Induction of estrogen receptor β-mediated autophagy sensitizes breast cancer cells to TAD1822-7, a novel biphenyl urea taspine derivative

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Abstract

Background Female breast cancer has become the most commonly diagnosed cancer worldwide. As a tumor suppressor, estrogen receptor β (ERβ) can be potentially targeted for breast cancer therapy.

Methods and results TAD1822-7 was evaluated for ERβ-mediated autophagy and cell death using cell proliferation assay, Annexin V/PI staining, immunofluorescence, western blotting, ERβ siRNA, ERβ plasmid transfection and hypoxia cell models. TAD1822-7 upregulated ERβ causing cell death and induced mitochondrial dysfunction and autophagy companied with mitochondrial located ERβ. Enhanced levels of microtubule associated protein1 light chain 3 (LC3)-II and p62/SQSTM1 (p62) indicated that TAD1822-7 blocked the late-stage autolysosome formation, leading to cell death. Mechanistically, TAD1822-7-induced cell death was mediated by phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathways. Moreover, TAD1822-7 modulated hypoxia inducible factor (HIF) functions and autophagy via the inhibition of HIF-1β in the context of hypoxia-induced autophagy. ERβ overexpression and ERβ agonist showed similar effects, whereas ERβ siRNA abrogated TAD1822-7-induced cell death, the inhibition of PI3K/AKT pathway and autophagy. The involvement of PI3K/AKT pathway and autophagy was also demonstrated in TAD1822-7-treated hypoxic breast cancer cells.

Conclusions These findings provide new insight into the mechanism underlying the inhibitory effects of TAD1822-7 via ERβ-mediated pathways in breast cancer cells.

Keywords Estrogen receptor β · Autophagy · Cell death · Breast cancer · TAD1822-7 · Hypoxia

Introduction

Female breast cancer, a malignancy originating from mammary epithelial tissue, has surpassed lung cancer as the most commonly diagnosed cancer in 2020 [1]. The discovery and characterizing of estrogen receptors (ER) in breast cancer, especially ERα, has greatly improved the determining of medication strategies [2]. On the contrary to the tumor-promoting ERα, ERβ has been shown to act as a tumor suppressing protein in breast cancer cells [3]. Since the widespread expression of ERβ is detected in luminal breast cancers but also in triple-negative breast cancers, ERβ has been proposed as a potential target for breast cancer therapy [4]. However, the exact function of ERβ in breast cancer carcinogenesis and progression still needs to be further elucidated.

Autophagy plays critical roles in the maintenance of cellular homeostasis, and the dysregulation of autophagy has been implicated in cancers [5]. Emerging studies have suggested that ERβ activation could trigger autophagy monitored by the conversion of LC3-I to LC3-II, which contributes to the prevention of breast cancer cell growth, migration and invasion [6, 7]. Bridging autophagy substrates with LC3 proteins, p62 acts as an autophagy adaptor that mediates the selective recognition and degradation of substrates by lysosomes. Following delivery of autophagosomes to the lysosome, p62 is degraded with its substrates, therefore p62 is used as another essential marker to study autophagy flow [8]. The role of ERβ in p62 regulation is yet to determine to clarify how ERβ modulate autophagy in breast cancer cells.
Hypoxia is a key feature of tumor local environment and hypoxia-induced autophagy has been well documented [9]. Breast cancers contain hypoxic areas due to aggressive growth and insufficient oxygen perfusion [10]. Under hypoxia, HIF-1α and HIF-1β heterodimers translocate to the nucleus and activate downstream genes. In breast cancer cells, ERβ suppresses HIF-1 heterodimers via the downregulation of HIF-1β levels [11], thus the regulation of ERβ may lead to the suppression of hypoxia-mediated autophagy and downstream pathways.

