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# Anticancer effect of celastrol on human triple negative breast cancer: Possible involvement of oxidative stress, mitochondrial dysfunction, apoptosis and PI3K/Akt pathways

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ABSTRACT

Signaling via the phosphatidylinositol-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) is crucial for divergent physiological processes including transcription, translation, cell-cycle progression and apoptosis. The aim of work was to elucidate the anti-cancer effect of celastrol and the signal transduction pathways involved. Cytotoxic effect of celastrol was assessed by MTT assay on human triple negative breast cancer cells (TNBCs) and compared with that of MCF-7. Apoptosis induction was determined by AO/EtBr staining, mitochondrial membrane potential by JC-1, Annexin binding assays and modulation of apoptotic proteins and its effect on PI3K/Akt/mTOR pathway by western blotting. Celastrol induced apoptosis in TNBC cells, were supported by DNA fragmentation, caspase-3 activation and PARP cleavage. Meanwhile, celastrol triggered reactive oxygen species production with collapse of mitochondrial membrane potential, down-regulation of Bcl-2 and up-regulation of Bax expression. Celastrol effectively decreased PI3K 110α/85α enzyme activity, phosphorylation of $Akt^{ser473}$ and p70S6K1 and 4E-BP1. Although insulin treatment increased the phosphorylation of $Akt^{ser473}$, p70S6K1, 4E-BP1, celastrol abolished the insulin mediated phosphorylation. It clearly indicates that celastrol acts through PI3k/Akt/mTOR axis. We also found that celastrol inhibited the Akt/GSK3β and Akt/NFkB survival pathway. PI3K/Akt/mTOR inhibitor, PF-04691502 and mTOR inhibitor rapamycin enhanced the apoptosis-inducing effect of celastrol. These data demonstrated that celastrol induces apoptosis in TNBC cells and indicated that apoptosis might be mediated through mitochondrial dysfunction and PI3K/Akt signaling pathway.



## 1. Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide (Siegel et al., 2014). Triple-negative breast cancer (TNBC), characterized by a lack of expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER)-2, accounts for 15% of all types of breast cancer (Corkery et al., 2009; Foulkes et al., 2010). Epidemiological studies demonstrate that TNBC, showing a tendency towards early metastasis and poor prognosis, represents a significant clinical challenge. Since TNBC does not respond to endocrine therapy or other available targeted agents, drug treatment options are limited to traditional chemotherapy which is frequently trapped in the drug resistance (Montagna et al., 2013). Therefore, to explore new drugs or treatments against TNBC has been very imperative and attracted extensive attention.

Apoptosis is a programmed cell death mechanism that can be driven by two major apoptotic pathways, the cell death receptor-mediated extrinsic pathway and the mitochondrial-mediated intrinsic pathway (Kasibhatla and Tseng, 2003; Xiao et al., 2013). The mitochondrial-mediated apoptotic pathway causes MMP loss, cytochrome c release, and the cleavage of executioner caspase-3, ultimately resulting in chromatin condensation, DNA fragmentation and the formation of apoptotic bodies (Li et al., 2009; Wang et al., 2013; Yuan et al., 2012). Additionally, the PI3K/Akt signaling pathway plays a critical role in apoptosis (Carnero et al., 2008; X. Zhang et al., 2011). Because the PI3K/Akt pathway reduces apoptosis and promotes tumor cell growth, the inhibition of this pathway may be a valid approach to treating human cancer.

