, 25.0 μg/ml) for 24 and 48 h. The CCK-8 assay kit (C0039, Beyotime Institute of Biotechnology, Haimen, China) was then used to measure the cell viability (%) in accordance with the manufacturer's instructions. Microplate reader (ThermoFisher, MA, USA) was used to measure the absorbance at 450 nm.

2.5 Combination studies

MDA-MB-468 cells were harvested and plated (2.5×10^4 per well) in 96-well plates for 24 h. Cells were treated with EPI and F-α-DDB-derivative at concentrations of 0.75, 1.5, 3.0, 6.0, 12.0 μg/ml and 5.75, 11.5, 23.0, 46.0, 92.0 μg/ml, respectively, for 24 h. determined by each drug's half-maximal inhibitory concentration (IC_{50}) value. By comparing the result to the control, the fractional inhibition of cell proliferation was computed. Using the CCK8 assay, cell proliferation was assessed after a 24 h incubation period. Chou and Talalay's [19] median-effect analysis served as the basis for the combination index (CI) approach, which was used to calculate the effects of the combination for each experimental condition. $CI > 1$, $CI = 1$, and $CI < 1$ indicates antagonism, additivity, and synergy, respectively.

2.6 Cell scratch assay

Cells were seeded into 6-well plates and incubated for 24 h to reach nearly 100% density. Then, two scratches were made in the wells with a 200 μl tip to form a cross. After capturing the images of wound healing with a 4 × objective lens using inverted microscope (Olympus Corporation, Tokyo, Japan), cells were treated with different concentrations of EPI (0.75, 1.5, 3.0, 6.0 μg/ml) and F-α-DDB-derivative (5.75, 11.5, 23.0, 46.0 μg/ml) monotherapy or in combination. After another 6 h incubation, the images of wound healing were also captured. Images were analyzed by ImageJ software (MD, USA).

2.7 Immunofluorescence

Cells were seeded in 24-well plates for 24 h. After 24 h of treatment with different concentrations of EPI (0.75, 1.5, 3.0, 6.0 μg/ml) and F-α-DDB-derivative (5.75, 11.5, 23.0, 46.0 μg/ml) monotherapy or in combination, Ki67 were stained with Ki67 Cell Proliferation Assay Kit (C2301S, Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's instructions. The red and blue fluorescence was observed by fluorescence microscope. Images were captured with a 10 × objective lens. Images were analyzed by ImageJ software (MD, USA).

2.8 Apoptosis assay

Cells were seeded in 6-well plates and treated with EPI (0.75, 1.5, 3.0, 6.0 μg/ml) and F-α-DDB-derivative (5.75, 11.5, 23.0, 46.0 μg/ml) monotherapy or in combination for 24 h. The cells were then collected, washed, and resuspended in PBS. Cells were stained with Annexin V Apoptosis Detection Kit APC (88–8007-72, Thermo Fisher Scientic, Waltham, MA, USA)

according to the manufacturer's instructions. Cell suspension was analyzed by the flow cytometer (Beckman Coulter, CA, USA).

2.9 In vivo studies

MDA-MB-468 cells (1×10^6) were suspended in a 1:1 ratio in DMEM medium with a Matrigel basement membrane matrix (Corning, NY, USA). Cell suspension (150µL) was injected subcutaneously into the right side of the fourth mammary gland of each mouse. The tumor length (L) and width (W) were measured and the tumor volume was calculated by using the formula: $V(mm^3) = (L \times W^2)/2$ [20]. After a four-week tumor inoculation, the size of tumors was approximately 95 mm^3. The mice were divided into four treatment groups randomly. The mice accepted treatments with either Nacl, EPI (2.0 mg/kg), F-α-DDB-derivative (20.0 mg/kg) or a combination of EPI/F-α-DDB-derivative by intraperitoneal injection once every 2 days for seven times. Weights and tumor sizes were recorded every other day. Blood was collected and the mice were sacrificed after 15 days. Serum ALT, AST, UREA, CREA, CK, CK-MB and LDH levels were measured by corresponding Kits (S03030, S03040, S03036, S03076, S03024, S03034, S03023, Rayto, Shenzhen, China) and Automatic Biochemistry Analyzer (Rayto, Shenzhen, China) according to the manufacturer's instructions. Tumors, hearts, kidneys and livers were removed, weighed and fixed in paraformaldehyde fixative. The fixed tumors were embedded in paraffin blocks for the H&E staining, and optical microscope was used to view the samples. Furthermore, the major organs were also H&E stained to identify the histological changes.

2.10 Statistical analysis

All data were obtained from at least three independent experiments in vitro and 6 mice in vivo. Comparisons of the different groups were performed with Dunnett's post hoc test and a one-way analysis of variance. The GraphPad PRISM software (GraphPad Software Inc., Avenida, CA, USA) was used for all statistical analyses. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ were regarded as significant differences.

3 Results

3.1 Effect of EPI and F-α-DDB-derivative on MDA-MB-468 cell viability

To evaluate the inhibitory effect of EPI and F-α-DDB-derivative on BC cells MDA-MB-468, dose–response experiments were performed. Cell line MDA-MB-468 were exposed to elevated EPI or F-α-DDB-derivative concentrations for 24 h and 48 h and CCK8 assays were conducted to assess cell viability. Epirubicin and F-α-DDB-derivative both had a cytotoxic effect on MDA-MB-468 in dose and time-dependent fashion. Following 24 and 48 h of treatment, the 50% maximum inhibitory concentrations (IC_{50}) in MDA-MB-468 were 3.146 µg/ml (95% CI [2.851, 3.468]) and 0.036 µg/ml (95% CI [0.027, 0.045]) for EPI (Fig. 1B) and 23.210 µg/ml (95% CI [14.720, 46.450]) and 0.005 µg/ml (95% CI [1.788×10^{-18}, 0.093]) for F-α-DDB-derivative (Fig. 1C), respectively. Following that, fixed ratios for upcoming combination experiments and the computation of combination indices (CIs) were produced using the IC_{50} concentrations.