Taspine is a natural alkaloid originally from the extracts of the root and rhizome of Caulophyllum robustum Maxim. (Hong Mao Qi in Chinese). It possesses many pharmacologic properties including antibacteria, antibiotic, antivirus and anti-inflammatory. TAD1822-7 (Fig. 1a) is a novel biphenyl taspine derivative designed in our laboratory [12]. Previous studies have revealed the antitumor and antiangiogenesis effects in breast cancer cells. However, the regulatory properties of TAD1822-7 in the context of ERβ-mediated pathways and autophagy have not been investigated. In this study, we found that TAD1822-7 could induce breast cancer cell death attributed to the upregulation of ERβ expression and inhibition of PI3K/AKT pathways. Furthermore, TAD1822-7 triggered mitochondrial dysfunction and autophagy but blocked the late-stage autolysosome formation, thereby leading to the inhibition of autophagic flux and the promotion of cell death. In addition, TAD1822-7 inhibited hypoxia-induced autophagy and PI3K/AKT pathway through the ERβ-mediated downregulation of HIF-1β.

Materials and methods

Cell lines and cell culture

Human breast cancer cell lines T47D and MDA-MB-231 were obtained from Shanghai Institute of Cell Biology in the Chinese Academy of Sciences and cultured as previously described [13]. Cells were incubated at a 5% CO₂ with 1% O₂ balanced with N₂ using a hypoxic chamber for hypoxic condition.

Western blotting

Cell lysate was prepared in RIPA buffer containing phosphatase and protease inhibitors (Roche Technology, Basle, Switzerland). Samples were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA), blocked with 5% milk and incubated with primary antibodies at 4 °C overnight. Then the membrane was incubated with secondary antibodies and the expression was visualized with Tanon5200 imaging system (Tanon, Shanghai, China).

Plasmid transfection

ERβ plasmid was obtained from Sino Biological (Beijing, China). Exponentially growing T47D and MDA-MB-231 cells were seeded in 6-well plates and transfected with ERβ plasmids using ExFect2000 transfection reagent (Vazyme, Nanjing, China).

siRNA transfection

T47D and MDA-MB-231 cells were transfected with ERβ-specific siRNA target sequences 5′-TAGCGACGCTCCTGCG CTTCAC-3′ synthesized by Vazyme using ExFect2000 transfection reagent.

Live/dead staining

Cell viability was assessed by the Live/Dead cytotoxicity tool kit (Invitrogen®). The fluorescence was observed under a fluorescent microscope (DM505, Nikon Co., Ltd., Ota-wara, Tochigi, Japan).

MTT assay

T47D and MDA-MB-231 cells were seeded into 96-well plates and cultured with indicated stimulations. After treatment, cells were cultured in 0.5 mg/mL MTT prepared in serum-free medium for 4 h. After the removal of medium,
Fig. 1 TAD1822-7 (TAD) induced ERβ-mediated cell death in breast cancer cells. 

a) Chemical structure of TAD. 
b) T47D and MDA-MB-231 cells were treated with different concentrations of TAD1822-7 for 24 h. ERβ protein levels were examined by western blotting. 
c) T47D and MDA-MB-231 cells were transfected with NC (negative control) or ERβ plasmid using ExFect2000 transfection reagent. After 24 h, ERβ protein levels were determined by western blotting. β-actin served as controls. 
d) T47D and MDA-MB-231 cells were transfected with ERβ plasmids or treated with DPN or TAD for 24 and 48 h, and the cell viability was examined by MTT assay (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 one-way ANOVA followed by Dunnett's posttest in comparison with untreated control or as indicated. 
e) T47D and MDA-MB-231 cells were transfected with ERβ siRNA using ExFect2000 transfection reagent. After 24 h, ERβ protein levels were determined by western blotting. β-actin served as controls. 
f) T47D and MDA-MB-231 cells were transfected with ERβ siRNA and treated with TAD for 24 h, and the cell viability was examined by MTT assay (n = 3). 
g) T47D and MDA-MB-231 cells were treated with DPN and TAD for 24 h, and the cell viability was examined by MTT assay (n = 3). Live and dead cells were stained with 1 µM calcein-AM and 1 mg/mL−1 PI, respectively. The results shown were representative of 3 independent experiments. 
h) Cell apoptosis was measured by Annexin V-FITC/PI staining. The percentage of apoptotic cells is indicated. The results shown were representative of 3 independent experiments.
DMSO was added to dissolve the crystal. Optical density was analyzed using a microplate reader (Bio-Rad, Hercules, CA, USA).

