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# Downregulation of TIGAR sensitizes the antitumor effect of physapubenolide through increasing intracellular ROS levels to trigger apoptosis and autophagosome formation in human breast carcinoma cells

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ABSTRACT

Physapubenolide (PB) is a cytotoxic withanolide isolated from *Physalis angulata* that was used as a traditional Chinese medicine. In this study, we investigated the role of TIGAR and ROS in PB-induced apoptosis and autophagosome formation in human breast carcinoma MDA-MB-231 and MCF-7 cells. PB induced apoptosis by decreasing mitochondrial membrane potential and elevating the Bax/Bcl-2 protein expression ratio in MDA-MB-231 and MCF-7 cells. Caspase inhibitor Z-VAD-FMK treatment partly blocked PB induced cytotoxicity, suggesting that apoptosis serves as an important role in the anti-proliferative effect of PB. Meanwhile, PB induced autophagosome formation, as characterized by increased acridine orange-stained positive cells, accumulation of punctate LC3B fluorescence and a greater number of autophagic vacuoles under electron microscopy. Furthermore, PB inhibited autophagic flux as reflected by the overlapping of mCherry and GFP fluorescence when MDA-MB-231 cells were transfected with GFP-mCherry-LC3 plasmid. Depletion of LC3B, ATG5 or ATG7 reduced PB-induced cytotoxicity, indicating that autophagosome associated cell death participated in the anti-cancer effect of PB. Moreover, PB-induced apoptosis and autophagosome formation were linked to the generation of intracellular ROS, and pre-treatment with the antioxidant NAC obviously mitigated the effects. Interestingly, PB treatment slightly increased TIGAR expression at low concentrations but decreased TIGAR expression drastically at high concentrations. Downregulation of TIGAR by small interfering RNA augmented low concentrations of PB-induced apoptosis and autophagosome formation, which contributed to the observed anti-cancer effect of PB and were reversed by NAC pre-treatment. Consistently, in MDA-MB-231 or MCF-7 xenograft mouse model, PB suppressed tumor growth through ROS induced apoptosis and autophagosome associated cell death accompanied with the downregulation of TIGAR. Taken together, these results indicate that downregulation of TIGAR increased PB-induced apoptosis and autophagosomes associated cell death through promoting ROS generation in MDA-MB-231 and MCF-7 cells.



## 1. Introduction

Among the most common cancers, breast cancer is the leading cause of cancer-related death among females across the world with an estimated 1.7 million cases and 521,900 deaths in 2012 [1]. In the United States, statistics suggested that there occurred 231,840 new cases of invasive breast cancer and 40,290 women died from the disease in 2015 and that about 12.5% women will develop breast cancer during their lifetime [2]. Although adjuvant systemic chemotherapy reduces the risk of distant recurrence and breast cancer mortality, there are serious irreversible effects associated with chemotherapy. Therefore, efforts are still underway to develop more effective and less toxic, preferably natural origin agents that could be successfully administered to breast carcinoma patients.

*Abbreviations:* PB, physapubenolide; ROS, reactive oxygen species; TIGAR, TP53-induced glycolysis and apoptosis regulator; NAC, *N*-acetylcysteine; PCD, programmed cell death; ADR, Adriamycin; MDA, Malondialdehyde; AO, acridine orange.

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Apoptosis is the most well described form of type I programmed cell death and involves the activation of catabolic enzymes (proteases in particular) in signaling cascades, which leads to the rapid demolition of cellular structures and organelles [3–5]. However, in tumor cells one of the hallmarks is the evasion of cancer cells from apoptosis [6]. So apoptosis targeted agents has been unable to meet the goal to completely ablate the mechanisms of cancer cell survival. Additionally, defect in apoptosis is a common cause of chemoresistance in clinic [7]. Therefore, it is important to explore novel way to trigger cancer cell death. Macroautophagy (referred to hereafter as autophagy) is another mechanism of cell death, which is a catabolic process that sequesters cytoplasmic proteins and organelles into autophagosomes and transports them to lysosomes for recycling and degradation [8,9]. So developing the autophagic cell death inducer and combination the apoptotic and autophagic cell death ways may have greater advantages during the treatment of cancer.

TIGAR, shares functional sequence similarities with the bisphosphatase domain (FBPase-2) of the bifunctional enzyme PFK-2/FBPase-2 (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase), which degrades Fru-2,6-P2 (fructose-2,6-bisphosphate), leading to an inhibition of glycolysis. In view of this, TIGAR functions to decrease intracellular reactive oxygen species (ROS) levels through directing glycolysis into the pentose phosphate shunt to produce NADPH [10,11]. ROS are known to be involved in the regulation of different physiological processes, including apoptosis and autophagy [12–15] and some mechanisms of autophagy and apoptosis modulated by free radicals have been established. For example, in response to ROS, different protein kinase signaling cascades (including Nrf2, p53, MAPK, and AKT) can simultaneously trigger autophagic and apoptotic responses [12,16]. However, little is known about how TIGAR regulates apoptosis and autophagy in specific pathological environments. Whether TIGAR could regulate apoptosis and autophagy through modulating ROS levels is also needed to be clarified. A more complete and deeper understanding the regulation role of TIGAR on apoptosis and autophagy as well as ROS will pave a new avenue for developing novel chemotherapeutic drugs.