3.2 Effect of EPI in combination with F-α-DDB-derivative on MDA-MB-468 cell viability

We investigated if a combination therapy with two compounds may further diminish the viability of BC cells, given that the use of numerous medications with various processes or modes of action can direct the effect to a particular target or provide a more effective treatment. Each substance was tested in pairs at varying concentrations while accounting for its IC_{50} values. Concentrations of 0.25, 0.5, 1.0, 2.0 and 4.0 times the IC_{50} were employed for this. Considering that EPI is less sensitive at 24 h than at 48 h, MDA-MB-468 cells were treated with 0.75, 1.5, 3.0, 6.0, 12.0 µg/ml of EPI in combination with F-α-DDB-derivative at concentrations of 5.75, 11.5, 23.0, 46.0 92.0 µg/ml, respectively for 24 h. Dose–response curves for MDA-MB-468 cell line exposed to EPI and F-α-DDB-derivative singly and in combination were shown. When MDA-MB-468 cells were cotreated with EPI (0.75, 1.5, 3.0, 6.0 µg/ml) and F-α-DDB-derivative (5.75, 11.5, 23.0, 46.0 µg/ml), the cell viability significantly decreased in comparison to monotherapy alone (Fig. 1D). We used media effect analysis, which determined the degree of synergy, additivity, or antagonism at different levels of cell death, to thoroughly assess the nature of the interaction between EPI and F-α-DDB-derivative.

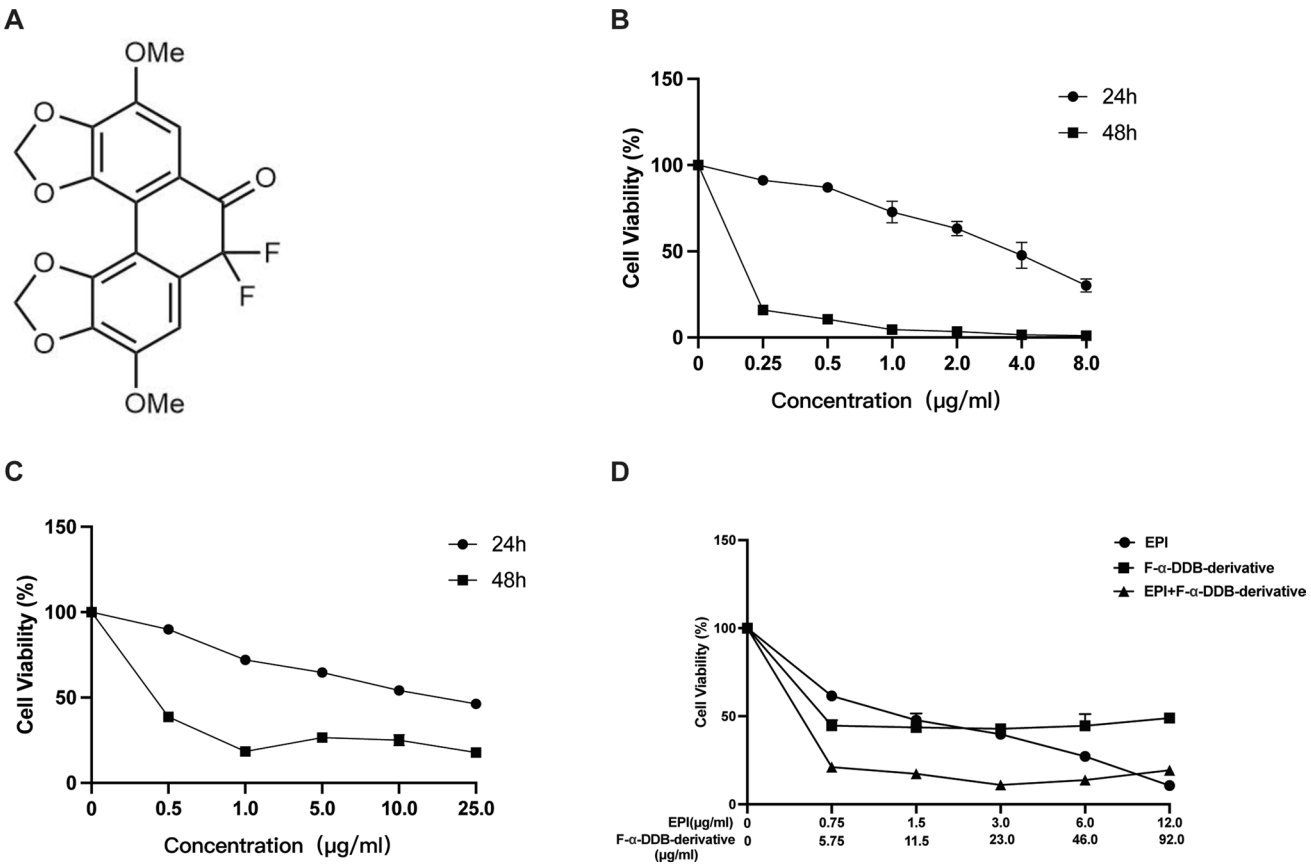


Fig. 1 Effects of EPI and F-α-DDB-derivative on MDA-MB-468 cell viability. **A** Chemical structure of F-α-DDB-derivative. **B** Effects of different concentrations of EPI (**B**) and F-α-DDB-derivative (**C**) on the viability of MDA-MB-468 cells for 24 h and 48 h, as measured by the CCK-8 assay. **D** Effects of EPI (0.75, 1.5, 3.0, 6.0 μg/ml) in combination with F-α-DDB-derivative (5.75, 11.5, 23.0, 46.0 μg/ml) on the viability of MDA-MB-468 cells for 24 h, as measured by the CCK-8 assay