**Annexin V/PI staining**

Cells were plated into 6-well plates. After treatment, cells were stained with AnnexinV-FITC and PI and analyzed by flow cytometry (ACEA NovoCyte, Agilent, USA). Data were analyzed with NovoExpress software.

**Immunofluorescence**

Cells were stained with Mitoorange (KeyGEN BioTECH, Jiangsu, China) and fixed with 4% paraformaldehyde and then blocked with 10% BSA. Then, the cells were incubated with primary antibodies (1:200) and then a CoraLite488-conjugated anti-rabbit secondary antibody (1:50). Nuclei were stained with DAPI. Fluorescent images were taken using an inverted fluorescence microscope.

**Mitochondrial membrane potential (ΔΨm) assay**

After indicated treatment in 6-well plates, cells were harvested and stained with 100 nM TMRE at 37 °C for 30 min. ΔΨm was detected by flow cytometry.

**Statistical analysis**

One-way analysis of variance and further Dunnett’s multiple comparison test were used, and \( P \) value < 0.05 was considered statistically significant (Prism 6.0, GraphPad, La Jolla, CA, USA). Data are presented as mean ± S.E.M.

**Results**

**TAD1822-7 induced ERβ-mediated cell death in breast cancer cells**

Tumor suppressing effects of ERβ have been found in breast cancer cells. To evaluate the potential modulation of ERβ by TAD1822-7 in breast cancer cells, the protein levels of ERβ were examined in TAD1822-7-treated T47D and MDA-MB-231 cells. As shown in Fig. 1b, TAD1822-7 increased ERβ expression in a concentration-dependent manner. To confirm the anti-tumor effects of ERβ, ERβ overexpressing T47D and MDA-MB-231 cells were established (Fig. 1c). Live/dead cell staining was performed in breast cancer cells with transfected ERβ plasmids or treated ERβ specific agonist, DPN, as well as TAD1822-7. The data from MTT assay indicated that overexpression and activation of ERβ as well as TAD1822-7 induced cell death in T47D and MDA-MB-231 cells (Fig. 1d). Additionally, ERβ knock down cells were also established by ERβ specific siRNA in T47D and MDA-MB-231 cells (Fig. 1e). In the presence of ERβ siRNA, the anti-proliferation effects of TAD1822-7 was abolished compared to control siRNA (Fig. 1f). Moreover, as shown in Fig. 1g, cell viability was evaluated after the treatment of both TAD1822-7 and DPN, and the further increased cell death indicated that TAD1822-7 increased ERβ expression which led to further cell death with the activation of ERβ by DPN. Moreover, significant cell death indicated by the red fluorescence was induced when ERβ was overexpressed and activated, suggesting TAD1822-7 might inhibit breast cancer cell growth via the induction of ERβ (Fig. 1h). Further analyses using Annexin V/PI staining were performed to confirm that TAD1822-7 induced ERβ-mediated apoptosis in breast cancer cells (Fig. 1i).

**TAD1822-7 induced ERβ-mediated mitochondrial dysfunction in breast cancer cells**

To further investigate the sub-cellular location of TAD1822-7-induced ERβ, immunostaining of ERβ was performed. Similar to DPN-stimulated cells, TAD1822-7-increased ERβ was found to localize in cytoplasm and colocalized with a mitochondrial marker (Fig. 2a). Moreover, TAD1822-7-induced mitochondrial localization of ERβ led to a decrease of mitochondrial membrane potential assessed by TMRE staining, which was in line with the decreased mitochondrial membrane potential in ERβ-transfected and DPN-stimulated T47D and MDA-MB-231 cells (Fig. 2b).