Natural compounds have been a fertile source of potential cancer chemotherapeutic and chemoprevention agents, and they have received great attention because they are considered to be safe and to reduce the risk of mutagenicity in normal cells. Celastrol, known as a triterpene, is a functional ingredient that was originally identified and extracted from traditional Chinese medicine named *Tripterygium wilfordii* Hook F. ("Thunder of God Vine") (Pang et al., 2010) and shows potential in treatment of chronic inflammatory disorders, such as arthritis (Venkatesha et al., 2011), lupus erythematosus, lateral

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sclerosis and Alzheimer's disease (Kannaiyan et al., 2011). It also potentiates apoptosis in numerous tumor cells through inhibition of IκBα kinase, proteasome (Dai et al., 2010), topoisomerase (Nagase et al., 2003), heat shock protein (Trott et al., 2008), and VEGF receptor expression (Pang et al., 2010). Recently several studies have demonstrated that celastrol exhibits anticancer activity against various cancers (e.g., cervical (Hu et al., 2013), prostate (Yang et al., 2006), gastric (Sha et al., 2014), breast (Y. Kim et al., 2010) and colon cancers (Yadav et al., 2010)). However, the anticancer effects and putative mechanisms of apoptosis induced by celastrol in TNBC cells remain unknown.

The objectives of the present study were to demonstrate the effect of celastrol-induced death in TNBC cells, and to investigate the possible underlying cellular mechanisms by evaluating mitochondrial dysfunction and PI3K/Akt signaling pathway activity after celastrol treatment. In addition, the production of ROS, and their role in celastrol-induced mitochondrial function and PI3K/Akt pathways were examined.

## 2. Material and methods

### 2.1. Chemicals and antibodies

Celastrol (≥97% purity) and mTOR inhibitor rapamycin were purchased from Santa Cruz Biotechnology, Inc. (Texas, USA). Dual PI3K/Akt and mTOR inhibitor, PF04691502 was obtained from Selleckchem, UK. Compounds were dissolved in dimethyl sulfoxide (DMSO) and stored as aliquots (20 mM) at −20 °C. Acridine orange, Ammonium acetate, Agarose, Acrylamide, Caspase-3-substrate, 2′,7′-dichlorofluorescein diacetate (DCFDA), Dithiothreitol, Dihydrogen sodium phosphate, EGTA, HEPES, Methylthiazolyldiphenyl-tetrazoliumbromide (MTT), Nonidet P-40, N1,N1-Dimethyl bis acrylamide, NADH, Propidium Iodide, Proteinase K, RNAse A, Sodium pyrophosphate, Tween 20, and Triton-X-100 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), Penicillin, Streptomycin, and Trypsin/EDTA were purchased from Invitrogen Life Technologies, USA. PI3K110α/85α enzyme assay kit was purchased from Gyrasol Technologies, Lawrence, KS, USA. Primary antibodies included Bcl-2 (28 kDa), Bax (20 kDa), Cytochrome C (14 kDa), p53 (53 kDa), Caspase-3 (17, 19, 35 kDa), poly (ADP-ribose) polymerase (PARP) (116/89 kDa), NF-κB (P65) (65 kDa), total Akt (60 kDa), Ser473 phospho Akt (p-Akt) (60 kDa), phospho-4E-BP1 (21 kDa), phospho-p70S6K1 (70 kDa), phospho-GSK-3β (46 kDa), GSK-3β (46 kDa), Cyclin D1 (37 kDa), c-Myc (57–70 kDa), phospho-IKKα/β (60 kDa), phospho-p65NFkB (65 kDa), p65NFkB (65 kDa), Actin (42 kDa), Horseradish peroxidase-conjugated secondary anti-mouse and anti-rabbit antibodies were purchased from Cell Signaling Technology, Danvers, MA, USA. VEGF (43 kDa) was purchased from Abcam, Cambridge, MA, USA.

### 2.2. PI3K110α/85α enzyme assay

Enzyme activity was performed as per manufacturer guidelines, Gyrasol Technologies, USA. Briefly, PI3K enzyme activity was determined in 15 μL of 25 mM HEPES, pH 7.5, 50 mM $MgCl_2$, 0.05% $NaN_3$ containing 8 μM substrate, 100 μM ATP (S − Enz − Inh) with the reaction started by addition of 50 nM enzyme (S + Enz − Inh) or addition of 50 nM enzyme and 1000 nM celastrol (S + Enz + Inh). After 90 min of incubation at room temperature the enzyme reaction was stopped by the addition of 5 μL of post-reaction buffer 40 μL of 1X Sensor (1:150) to each well. Plate was incubated for 90 min at room temperature. Fluorescence was monitored with the Multimode Plate Reader at wavelengths of 540 (excitation) and 580 nm (emission). Phosphorylation was measured by change in fluorescence of a dye-labeled and phosphorylated substrate when bound by the sensor. The decrease in fluorescence directly correlates to the level of substrate conversion.