The combination of EPI and F-α-DDB-derivative at concentrations of 0.75, 1.5, 3.0, 6.0 μg/ml and 5.75, 11.5, 23.0, 46.0 μg/ml respectively demonstrated CI values less than 1.0, indicating the synergistic interactions of two medications. On the other hand, CI value of cotreatment of EPI (12.0 μg/ml) and F-α-DDB-derivative (92.0 μg/ml) more than 1.0 was obtained, suggesting the antagonism (Table 1). As EPI (0.75, 1.5, 3.0, 6.0 μg/ml) and F-α-DDB-derivative (5.75, 11.5, 23.0, 46.0 μg/ml) interacted synergistically to prevent the growth of MDA-MB-468 cell line, while EPI (12.0 μg/ml) in combination with F-α-DDB-derivative (92.0 μg/ml) just showed antagonistic effects, we further explored the effective dose and mechanism of EPI and F-α-DDB-derivative at concentrations of 0.75, 1.5, 3.0, 6.0 μg/ml and 5.75, 11.5, 23.0, 46.0 μg/ml respectively on MDA-MB-468 cells.

Table 1 CI values of cotreatment of EPI and F-α-DDB-derivative

Regimen		CI value
EPI (μg/ml)	F-α-DDB-derivative (μg/ml)	
0.75	5.75	0.16289 ± 0.01877
1.5	11.5	0.15665 ± 0.03157
3.0	23.0	0.19917 ± 0.00498
6.0	46.0	0.52113 ± 0.03485
12.0	92.0	2.2494 ± 0.1125

EPI epirubicin

Results are the mean ± SD of at least three independent experiments

Fig. 2 Cotreatment of EPI and F-α-DDB-derivative inhibits MDA-MB-468 cell proliferation. **A** MDA-MB-468 cells were treated with different concentrations of EPI, F-α-DDB-derivative, EPI/F-α-DDB-derivative or DMSO for 24 h. To assess cell proliferation, expression levels of Ki67 were detected by immunofluorescence assay after treating cells with different concentrations of monotherapy, co-administration, DMSO or control for 24 h. Nuclei were stained with DAPI (blue), and Ki67 was stained with a specific antibody (red). Scale bars, 100 μm. **B** Quantification of Ki67-positive cells in each treatment group. Data represented as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 compared with the control group, ns not significant

3.2.1 EPI in combination with F-α-DDB-derivative decrease the expression level of Ki67 of MDA-MB-468 cell line

Cell mitosis is strongly associated with the nuclear antigen Ki67, which is expressed to varied degrees in all cells except G0 phase cells. Since Ki67 and cell mitosis are tightly connected, elevated Ki67 expression indicates that tumor cells are actively proliferating. In the case of BC, Ki67 immunohistochemical staining has become a standard postoperative examination index to evaluate the proliferation ability of tumor and serves as the foundation for BC genotyping, being great value in the therapeutic decision-making and prognosis assessment of BC [21]. To investigate the effect of EPI, F-α-DDB-derivative, and cotreatment with both on the expression level of Ki67, Ki67 immunofluorescence assays were conducted. Cells were treated with 0.75, 1.5, 3.0, 6.0 μg/ml of EPI, 5.75, 11.5, 23.0, 46.0 μg/ml of F-α-DDB-derivative, and with EPI/F-α-DDB-derivative for 24 h. There was no significant difference in the effect of either 0.75 μg/ml EPI, 5.75 μg/ml F-α-DDB-derivative or EPI/F-α-DDB-derivative on Ki67, whereas in the 1.5 μg/ml EPI coupled with 11.5 μg/ml F-α-DDB-derivative group and the 3.0 μg/ml EPI in combination with 23.0 μg/ml F-α-DDB-derivative group outperformed the monotherapy group, compared with control group ($P < 0.05$ and $P < 0.001$, respectively). In addition, 6.0 μg/ml EPI in combination with 46.0 μg/ml F-α-DDB-derivative demonstrated that the combination effect was superior to that of the F-α-DDB-derivative monotherapy group, but similar to that of the EPI monotherapy group ($P < 0.0001$) (Fig. 2A, B). Taken together, our findings imply that combination of EPI and F-α-DDB-derivative could lower the level of Ki67 and inhibit cell proliferation.