**TAD1822-7 inhibited ERβ-mediated PI3K-AKT pathways in breast cancer cells**

The expression of PI3K catalytic subunits, p110α and p110β, regulatory subunit p85, as well as AKT in both T47D and MDA-MB-231 cell lines were examined to further explore the regulatory mechanisms of TAD1822-7 in breast cancer cells. In accordance with ERβ plasmid-transfected and DPN-stimulated cells (Fig. 2c, d), TAD1822-7 reduced the protein levels of p110α, p110β, p-p85 and p-AKT in a concentration-dependent manner (Fig. 2e). Furthermore, ERβ siRNA negated the regulatory effects of TAD1822-7 on p110α, p110β, p-p85 and p-AKT expression (Fig. 2f). These results indicated that TAD1822-7 inhibited ERβ-mediated PI3K-AKT pathways in breast cancer cells.

**TAD1822-7 inhibited autophagy in breast cancer cells**

As mitochondrial dysfunction is likely to result in the modulation of autophagy [14], we speculated that TAD1822-7
Fig. 2 TAD induced ERβ-mediated mitochondrial dysfunction and inhibited ERβ-mediated PI3K-AKT pathways in breast cancer cells. a MDA-MB-231 cells were treated with DPN or TAD for 24 h. Mitotracker (red), ERβ (green), DAPI (blue) staining and 3-channel merged images indicated the colocalization of mitochondria and ERβ. Scale bars, 50 μm. b T47D and MDA-MB-231 cells were transfected with ERβ plasmids or treated with DPN or TAD for 48 h. Mitochondrial membrane potential was measured by TMRE staining. The results shown were representative of 3 independent experiments. c T47D and MDA-MB-231 cells were transfected with ERβ plasmids for 24 h. d T47D and MDA-MB-231 cells were treated with DPN for 24 h. e T47D and MDA-MB-231 cells were treated with TAD for 24 h. p110α, p110β, p-p85, p-AKT and AKT protein levels were assessed by western blotting. β-actin served as controls. n = 3. Quantification plots are shown on the right. f T47D and MDA-MB-231 cells were transfected with ERβ siRNA and treated with TAD for 24 h, and p110α, p110β, p-p85, p-AKT and AKT protein levels were assessed by western blotting. β-actin served as controls. n = 3. *p < 0.05, **p < 0.01, ***p < 0.001 one-way ANOVA followed by Dunnett’s posttest in comparison with control or as indicated.
regulates autophagy in breast cancer cells. As shown in Fig. 3a, increased LC3-II protein levels suggested the accumulation of LC3-II and formation of autophagosomes. While, elevated levels of p62 indicated the inhibition of autophagy by TAD1822-7 in T47D and MDA-MB-231 cells. Similarly, ERβ transfection and DPN also inhibited autophagy in breast cancer cells (Fig. 3b, c). Consistently, ERβ siRNA negated TAD1822-7-enhanced LC3-II and p62 expression (Fig. 3d).
Inhibition of autophagy contributed TAD1822-7-induced cell death in breast cancer cells

Different roles of autophagy have been increasingly recognized that autophagy either inhibits or supports tumor cell growth [15]. To further identify the role of TAD1822-7-induced autophagy in cell death, cell viability was determined in TAD1822-7 and in the presence of autophagy inhibitors, including vacuolar H + -ATPase inhibitor, Baf, lysosomal inhibitor, CQ, and mitophagy inhibitor, Mdv. In both T47D and MDA-MB-231 cells, significant stronger inhibition of cell viability was observed in TAD1822-7 and Mdv combined treated cells in comparison with TAD1822-7 alone treated cells (Fig. 3e). We measured autophagic flux by comparing LC3 and p62 levels in the presence of Mdv, and the combination of increase in LC3-II and p62 both in the presence and absence of Mdv suggested that TAD1822-7 stimulated autophagy but simultaneously inhibited the late stages of autophagy (Fig. 3f). We further assess cell apoptosis in T47D and MDA-MB-231 cells treated with the combination of TAD1822-7 and Mdv. Consistently, further elevated apoptotic cell numbers were found in TAD1822-7- and Mdv-treated cells (Fig. 3g). Taken together, TAD1822-7-inhibited autophagy contributed TAD1822-7-induced cell death in breast cancer cells.