### 2.3. Cell lines

The cell lines used were: MCF-7 (ER + ve), MDA-MB-231(ER − ve) and BT-549 (ER − ve) (Kind gift samples by Dr. Radha, Centre for Cellular and Molecular Biology, Hyderabad, Telangana) and MCF-10A (non-malignant breast epithelial cells) was purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin–streptomycin whereas MCF-10A cells were cultured in MEBM media along with 100 ng/mL cholera toxin. The cells were maintained in a humidified incubator at 37 °C with 5% $CO_2$. Cell lines were subcultured by enzymatic digestion with 0.25% trypsin/1 mM EDTA solution when they reached approximately 70–80% confluency.

### 2.4. Cytotoxicity studies

Cell viability was determined by MTT colorimetric assay (Naidu et al., 2013). The breast cancer cells (MCF-7, MDA-MB-231 and BT-549) were seeded at a density of $1 \times 10^4$ cells/well, in 100 μL DMEM medium and were allowed to attach for overnight in a $CO_2$ incubator. Cells were treated with 0–25 μM celastrol for 24 and 48 h. After the treatment, 10 μL of MTT (5 mg/mL) in 100 μL medium was added and incubated at 37 °C for 4 h after aspirating the medium with celastrol. Then the media with MTT were removed and the formed purple formazan crystals were dissolved in 200 μL of dimethyl sulphoxide and read at 570 nm in a multidetection plate reader (Spectramax M4, Molecular devices, USA). Cytotoxicity was expressed as the concentration of celastrol inhibiting cell growth by 50% (IC50 value).

### 2.5. Colony formation assay

MDA-MB-231 cells (250 cells/well) were cultured in six well plates overnight and then exposed to designated concentration of celastrol for 24 h. After rinsing with fresh medium, cells were allowed to grow for 12 days to form colonies. The resulting colonies were fixed and stained with 1% crystal violet (in methanol) for 4 h. The size and number of stained colonies with >50 cells were counted under inverted phase contrast microscope. Colony formation was calculated as a percentage to control cultures (Arbab et al., 2013).

### 2.6. Lactate dehydrogenase (LDH) release assay

LDH activity was evaluated spectrophotometrically in the culture media after drug treatment as described previously (Tikoo et al., 2011). Briefly, 100 μL of culture media was added to 100 μL NADH (2.5 mg/mL, in phosphate buffer, pH 7.4) in the total reaction volume of 2.6 mL. To it, 100 μL of sodium pyruvate (2.5 mg/mL, in phosphate buffer pH 7.4) was added after an incubation period of 2 min. The absorbance of resulting solution was measured at 340 nm for a period of 5 min at 1 min interval. The amount of LDH was calculated as: $LDH = (\Delta E \times 0.35 \times 27) / (0.347 \times 2)$, where, ΔE is the change in absorbance/min, 0.35 is the concentration of NADH, 27 is the dilution factor, 0.347 is the molar extinction coefficient of sodium pyruvate and 2 is the time of incubation with sodium pyruvate.

### 2.7. Assessment of cell morphology

Cells ($1 \times 10^6$ cells/well) were grown in 6-well plates and treated with or without celastrol at concentrations ranging from 0.5 to 1.5 μM for 24 h. Morphological changes were observed with an inverted phase contrast microscope (Model: Nikon, Japan) and photographs were taken with digital camera (Nikon Inc., Japan) at 200× magnification. The acridine orange/ethidium bromide (AO/EtBr) staining procedure was followed to differentiate the live, apoptotic and necrotic cells (Ribble et al., 2005). Briefly, treated or untreated cells were stained with acridine orange (10 μg/mL) and ethidium bromide (10 μg/mL)



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