3.3 EPI, F-α-DDB-derivative, and cotreatment with both inhibit migratory capacity of MDA-MB-468 cell line

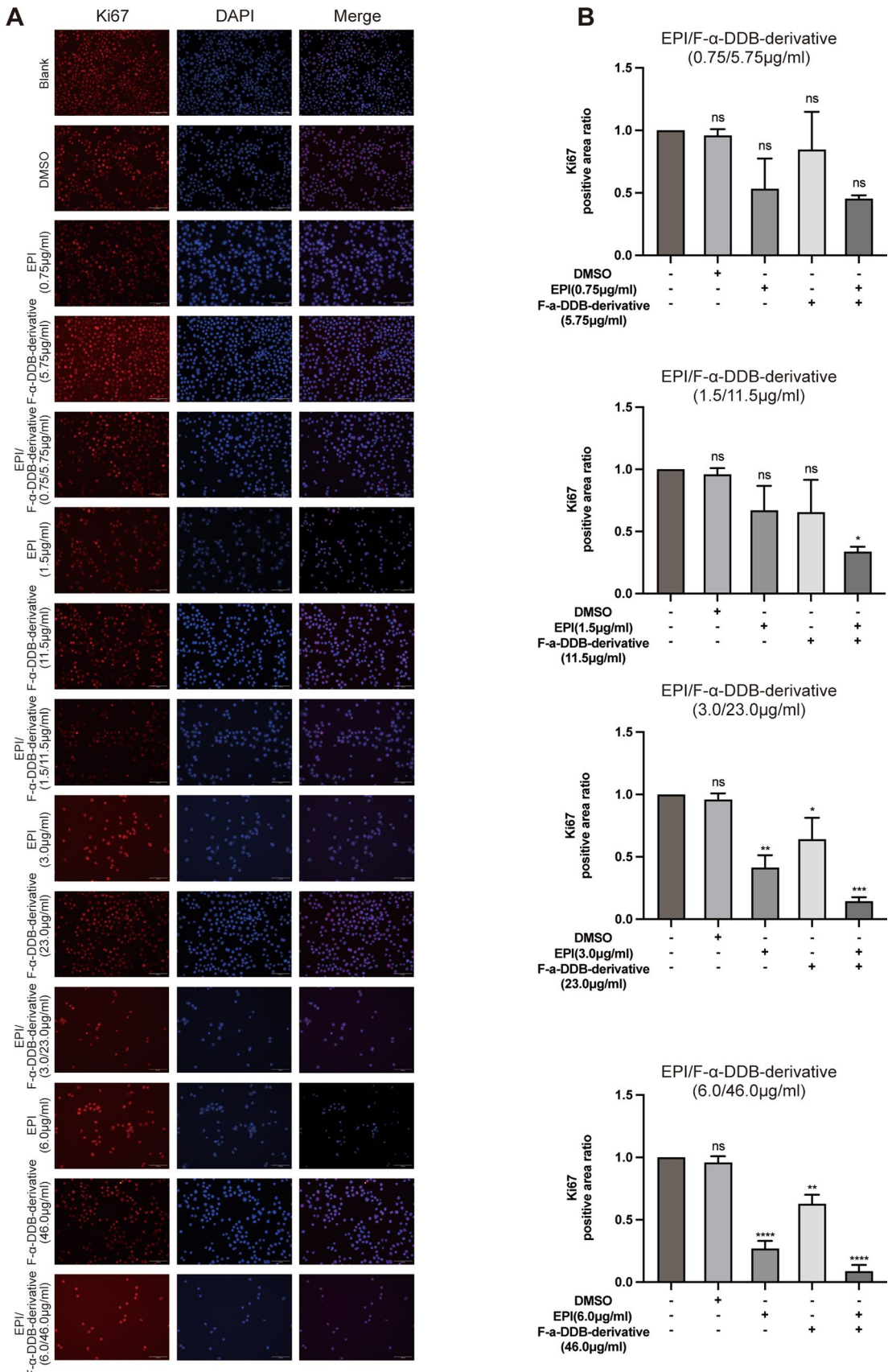
Cell scratch assay was employed to determine the effect of co-administration on migratory capacity of MDA-MB-468 cells. We found that cell migration rate was significantly reduced in the group treated with 1.5, 3.0, 6.0 μg/ml of EPI in combination with 11.5, 23.0, 46.0 μg/ml of F-α-DDB-derivative compared with that in control group ($P < 0.05$, $P < 0.001$ and $P < 0.0001$ respectively) except that in 0.75 μg/ml of EPI in combination with 5.75 μg/ml of F-α-DDB-derivative. Moreover, cell migration rate decreased more in the 3.0ug/ml EPI combined with 23.0ug/ml F-α-DDB-derivative group than in the 3.0ug/ml EPI or 23.0ug/ml F-α-DDB-derivative monotherapy groups. The results implicate that co-treatment of EPI (1.5, 3.0, 6.0 μg/ml) and F-α-DDB-derivative (11.5, 23.0, 46.0 μg/ml) could suppress the capacity of cell migration (Fig. 3A, B).

3.4 Impact of EPI and F-α-DDB-derivative alone, and EPI plus F-α-DDB-derivative on cell apoptosis

To further assess the synergism of EPI and F-α-DDB-derivative, the effect of co-treatment on apoptosis in MDA-MB-468 cells was examined by using Annexin V-APC and DAPI staining and was analyzed by flow cytometry. Data demonstrated that apoptosis rate was higher in combination group than in EPI or F-α-DDB-derivative monotherapy group at all four concentrations. Furthermore, the highest apoptosis rate was observed in the combination of 6.0 μg/ml EPI and 46.0 μg/ml F-α-DDB-derivative group and the lowest apoptosis rate was obtained in the combination of 0.75 μg/ml EPI and 5.75 μg/ml F-α-DDB-derivative group. Apoptosis rates were similar in 1.5 μg/ml EPI and 11.5 μg/ml F-α-DDB-derivative group and 3.0 μg/ml EPI and 23.0 μg/ml F-α-DDB-derivative group (Fig. 4A, B).

3.5 Inhibitory effect of EPI in combination with F-α-DDB-derivative in vivo

To evaluate the synergistic effect of EPI and F-α-DDB-derivative and ascertain whether in vitro results could be reproduced in vivo, we established nude mice model. Once the implanted MDA-MB-468 cells had developed into tumors after 30 days of inoculation, mice were received injections of either EPI, F-α-DDB-derivative or both medications in combination for 14 days. Compared to Nacl treatment, EPI or F-α-DDB-derivative single-agent treatment did not significantly decrease tumor volume. On the other hand, compared to treatment alone, co-treatment resulted in a


A
Ki67
DAPI
Merge
Blank
DMSO
EPI (0.75μg/ml)
F-α-DDB-derivative (5.75μg/ml)
EPI/ F-α-DDB-derivative (0.75/5.75μg/ml)
EPI (1.5μg/ml)
F-α-DDB-derivative (11.5μg/ml)
EPI/ F-α-DDB-derivative (1.5/11.5μg/ml)
EPI (3.0μg/ml)
F-α-DDB-derivative (23.0μg/ml)
EPI/ F-α-DDB-derivative (3.0/23.0μg/ml)
EPI (6.0μg/ml)
F-α-DDB-derivative (46.0μg/ml)
EPI/ F-α-DDB-derivative (6.0/46.0μg/ml)
B
EPI/F-α-DDB-derivative (0.75/5.75μg/ml)
Ki67 positive area ratio
DMSO
EPI(0.75μg/ml)
F-a-DDB-derivative (5.75μg/ml)
EPI/F-α-DDB-derivative (1.5/11.5μg/ml)
EPI(1.5μg/ml)
F-a-DDB-derivative (11.5μg/ml)
EPI/F-α-DDB-derivative (3.0/23.0μg/ml)
EPI(3.0μg/ml)
F-a-DDB-derivative (23.0μg/ml)
EPI/F-α-DDB-derivative (6.0/46.0μg/ml)
EPI(6.0μg/ml)
F-a-DDB-derivative (46.0μg/ml)