TAD1822-7 controlled HIF-1β pathways by ERβ induction in hypoxic breast cancer cells

Hypoxia chamber was used to mimic the hypoxic environment of tumor area. Oxygen level was set to 1% based on the reported physical oxygen level in breast cancer [10]. Consistent with the literature that ERβ suppresses HIF-1 activation via the downregulation of HIF-1β [11], the evaluation of HIF-1α and HIF-1β protein levels revealed that ERβ transfection and ERβ agonist treatment reduced the protein levels of HIF-1β but not HIF-1α (Fig. 4a, b). TAD1822-7 treatment remained consistent downregulation of HIF-1β in company with the upregulation of ERβ expression (Fig. 4c). Again, downregulation of HIF-1β by TAD1822-7 was negated by combining ERβ siRNA with the treatment of TAD1822-7 under hypoxia (Fig. 4d).

TAD1822-7 inhibited hypoxia-induced autophagy and PI3K-AKT pathways by ERβ induction in breast cancer cells

The levels of autophagy related proteins were evaluated in hypoxic T47D and MDA-MB-231 cells under the treatment of TAD1822-7 and ERβ transfection. As shown in Fig. 5a, both treatments resulted in an inhibition of hypoxia-induced autophagy indicated by the enhancement of LC3-II levels and p62. Furthermore, as hypoxia regulates cellular functions through PI3K-AKT signaling, the regulation of p110α, p110β, p-p85 and p-AKT by TAD1822-7 and ERβ transfection in hypoxic cells was determined and the data indicated that TAD1822-7 and ERβ transfection inhibited hypoxia-induced PI3K-AKT pathway proteins (Fig. 5b). These data suggested that TAD1822-7 inhibited hypoxia-induced autophagy and PI3K-AKT pathways by ERβ induction in breast cancer cells.

Discussion

ERβ has been recognized to play a critical role in the prevention of breast cancer development and metastasis. In this study, we found that TAD1822-7 could hinder breast cancer cell growth via the upregulation of ERβ levels and the inhibition of PI3K/AKT pathways. Further studies revealed that TAD1822-7 initiated mitochondrial dysfunction and autophagy but blocked the late-stage autolysosome formation, thereby resulted the inhibition of autophagy and the promotion of cell death. Moreover, TAD1822-7 inhibited HIF-1β expression and autophagy through the upregulation of ERβ in hypoxic breast cancer cell models, and the underlying mechanism was further demonstrated to be PI3K/AKT pathway mediated actions. These results suggest that TAD1822-7 could inhibit breast cancer cell growth under both normoxia and hypoxia through ERβ upregulation and the inhibition of PI3K/AKT pathways, autophagy and HIF functions.

Concerning the expression level of ERβ in clinical samples, ERβ decreases from the highest values in normal tissue to almost absent in advanced tumors during the development of breast cancer. To date, the prevalent treatment strategies of hormone-sensitive tumors (~75% of breast cancer) are
based on the use of ERα-targeting drugs. Whereas, lacking of therapeutic targets, triple-negative breast cancer, accounting for around 15% of all breast cancer, is known to be more aggressive compared to ERα-positive breast cancer [16]. Recently, great efforts have been focused on the identification of new ERβ agonists, and some natural molecules have showed selectivity for ERβ and promising effects on proliferation inhibition and invasiveness suppression against breast cancer [17–19]. In this work, we demonstrate that TAD1822-7 inhibits the cell growth in both T47D (ERα+/ERβ+) and MDA-MB-231 (ERα-/ERβ+) cells via the upregulation of ERβ. ERβ induces apoptosis via the regulation of apoptotic proteins in BCL-2 family and caspasas [20, 21]. Moreover, ERβ-modulated autophagy has

