

Eriocalyxin B induces apoptosis in human triple negative breast cancer cells via inhibiting STAT3 activation and mitochondrial dysfunction

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Abstract: Eriocalyxin B (EriB), a potent ent-kaurene extracted from *Isodon eriocalyx*, has turned up as novel anti-cancer agent during recent years against a range of cancer types. TNBC (Triple negative breast cancer) is highly aggressive breast cancer, which is resistant towards current therapeutics due to absence of drug targets. Here, we have probed the molecular mechanism of EriB-induced apoptosis in TNBC (MDA-MB231) cells to check whether its anticancer activity is mediated by modulation of STAT3 and NF- κ B. EriB induced apoptosis in MDA-MB231 cells via inhibiting NF- κ Bp65, STAT3 phosphorylation, increasing Bax/Bcl-2 ratio, MMP dissipation, and activation of caspase-3. These results provide a rationale for further *in vivo* investigations on EriB, which might also prove to be a potential drug candidate for developing novel therapeutics against TNBC.

Keywords: Eriocalyxin B, apoptosis pathways, STAT3, Bcl-2, Bax.

INTRODUCTION

Breast cancer has been reported as most prevalent malignancy in women responsible for 25% of all the cancers around the world (Ghoncheh *et al.*, 2016). TNBC (triple negative breast cancer) lacks estrogen receptor (ER), human epidermal growth factor receptor-2 (HER2) and progesterone receptor (PR) on their cellular surfaces (Austin *et al.*, 2018). High metastatic potential, aggressive nature, absence of drug targets and poor prognosis have restricted the development of targeted therapies against TNBC (Shao *et al.*, 2017). Moreover, tumor recurrence and emergence of resistance against conventional chemotherapies has further limited the treatment options for this malignancy (O'Reilly *et al.*, 2015; Han *et al.*, 2019). Therefore, novel therapeutics for the effective treatment of TNBC's are urgently required.

Screening of natural product libraries has been emerged as an effective tool in cancer drug discovery in recent decades because of biosafety profile of nature-derived compounds (Richards *et al.*, 2018). In this study, we have performed screening of natural compound library against breast cancerous cells (MCF-7) and TNBC cell line (MDA-MB231) (Chavez *et al.*, 2010; Mander *et al.*, 2018). After this screen, a potent hit, Eriocalyxin B (EriB), was selected for further experimentation based upon selective anti-proliferative potential of EriB against MDA-MB231.

EriB, a bioactive ent-kaurene, is isolated from *Isodon eriocalyx* (Zhou *et al.*, 2017). This herb belongs to Labiatae family, a native plant family of south-west China (Lu *et al.*, 2016). EriB has potential to halt the growth and induce apoptosis in several types of cancers including pancreatic adenocarcinoma (Li *et al.*, 2014), ovarian cancer (Leizer *et al.*, 2011) and colon carcinoma (Lu *et al.*, 2016; Riaz *et al.*, 2019). However, the molecular mechanism responsible for cytotoxic effects of EriB demands further investigations. Our recent study is an effort to determine the mechanism of eriocalyxin-induced apoptosis in MDA-MB231 cells. Our study demonstrated that EriB inhibits cellular proliferation in MDA-MB231 cells via apoptosis induction through down regulation of NF- κ Bp65 (Nuclear factor kappa-B) and STAT3 (signal transducer and activator of transcription 3) phosphorylation.

MATERIALS AND METHODS

Cell culture

Human cancerous cells, MDA-MB231 and MCF-7, was cultured and maintained in DMEM consisting of 10% FBS and 1% antibiotics. Cells were incubated in CO₂ incubator and allowed to grow to attain 70-80% confluency.