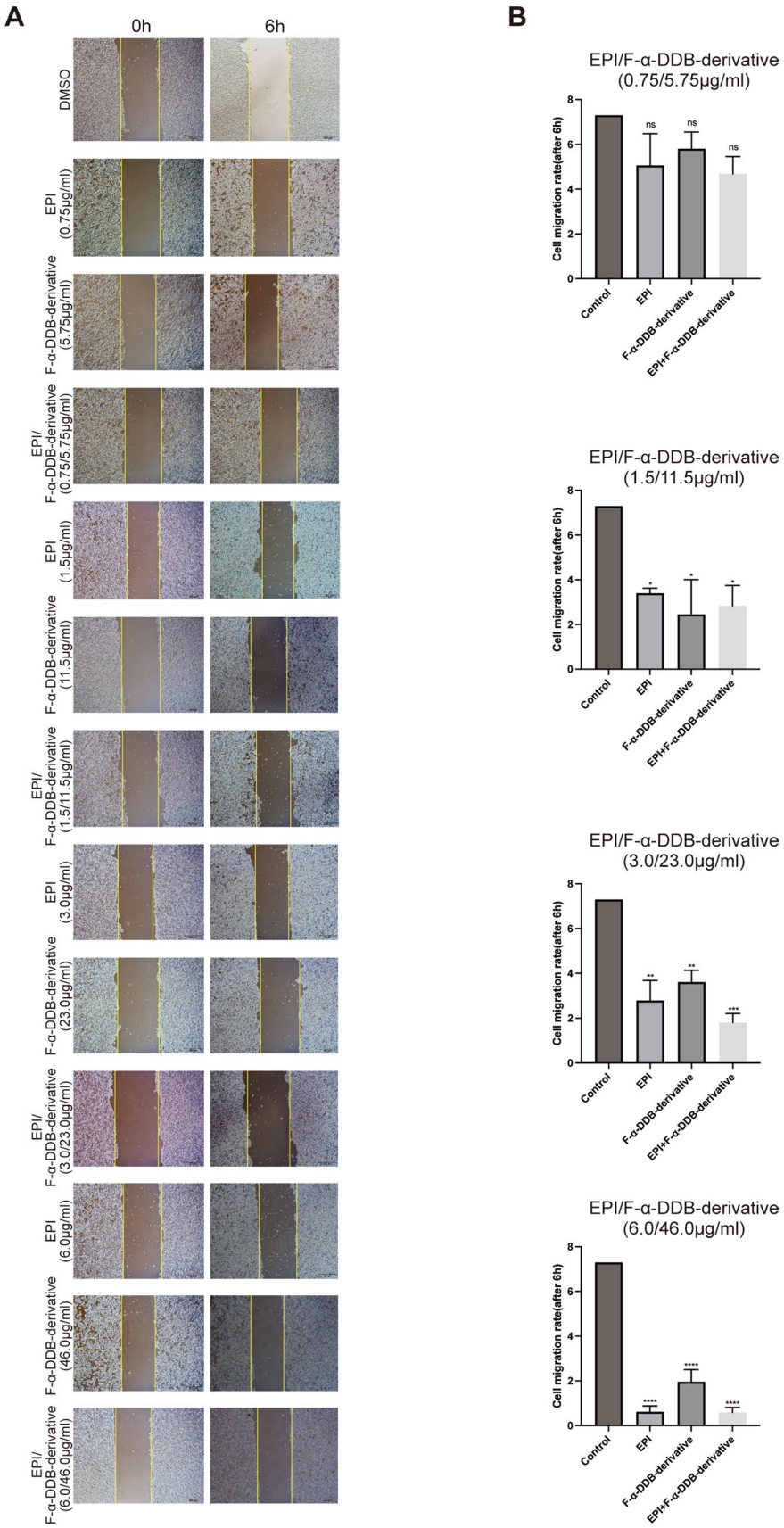


Fig. 3 Cotreatment of EPI and F-α-DDB-derivative inhibits MDA-MB-468 cell migration. **A** MDA-MB-468 cells were treated with different concentrations of EPI, F-α-DDB-derivative, EPI/F-α-DDB-derivative or DMSO for 6 h. The scratch assays were performed to detect the effects on migration. Scale bars, 500 μm. **B** Quantification of cell migration in each treatment group. Data represented as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 compared with the control group, ns not significant