Fig. 4 TAD1822-7 controlled HIF-1β pathways by ERβ induction in hypoxic breast cancer cells. a T47D and MDA-MB-231 cells were transfected with ERβ plasmids and incubated in 1% O2 for 24 h. b T47D and MDA-MB-231 cells were treated with DPN for 24 h. c T47D and MDA-MB-231 cells were treated with TAD and incubated in 1% O2 for 24 h. HIF-1α and HIF-1β protein levels were determined by western blotting. n = 3. β-actin served as controls. Quantification plots are shown on the right. d Hypoxic T47D and MDA-MB-231 cells were transfected with ERβ siRNA and treated with TAD for 24 h. HIF-1β and ERβ protein levels were assessed by western blotting. β-actin served as controls. n = 3. Quantification plots are shown on the right. *p<0.05, **p<0.01 one-way ANOVA followed by Dunnett’s posttest in comparison as indicated.
also been noticed in some studies reporting the increase of LC3-II indicating the initiation of autophagy and formation of autophagosomes. However, the regulation of p62 is much less documented. Our results demonstrated that ERβ increased LC3-II and the expression of p62, suggesting TAD1822-7 triggers autophagy and blocks the late-stage autolysosome formation, which increases cellular stress and eventually leads to cell death. In consistency with the literature [6], our findings also demonstrate that ERβ affects PI3K/AKT pathway to regulate autophagy.

Rapid growth of tumor cells generates a hypoxic local environment which influences many cellular functions, such as angiogenesis, erythropoiesis, energy metabolism, metastasis and autophagy [22]. HIF-1 complexes are responsible for the regulation of these cellular functions via the control of target genes, including erythropoietin, vascular endothelial growth factor (VEGF) and chemokine receptor CXCR4 [23]. Our finding confirmed the study showing that TAD1822-7-induced ERβ inhibits HIF-1-mediated functions through the downregulation of HIF-1β. These results may further reveal the mechanism of our previous finding, TAD1822-7 inhibits the invasion of breast cancer via CXCR4 inhibition [9], is that TAD1822-7 inhibits HIF-1β so as to downregulate HIF-1 target gene CXCR4. Probably, for the same reason, TAD1822-7 inhibits VEGF and therefore angiogenesis in tumor cells.

Our study reports new findings that TAD1822-7 upregulates ERβ leading to breast cancer cell death. Mechanistically, we demonstrate that the inhibitory effects of TAD1822-7-upregulated ERβ on the cell proliferation and apoptosis of breast cancer cells are mediated by PI3K/AKT on the cell proliferation and apoptosis of breast cancer cells are mediated by PI3K/AKT signaling pathways which induce mitochondrial dysfunction and autophagy. Moreover, TAD1822-7 modulates HIF functions and autophagy via the inhibition of HIF-1β in the context of hypoxia-induced autophagy. These findings provide new insights into the mechanism underlying the inhibitory effects of TAD1822-7 in breast cancer cells.
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Author contributions Conceptualization: QS, YZ; Methodology: QW, KC; Formal analysis and investigation: QW, KC, JW, AS; Writing—original draft preparation: QS, YZ; Writing—review and editing: QS, YZ; Funding acquisition: QS, YZ; Resources: YZ; Supervision: YZ.

Data availability The analyzed data are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval Present study was conducted after the prior approval by the biomedical ethics committee of Xi’an Jiaotong University Health Science Center and conform to ethical principles.

Consent to participate All authors have seen the manuscript and approved to submit the manuscript.

Consent for publication All authors consent to the publication of the manuscript.

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