MTT assay

To access cytotoxicity of EriB, MTT assay was performed by firstly seeding cells in 96-well plate in DMEM media consisting of 10% FBS and 1% antibiotics. EriB was purchased from Chem Faces, Wuhan, China (Catalogue. No. CFN97402). 20mM stock concentration of EriB was

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prepared in DMSO. EriB-treated cells were incubated for 24 hours. After that 10 μ l of MTT with final concentration of 500 μ g/ml was mixed in each well and further incubated for 4h. The media was discarded and in each well, 150 μ l of DMSO was mixed. The wavelength of plates was taken at 450nm utilizing microplate reader while 620nm was used as reference (Thermo Scientific, USA) (Rasul *et al.*, 2011). The inhibition percentage (I %) was calculated by the equation as given below:

$$I \% = [A450 (\text{control}) - A450 (\text{treated})] / A450 (\text{control}) \times 100$$

Where, "I" refers to the inhibition rate and A is the absorbance at 450 nm.

Measurement of cell apoptosis

Annexin V/PI assay followed by flow cytometric analysis was performed for the analysis of cellular apoptosis (Rasul *et al.*, 2012b). After 12h of EriB treatment (0, 1.5 and 3 μ M), cells were harvested. PBS was used for washing of the cells and this step was repeated twice. Cells were resuspended in 10 μ l of Annexin V-FITC/PI in binding buffer (500 μ l) for 15 minutes in dark. Samples were analyzed by Flow cytometry.

Mitochondrial $\Delta\psi_m$ assay

To analyze the changes in $\Delta\psi_m$, JC-1 staining solution (10 μ l) was mixed into each well after EriB treatment and incubation was done for 30mins. After centrifugation at

400 \times g for about 5 minutes, the collected cells were subjected to washing for two times with assay buffer (200 μ l/well). After addition of 200 μ l assay buffer in all wells, FACs analysis was performed (Rasul *et al.*, 2012a).

Western blot (WB) analysis

EriB-induced changes in various apoptotic proteins were probed by western blot analysis (Rasul *et al.*, 2012a) In brief, EriB treated (0, 1.5 and 3 μ M) cells were harvested and subjected to lysis in RIPA buffer. Afterward, protein lysate was separated by centrifugation for 15 mins at 1350 rpm at 4 $^{\circ}$ C. Protein was loaded on SDS-PAGE electrophoresis gel (10 or 12% according to the protein size), further, the gel was transferred on polyvinylidene fluoride membrane (PVDF). After necessary transfer time, membranes were blocked by using 5% low-fat milk and incubated for 2h. Next, incubation of membrane with appropriate primary antibodies caspase-3, STAT3, p-STAT3, Bcl-2, Bax, Bcl-XL and NF- κ B, was completed at 4 $^{\circ}$ C for overnight and washed for 6 times with TBST. Finally, blots were incubated with appropriate secondary antibody (anti-mouse horseradish peroxidase conjugated) for 1h at room temperature, then TBST was used for washing for 30 minutes (6 times) and signals were detected on X-ray film using ECL plus chemiluminescence kit (Millipore Corporation, Billerica, USA).