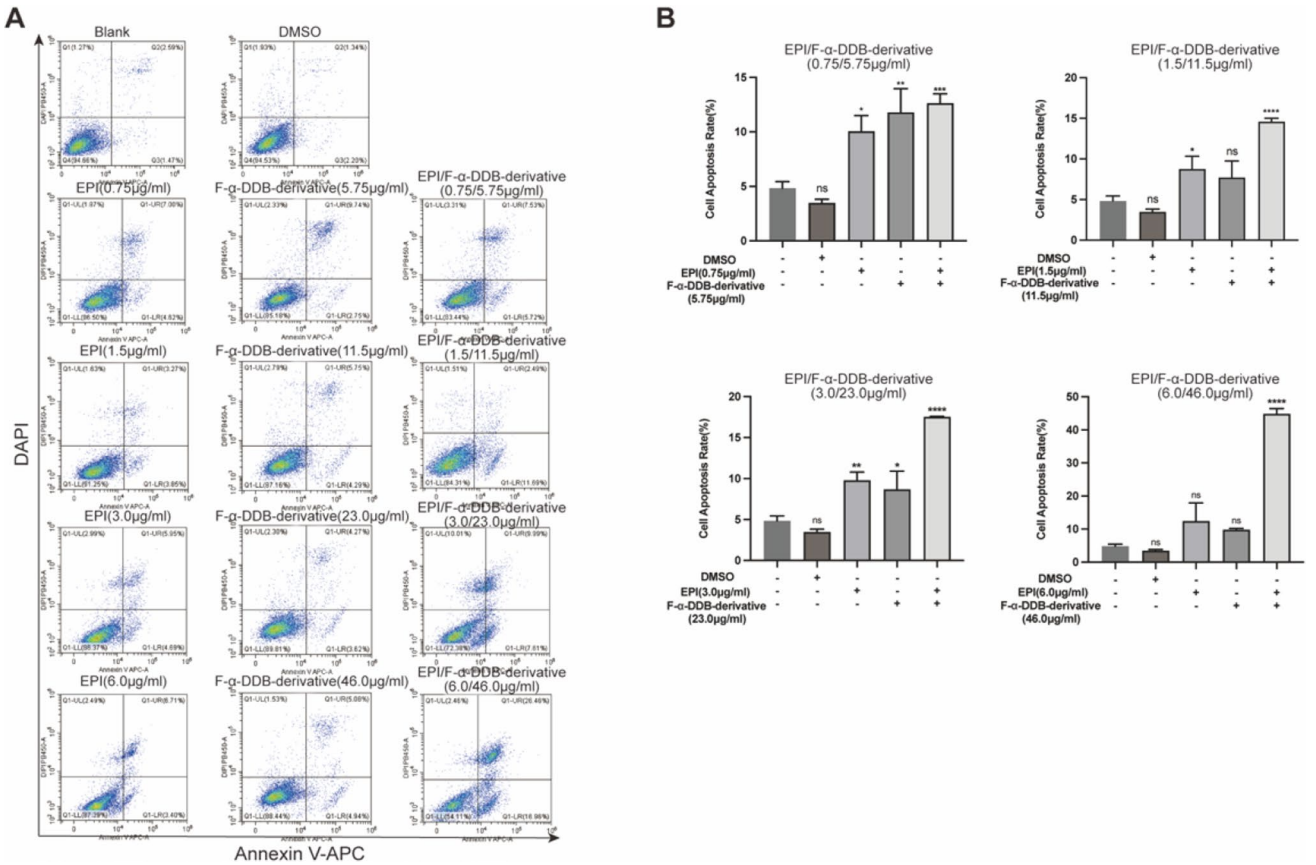


Fig. 4 EPI/F-α-DDB-derivative combination promotes cell apoptosis. **A** Apoptosis was assessed in MDA-MB-468 cells treated with different concentrations of EPI, F-α-DDB-derivative, or their combination for 24 h using Annexin V-APC/DAPI staining followed by flow cytometry analysis. **B** Quantitative analysis of apoptosis rates in each treatment group. Data represented as mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 compared with the control group, ns not significant

significant decrease in tumor volume at day 15 (P < 0.05) (Fig. 5A). However, significant decrease in body weight was observed at day 9, 11, 13 and 15 in combination of EPI and F-α-DDB-derivative compared to control group (P < 0.05) (Fig. 5B). H&E staining of sections of the tumors from mice given combination of EPI and F-α-DDB-derivative showed significant morphological alterations, including necrosis and fibrosis. Additionally, it was found that there was fewer nuclear schizophrenia than in the single-agent group and the control group (Fig. 5C).

3.6 Effects of EPI and F-α-DDB-derivative monotherapy and co-treatment on cardiac, hepatic and renal functions in vivo

To investigate whether co-treatment would induce additional cardiotoxicity, hepatotoxicity and nephrotoxicity, serum AST, ALT, UREA, CREA, CK, CK-MB and LDH levels were measured. In EPI and F-α-DDB-derivative monotherapy and co-treatment groups, there were no significant differences in serum AST and ALT levels in contrast to the control group (Fig. 5D, E). As for cardiac function, serum CK level was higher in the EPI and F-α-DDB-derivative combination group than in control group (P < 0.05), but was not considerably different from that in the EPI monotherapy group while serum CK-MB and LDH levels did not significantly differ across the EPI monotherapy and co-treatment groups (Fig. 5F, G, H). And for renal function, serum CREA level was higher in the EPI and F-α-DDB-derivative combination group than in the control group (P < 0.05), but there was no significant difference between EPI monotherapy and combination groups (Fig. 5I). However, UREA level in combination group was significantly different from that in the control group and EPI monotherapy group (P < 0.05) (Fig. 5J). Considering the effects of EPI and F-α-DDB-derivative on heart and liver, H&E staining of sections of hearts and livers from mice treated with control, monotherapy or co-treatment didn't demonstrate evident histopathological changes (Fig. 5C).