*Physalis angulata* (Solanaceae), known as the traditional Chinese medicine "Kuzhi", is widely distributed throughout tropical and subtropical regions of the world. In some countries, the extracts or infusions from this medicinal plant in folk medicines have been used as a treatment for different illnesses, such as diuretic, malaria, hepatitis, asthma, dermatitis, rheumatism and liver problems. These plant extracts have also been utilized as anti-cancer, antipyretic, anti-leukemic, anti-mycobacterial and immunomodulatory agents [17–22]. Here we show that physapubenolide (PB, Fig. 1A), isolated from *Physalis angulata* exhibits significant anti-cancer effects in human breast carcinoma cells. In our previous published manuscript, we demonstrated PB inhibited glycolysis through the inhibition role of TIGAR on glycolysis and inhibiting glycolysis increased apoptosis [23]. However, the role of TIGAR in apoptosis during PB treatment is still not clear. Additionally, in this experiments, we found an interesting phenomenon that PB decreased TIGAR expression at higher concentrations which seemed to be inconsistent with our previous study that PB inhibited glycolysis by increasing TIGAR levels. However, there are also reports that TIGAR protects cells from ROS-associated apoptosis [24]. Then the role of TIGAR urgently needs to be identified during PB treatment. In the present study, we firstly report the role of TIGAR in PB induced apoptotic and autophagosome associated cell death. Our results demonstrate that PB induced downregulation of TIGAR increases the level of ROS and triggers apoptosis and autophagosome formation, which contributes to the anti-cancer effect of PB. These inspiring findings may provide strong evidence to determine the regulation mechanism of TIGAR in the two physiological processes, which could subsequently be developed as a novel breast carcinoma therapeutic target.

## 2. Materials and methods

### 2.1. Cell type and reagents

Human breast carcinoma MDA-MB-231 and MCF-7 cells were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). PB was isolated from *Physalis angulata* in our laboratory. Samples containing at least 95% concentrations of PB were used in all of our experiments. PB was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution of 50 mmol/L and stored at room temperature. Before each experiment, the stock solution of PB was diluted with medium to obtain different working concentrations. Control groups were treated with an equal amount of DMSO (<0.1%) in the corresponding experiments. Cleaved caspase-3 (Asp175) (5A1E) Rabbit mAb, caspase-3 (8G10) Rabbit mAb, caspase-9 (C9) Mouse mAb, cleaved caspase-9 (Asp330) (D2D4) Rabbit mAb, caspase-7 (D2Q3L) Rabbit mAb, cleaved caspase-7 (Asp198) (D6H1) Rabbit mAb, PARP, cleaved PARP (Asp214) (D64E10) Rabbit mAb, Beclin-1 (D40C5, rabbit mAb), ATG5 (D5F5U, rabbit mAb), ATG7 (D12B11, rabbit mAb), LC3B (D11, XP rabbit mAb), SQSTM/p62 (D5E2, rabbit mAb), Bax (D2E11, rabbit mAb) and Bcl-2 (D55G8, rabbit mAb) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-TIGAR antibody (ab62533) was purchased from Abcam (Cambridge, UK), β-actin and HPR-conjugated Goat Anti-Rabbit lgG antibodies were purchased from Vazyme Biotech Co., Ltd (Nan Jing, China).

### 2.2. Isolation and identification of PB

The air-dried aerial parts of P. *angulata* (1 kg) were powdered and extracted with $CH_2Cl_2$-MeOH (1:1) at room temperature for three times. After removing the solvents under vacuum, the residue (70 g) was subjected to column chromatography (CC) on D-101 macroporous resin and eluted with a step gradient of EtOH-$H_2O$ solvent system (20:80, 40:60, 60:40, 80:20, 95:5 v/v) to yield five fractions: Fr. A-E. Fr. C (7 g) was then chromatographed over silica gel with increasing polarities of $CH_2Cl_2$-MeOH solvent system (40:1, 20:1, 10:1, 1:1 v/v) to obtain four subfractions (Fr. C1-C4). Fr. C1 was applied to ODS MPLC eluted with isocratic MeOH-$H_2O$ (50:50 v/v) to afford eight subfractions (Fr. C3A-H). Fr. C3D was separated by preparative HPLC with MeOH-$H_2O$ (60:40 v/v) to give three subfractions (Fr. C3D1-3). Fr. C3D2 was further purified by preparative HPLC with $CH_3CN$-$H_2O$ (37:63 v/v) to obtain compound PB (170 mg). The structure of PB was identified using the MS, $^1H$ NMR, and $^{13}C$ NMR data by comparison with literature [25].

### 2.3. Cell viability and tumor colony forming assays

The cell viability of MDA-MB-231 and MCF-7 cells was measured via MTT assay. Cells were seeded in 96-well culture plates at a density of $5 \times 10^3$ cells per well. After incubation overnight, the cells were administrated with various concentrations of PB for 24, 48 or 72 h. MTT (5 mg/mL) was dissolved in PBS and filter-sterilized. Then, 20 μL of the prepared MTT solution was added to each well and the cells were incubated for 4 h until a purple precipitate was visible. The formed formazan crystals were dissolved in DMSO (150 μL/well) by continuous shaking for 10 min. Absorbance was measured using an ELISA reader (Spectra Max Plus384, Molecular Devices, Sunnyvale, CA) at a test wavelength