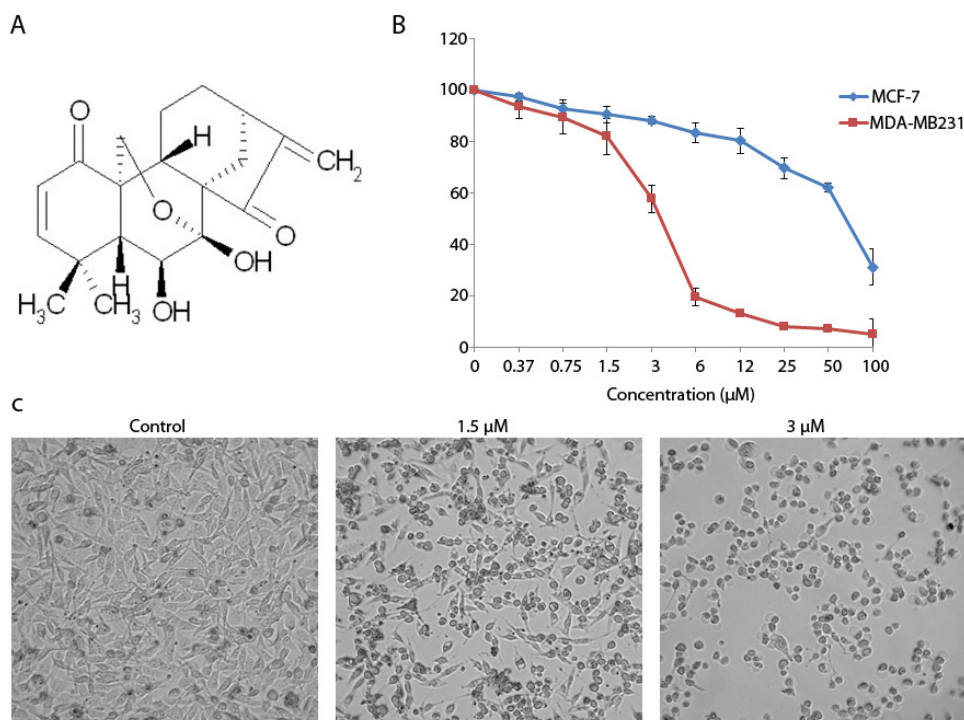


Fig. 1: Structural representation of EriB and its effects on the growth of MCF-7 and MDA-MB231 cells: (A) Structure of EriB (B) EriB caused significant inhibition of growth in MDA-MB231 cells selectively in comparison to MCF-7 cells. Cancer cells were given treatment of 0, 0.37, 0.75, 1.5, 3, 6, 12, 25, 50 and 100 μ M of EriB for 24 h. The percentage of viability was quantified by MTT assay (C) Morphologic variations induced by the treatment of 0, 1.5, 3 μ M of EriB in the MDA-MB231 cells observed by inverted phase contrast microscopy.

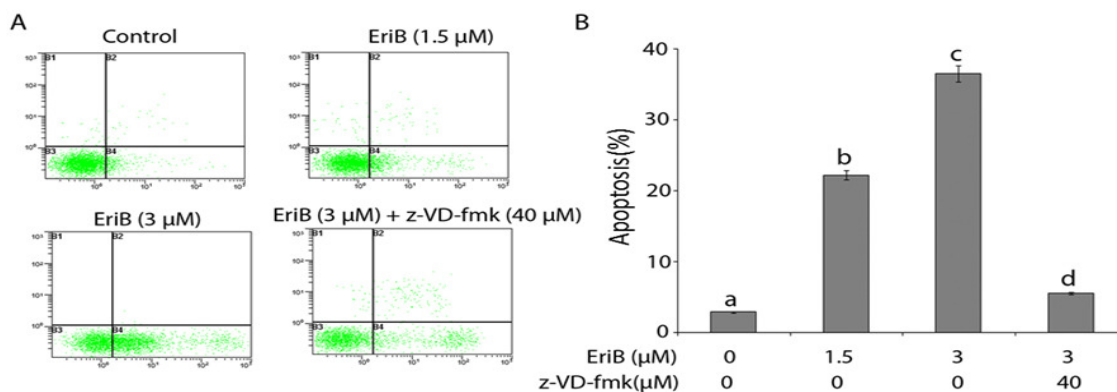


Fig. 2: Analysis of apoptosis in EriB treated-MDA-MB231 cells by flow cytometry (A) FACS analysis showing percentage of cancer cells in various phases of apoptosis in EriB treated MDA-MB231 cells (0, 1.5 and 3μM) and EriB + Z-VAD-FMK treated cells (3μM + 40μM) (B) Graph showing the percentage of cellular apoptosis in EriB treated MDA-MB231 cells (0, 1.5 and 3μM) and EriB + Z-VAD-FMK treated cells (3μM + 40μM). The data are expressed as the means ± SEM (n=3). * P<0.05 and ** P <0.01 compared to the control.

STATISTICAL ANALYSIS

Data was statistically analyzed by Origin lab 8 software. Data is represented as the mean ± standard deviation. For the evaluation of statistical significance, paired *t*-test was used. Differences were contemplated to be statistically significant at P<0.05.