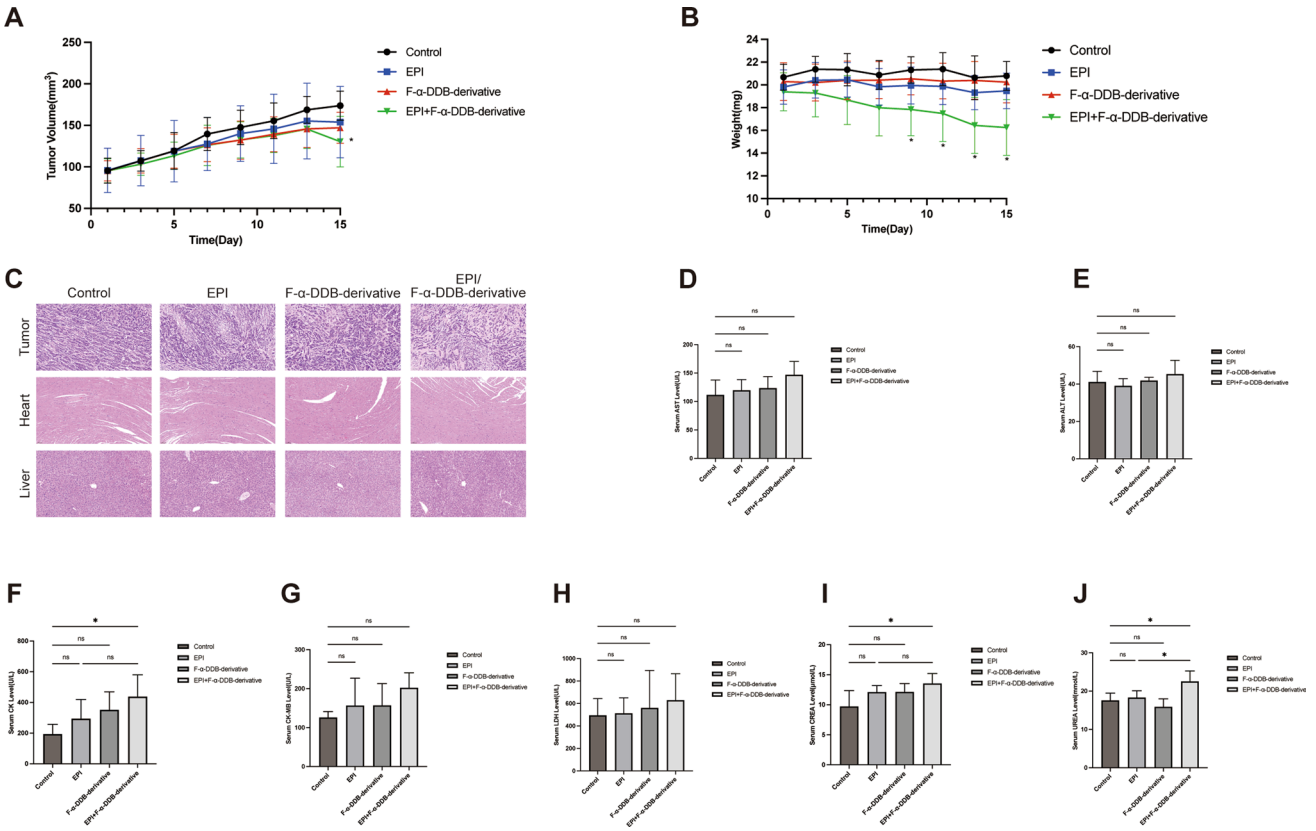


Fig. 5 The therapeutic efficacy of cotreatment of EPI and F-α-DDB-derivative without additional cardiotoxicity in vivo. Twenty-four BALB/c nude mice were inoculated subcutaneously with MDA-MB-468 cells and randomly divided into four groups (6 mice per group). **A**, **B** Changes in tumor volume and bodyweight during drug intervention. **C** Representative images of H&E staining for tumors, hearts and livers in four groups. Scale bars, 100 μm. **D**–**J** Serum cardiac, liver and renal function results. Data represented as mean ± SD of three independent experiments. ns not significant, *P < 0.05 compared with the control group

4 Discussion

TNBC is a subtype BC with low OS, high rate of distant metastases, and high rate of recurrence [22]. EPI has been shown to be therapeutic as it improves patient survival and cure rates and reduces recurrence rates. However, dose-dependent cardiotoxicity limits the clinical effectiveness of EPI and can cause patients to develop permanent heart failure [7]. Therefore, it's vital to explore strategies to balance EPI-induced cardiotoxicity and anti-tumor efficacy. In our previous study, we found that F-α-DDB-derivative, a novel bifendate derivative, inhibited cell viability, proliferation and migration in human TNBC cell line though it was less effective than EPI. In vivo study, F-α-DDB-derivative also demonstrated anti-TNBC efficacy. According to these results, F-α-DDB-derivative could potentially be further developed as a novel therapeutic approach for treating TNBC in combination with EPI. Here we report the synergistic effect of EPI and F-α-DDB-derivative on the treatment of TNBC. We also found that the effect of EPI (6.0 μg/ml) in combination of F-α-DDB-derivative (46.0 μg/ml) on cell viability, proliferation, migration and apoptosis was potent than that of EPI (0.75, 1.5, 3.0 μg/ml) and F-α-DDB-derivative (5.75, 11.5, 23.0 μg/ml). As co-administration of EPI and F-α-DDB-derivative didn't demonstrate increased cardiotoxicity, F-α-DDB-derivative is a promising anticancer treatment that can be used in combination with the traditional chemotherapy drug EPI.

In vitro study, we initially examined the cytotoxicity of EPI and F-α-DDB-derivative in MDA-MB-468 cell line. EPI and F-α-DDB-derivative were shown to have the potential to reduce the viability of TNBC cell line with dose-dependent inhibition of growth with IC_{50} value of 3.146 μg/ml and 23.21 μg/ml for 24 h, respectively. CDI values of cotreatment of EPI (0.75, 1.5, 3.0, 6.0 μg/ml) and F-α-DDB-derivative (5.75, 11.5, 23.0, 46.0 μg/ml) were less than 1, indicating the synergistic effect on antitumor activities. The combination effect of EPI and F-α-DDB-derivative was confirmed by cell scratch assay and detecting the expression level of Ki67.

In addition to unregulated proliferation, tumor growth is also caused by a decline in apoptosis. Therefore, apoptosis induction was a promising tumor therapeutic strategy. Our results showed that the combination of EPI and F-α-DDB-derivative significantly induced apoptosis.

We found that the minimum effective dose of cotreatment was 1.5 μg/ml for EPI and 11.5 μg/ml for F-α-DDB-derivative in cell proliferation and migration assays while EPI (0.75 μg/ml) in combination with F-α-DDB-derivative (0.75 μg/ml) was effective in apoptosis assays. Together, F-α-DDB-derivative inhibits cell proliferation, and migration and also promotes apoptosis, but is seemly more sensitive to apoptosis-inducing signaling pathways.