RESULTS

EriB inhibits the proliferation of MDA-MB231 cells

During this study, natural compounds library was screened against human MDA-MB231 and MCF-7 cells to find out potent therapeutic compounds. EriB showed significant anti-proliferative effect against TNBC, MDA-MB231, cells among compound library. In the next step, the effects of EriB (fig. 1A) were determined on the growth of MDA-MB231 and MCF-7 cells by MTT assay via quantification of viable cells. EriB caused significant inhibition of the growth of TNBC, MDA-MB231, cells in a dose-mediated mode as compared to MCF-7 cells (fig. 1B). Cytotoxic potential of EriB showed the morphological alterations of cells as noticed under a phase-contrast microscope (fig. 1C), it can be clearly seen that control cells had normal cell morphology and are adhered. After treating the cells with EriB, they displayed notable morphological alterations, i.e., they acquired a shrunken and rounded shape and increased floating cells was also noticed with remarkable decrease in cell number, which occurred in a dose-dependent mode.

EriB inhibits proliferation via induction of apoptosis in MDA-MB231 cells

The results of FACS analysis show that EriB induces apoptosis dose-dependently (fig. 2). Fig. 2 displays the percentage of EriB treated MDA-MB231 cells in early

and late phases of apoptosis (fig. 2A). However, EriB-induced apoptosis was markedly reduced in Z-VAD-FMK-treated cells, deciphering that the EriB-induced apoptosis is mediated by the caspases in MDA-MB231 cells (fig. 2B).

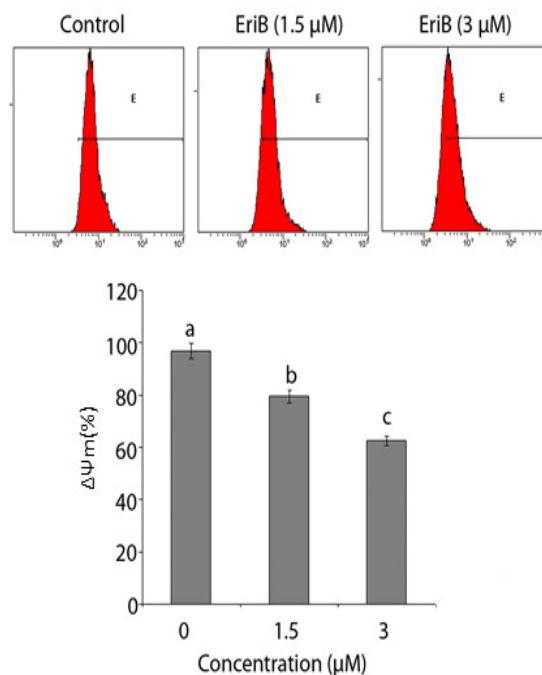


Fig. 3: The effect of EriB on the MMP in MDA-MB231 cells (A) FACS analysis showing MMP in EriB treated (0, 1.5 and 3μM) MDA-MB231 cells (B) Graph showing the loss of the MMP in MDA-MB231 cells after EriB treatment in a dose-mediated manner. The data are expressed as the means ± SEM (n=3). * P<0.05 compared to the control.