In vivo study, MDA-MB-468-bearing mice were injected with EPI (2.0 mg/kg) and/or F-α-DDB-derivative (20.0 mg/kg). The obvious reduction in size of tumors were demonstrated in the co-administration group compared with control group. Results from H&E staining confirm the combined impact, showing that MDA-MB-468 tumors treated in combination had higher levels of necrosis and fibrosis than those treated alone or as a control. A significant decrease in body weight of mice was also observed in the combination group after four intraperitoneal injections. Certain anti-tumor drugs may cause weight loss as one of their side effects. For example, cyclophosphamide showed some anti-tumor effects in the H22 hormonal mouse model, but may also have an effect on the body weight of the mice [23]. However, it was observed that the mice in the combination group were in good mental condition during the experiment and did not show any digestive reactions such as jaundice and diarrhea. To explore the reason for reduction in body weight and whether impact of drug toxicity worked, serum biochemistry and H&E staining of hearts and livers were examined. Results showed no additional cardiotoxicity or hepatotoxicity with the combination relative to EPI monotherapy. We speculated that it might be due to dehydration. The mice in the combination treatment group showed signs of dehydration, such as wrinkled skin, reduced skin elasticity, and yellow urine. Dehydration can lead to weight loss due to fluid loss. In addition, tumor may affect the body's metabolic pathways, leading to changes in body weight even when biochemical indicators do not yet show significant abnormalities [24]. We are now conducting tests to further investigate the underlying mechanism.

We've also noticed abnormalities in kidney function and speculated that this might be due to the direct toxicity of the drug, or the effect of the tumor itself on the body's metabolism. In cancer cachexia, muscle wasting leads to increased creatinine release, while the liver's ability to synthesize urea is reduced and the body's protein catabolism is enhanced, leading to elevated CREA and UREA. Furthermore, tumor progression and drug side effects may affect appetite and water intake in mice, leading to dehydration, which in turn affects renal function [25]. There is no denying that the impact of cotreatment may account for the renal dysfunction. The kidneys play a significant role in the removal of numerous antitumor medications and their byproducts. Chemotherapeutic drugs can impact the renal glomerulus, tubules, interstitium, and microvasculature. Cisplatin is the most nephrotoxic chemotherapy medication, frequently linked to acute kidney injury [26]. Overdosing can also result in increased toxicity [27]. Whether it is drug-induced or dose-dependent renal function abnormality needs further experimental verification.

Our study demonstrates that the combination of EPI and F-α-DDB-derivative significantly enhances the antitumor efficacy against TNBC without additional cardiotoxicity. This finding is of great significance to patients with TNBC, as it suggests a potential new treatment strategy that could improve therapeutic outcomes while minimizing the risk of severe side effects. The reduced cardiotoxicity associated with the combination therapy is particularly important, as it could lead to better quality of life for patients undergoing treatment. Future research will focus on further elucidating the molecular mechanisms underlying the synergistic effect of EPI and F-α-DDB-derivative, optimizing the dosage and administration schedule to maximize efficacy and minimize side effects, and evaluating the long-term effects and potential late toxicities of the combination therapy in animal models.

However, our study has some limitations. The molecular mechanisms by which bifendate and its derivatives regulate breast cancer proliferation, migration and apoptosis are less well studied. Existing studies have shown that bifendate and its derivatives can inhibit the migration and invasive ability of breast cancer cells by regulating matrix metalloproteinases (MMPs), and induce apoptosis of hepatocellular carcinoma cells through the mitochondrial pathway and MAPK signaling pathway [14, 28]. However, whether F-α-DDB-derivative has similar effects remains unclear, and its molecular mechanisms in regulating the proliferation, migration and apoptosis of TNBC need to be further investigated. Furthermore, the sample size used in the experiments may be limited, which could affect the generalizability and reliability of the results. Additionally, the animal model used in the study may not fully replicate the complexity of human TNBC, and further validation in clinical trials is needed. The study duration was relatively short, and long-term effects and potential late toxicities of the combination therapy were not fully assessed, which will be addressed in future long-term studies.

5 Conclusions

In conclusion, the present results demonstrate that the inhibition of cell proliferation, and migration can be enhanced by cotreatment. Furthermore, the combination of EPI and F-α-DDB-derivative would not result in further cardiotoxicity, suggesting that the dosage of EPI might be lowered and its undesirable side effects could be minimized. Based on the promising findings of this study, our subsequent preclinical research will focus on further elucidating the underlying molecular mechanisms of the synergistic effect between EPI and F-α-DDB-derivative. We plan to investigate the specific signaling pathways involved in the enhanced antitumor activity and apoptosis induction. Additionally, we aim to explore the potential for optimizing the dosage and administration schedule of the combination treatment to maximize efficacy while minimizing side effects. Future studies will also include more comprehensive evaluations of the long-term effects and potential late toxicities of the combination therapy in animal models.

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Author contributions YJ designed the study; HYH, LZYL and XYL performed the experiments; YJ, HYH and LZYL analyzed the data, wrote and revised the manuscript; All authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate All animal procedures were approved by the Shanghai Model Organisms Center Institutional Animal Care and Use Committee (SRCMO-IACUC) (NO.2024-0003), with the maximal permitted tumor burden not exceeding 1000 mm^3 in volume (calculated as length × $width^2$ × 0.5) or 10% of body weight. Throughout the study, tumor dimensions were monitored using calipers, and no animals exceeded the approved tumor size limits. At the experimental endpoint, mice were rapidly euthanized by cervical dislocation. This method was selected for its rapidity (< 5 s to unconsciousness) and compatibility with tissue collection requirements. Death was confirmed by absence of corneal reflex and cessation of breathing for > 1 min. All procedures adhered to the SRCMO-IACUC guidelines.

Competing interests The authors declare no competing interests.

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