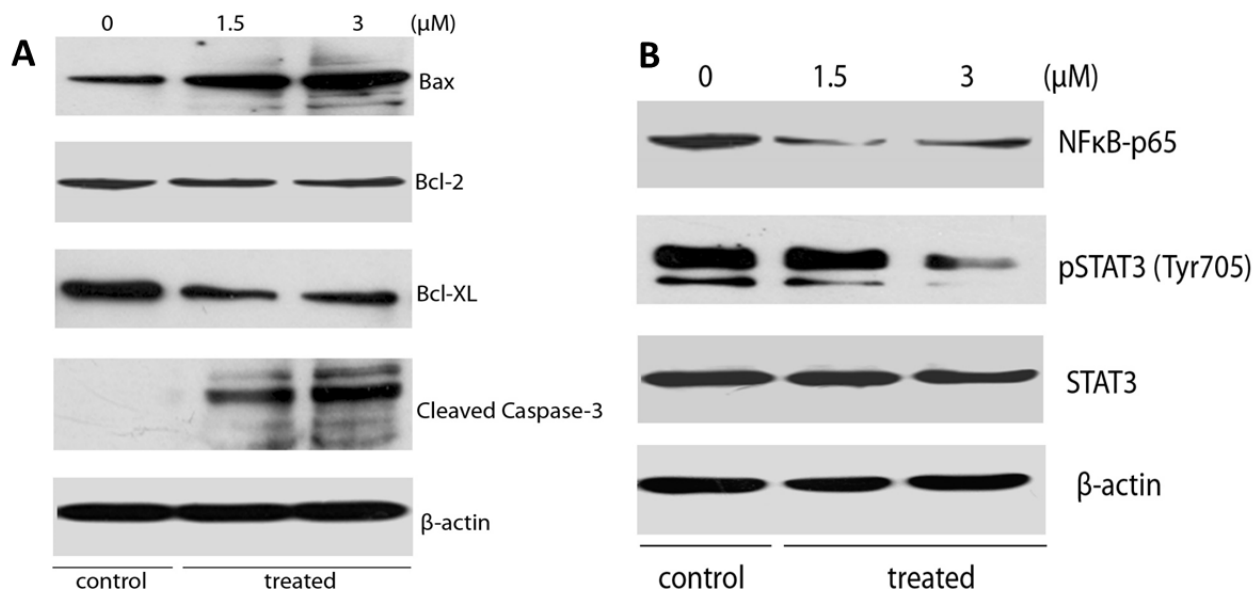


Fig. 4: (A) WB analysis showing the effects of EriB on the expression of intrinsic apoptotic pathway-associated proteins (Bax, Bcl-XL, Bcl-2, cleaved caspase-3) in MDA-MB231 cells (B) WB analysis representing the levels of NF-κBp65, pSTAT3 (tyr705) and STAT3 in EriB treated (0, 1.5 and 3μM) cells. β-actin was utilized as loading control.

Effect of EriB on mitochondrial membrane potential (MMP)

During our study, we have also determined whether EriB has potential to mediate MMP in MDA-MB231 cells. Fig. 3 displays the level of MMP in the EriB-treated cells (0, 1.5 and 3μM). The level of MMP observed was (99.97%, 88.77%, 73.81%) with a significant dissipation of MMP in MDA-MB231 treated cells as observed in dose-dependent mode. The results clearly show that EriB-induced apoptosis is linked to MMP dissipation (fig. 3).

Modulation of Bcl-2 family proteins by EriB

For further characterization of apoptotic effect of EriB, EriB-treated (0, 1.5 and 3μM) MDA-MB231 cells were analyzed for the levels of protein from Bcl-2 family. It was found that Bcl-2 and Bcl-XL expressions were reduced, however, Bax expression was markedly up-regulated (fig. 4A). To explore whether EriB induced apoptosis occurs in caspase-3-mediated mode, we further explored the expression of caspase-3 in 0, 1.5 and 3μM EriB-treated cells. As shown in fig. 4A, EriB treatment lead to a remarkable increase in the cleavage of caspase-3, which is an indication of caspase-mediated apoptosis.

STAT3 and NF-κB pathways are modulated in EriB-treated MDA-MB231 cells

Furthermore, to delineate the mechanism involved in EriB-induced apoptosis, we explored the impacts of EriB on NF-κBp65. It was revealed by WB analysis that treatment of these cells with EriB alleviated the nuclear NF-κBp65 levels dose-dependently. As EriB performs

role in reducing the expression of nuclear NF-κB in MDA-MB231 cells, thus, further we want to study whether EriB could inhibit STAT3 activation or not. As indicated in fig. 4B, treatment of cells by EriB lowered the expression of pTyr705 STAT3 dose-dependently while total STAT3 was found be un-affected (fig. 4B).

DISCUSSION

Breast cancer has been emerged as leading cause of human deaths in recent years (Zheng *et al.*, 2019). One of the aggressive kind of breast cancer is TNBC in which three receptors (PR, ER and HER2) are missing on their cellular surfaces (Austin *et al.*, 2018). Resistance of TNBC against currently available therapeutics as well as absence of drug targets has limited the treatment of this highly metastatic cancer (Han *et al.*, 2019). Thus, there is dire need to find out potent, selective and safer drug candidates and in this regard natural products has served humans since times as safer drug of choice (Veeresham, 2012).

Our results demonstrate that EriB, a potentially active nature-derived entity, has potential to markedly reduce the growth of TNBC cell line, MDA-MB231, via induction of apoptosis. The results are found to be consistent with the previous studies that have reported EriB as potent anticancer agent against pancreatic (Li *et al.*, 2018), colon (Lu *et al.*, 2016), breast (Zhou *et al.*, 2017) and ovarian cancer cells (Leizer *et al.*, 2011).

Anti-cancer mechanism of action of natural compounds proceeds via apoptosis induction in cancer cells (Safarzadeh *et al.*, 2014). Apoptosis is an important phenomenon regarding cell death. It comprises of multiple steps but the major pathways governing apoptosis are intrinsic and extrinsic pathways (Xu *et al.*, 2019). The intrinsic cell-death pathways are stimulated when Bcl-2 family proteins are modulated, MMP is mediated and cytochrome c is released, all of these factors ultimately cause activation of caspase-3 which induces apoptosis (Plati *et al.*, 2011). Mechanism of EriB-induced apoptosis in MDA-MB231 found to be similar to previous data, which reports the apoptosis induction by EriB in lymphoma cells via enhancing Bax while reducing the levels of Bcl-xl and Bcl-2 that is linked with the activation of caspases (Zhang *et al.*, 2010).

NF- κ B, a transcription factor, is associated with the survival as well as the proliferation of cells but its constitutive activation is reported to be responsible for several types of metastatic anomalies (Bharti *et al.*, 2003). The results of this research elaborated the reduced levels of nuclear protein NF- κ Bp65. Inhibition of NF- κ B in many metastatic conditions leads to the induction of apoptosis (Guzman *et al.*, 2013). Previously reported data also support our results that NF- κ B is inhibited by EriB treatment, which ultimately lead cells toward apoptosis (Wang *et al.*, 2007).

STAT3 is another transcriptional factor and its constitutive activation has been reported in more than 50% of breast cancers (Hsieh *et al.*, 2005; Kim *et al.*, 2013). Recently reported studies are of the view that oxidative stress conditions lead to the glutathionylation of STAT3 and this condition inhibits the phosphorylation of STAT3 concomitantly. NF- κ B is also associated with STAT3 activation. Phosphorylation of tyrosine (Tyr705) of STAT3 leads toward translocation of STAT3 to nucleus where it increases the expression of genes associated with cellular proliferation e.g., Bcl-2 and cyclin D1. Down regulation of STAT3 phosphorylation lowers the expression of anti-apoptotic proteins and resultantly induces apoptosis in tumor cells (Danial, 2007; Sui *et al.*, 2014). Our results present similar findings to a previous study, which reports the inhibition of STAT3 activation by EriB in STAT3-dependent cancers (Yu *et al.*, 2015).

CONCLUSION

In conclusion, our data provide evidence for EriB-induced inhibition of growth in MDA-MB231 cells. This is first study to elaborate the modulation of STAT3, NF- κ B and Bcl-2 family proteins by EriB in MDA-MB231 cells as mechanism of apoptosis. The present study concluded that EriB-induced apoptosis in MDA-MB231 cells involves modulation of Bcl-2 family proteins, inhibition of the levels of nuclear NF- κ Bp65 and caspase-3 activation. It is

anticipated that EriB may serve as a lead compound for the development of anti-TNBC therapies. Thus, more investigations are encouraged to validate the contribution of EriB in *in vivo* tumor therapy.

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