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# Psammaplin A induces Sirtuin 1-dependent autophagic cell death in doxorubicin-resistant MCF-7/adr human breast cancer cells and xenografts

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ABSTRACT

*Background:* Psammaplin A (PsA) is a natural product isolated from marine sponges, which has been demonstrated to have anticancer activity against several human cancer cell lines via the induction of cell cycle arrest and apoptosis. New drugs that are less toxic and more effective against multidrug-resistant cancers are urgently needed.
*Methods:* We tested cell proliferation, cell cycle progression and autophagic cell death pathway in doxorubicin-resistant MCF-7 (MCF-7/adr) human breast cancer cells. The potency of PsA was further determined using an in vivo xenograft model.
*Results and conclusion:* PsA significantly inhibited MCF-7/adr cells proliferation in a concentration-dependent manner, with accumulation of cells in G2/M phase of the cell cycle. PsA significantly decreased SIRT1 enzyme activity and reduced expression of SIRT1 protein in the cultured cells with greater potency than sirtinol or salermide. Acetylation of p53, a putative target of SIRT1, increased significantly following PsA treatment. In addition, PsA markedly increased the expression levels of autophagy-related proteins. In support of this, it was found that PsA significantly increased the expression of damage-regulated autophagy modulator (DRAM), a p53-induced protein.
*General significance:* The results of this study suggest that PsA is sufficient to overcome multidrug-resistant cancer via SIRT1-mediated autophagy in MCF-7/adr breast cancer cells, indicating that PsA has therapeutic potential for clinical use.



## 1. Introduction

Silent mating type information regulation 2 homolog 1 (SIRT1) belongs to the class III histone deacetylase (HDAC) family. Best known as a regulator in stress signaling, [1,2] SIRT1 also exerts control on tumor suppressor genes, [3,4] and influences lifespan in many organism [5–7]. In both cell lines and tumor specimens from diverse malignancies, SIRT1 is highly expressed [8] and evidence for epigenetic modification of SIRT1 suggests it may contribute to emergence of drug resistance in cancer [9,10]. SIRT1 deacetylates p53 as a component of HDAC complexes [11], possibly by acting at major p53 acetylation sites to prevent p53-dependent transactivation of *CDKN1A* (which encodes p21) and Bcl-2-associated X protein (BAX) [12]. The p53 tumor suppressor protein protects the cell from malignant transformation and the organism from cancer by inducing cell cycle arrest or apoptosis [13,14]. Because of its critical functions, p53 presents a point of vulnerability in cell growth regulation, as the cell cannot easily compensate for p53 dysfunction. Approximately 50% of all malignancies contain a p53 mutation or a functionally inactivated p53 [15,16]. In addition to its role in tumor suppression, inducing senescence and apoptosis of damaged cells, p53 may contribute to maintenance of intracellular homeostasis as a regulator of autophagy [17]. However, cytoplasmic p53 may not show tumor suppressor function, and some mutant p53 proteins may inhibit autophagy through extended residence in the cytoplasm [18,19]. Chemotherapeutic agents may induce autophagy by p53-dependent

*Abbreviations:* AMPK, AMP-activated protein kinase; Atg, autophagy-related gene; BAX, Bcl-2-associated X protein; DNMT, DNA methyltransferase; Dox, doxorubicin; DRAM, damage-regulated autophagy modulator; HDAC, histone deacetylase; LC3, microtubule-associated protein 1 light chain 3; MCF-7/adr, doxorubicin-resistant MCF-7; mTOR, mammalian target of rapamycin; PCNA, proliferating cell nuclear antigen; PsA, Psammaplin A; PUMA, p53-upregulated modulator of apoptosis; SIRT1, silent mating type information regulator 2 homolog 1; 3-MA, 3-methyladenine.

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mechanisms involving AMP-activated protein kinase (AMPK) activation and mammalian target of rapamycin (mTOR) inhibition [20,21]. Another p53-induced autophagy requires transcriptional activation of DRAM, a lysosomal protein that mediates autophagy [22,23]. Inhibitors of SIRT1 influence the functional status of p53, evident as an increase in acetylated p53 [24,25]. However, little is known of SIRT1-mediated autophagic cell death in the context of p53 mutation.

Psammaplin A (PsA) is a natural bromotyrosine-derived disulfide dimer that was originally isolated from the *Psammaplysilla* sponge in 1987 (Fig. 1) [26]. PsA was reported to have antibacterial and antitumor properties, and to inhibit various enzymes, including topoisomerase, farnesyl protein transferase, and chitinase [27,28]. By inhibiting HDAC and DNA methyltransferase (DNMT), PsA is proposed to influence the epigenetic modification of tumor suppressor genes [29]. However, the effects of PsA on SIRT1-mediated p53 target genes involved autophagy cell death pathway are not known.

The study investigated the antitumor effects of PsA as a SIRT1 inhibitor using a doxorubicin (Dox)-resistant MCF-7/adr human breast cancer cell lines. A principal finding from this study, that PsA induced autophagic cell death through SIRT1 inhibition, supports further investigation of PsA as a therapeutic agent for treating chemotherapy-resistant cancers.

## 2. Materials and methods

### 2.1. Chemicals and antibodies

Dox (50 mg/25 ml, Boryung Pharmacy) was kindly provided by the National Cancer Center in Korea. Psammaplin A (Santa Cruz, sc-258049), dissolved in dimethylsulfoxide (DMSO) (Sigma, D2650); Dox, in phosphate-buffered saline (PBS) (GIBCO, 21600-051); sirtinol (Sigma, S7942), in DMSO; and salermide (Sigma, S8825), in DMSO, were added directly to the culture medium. 3-Methyladenine (3-Ma) (Sigma, M9281) was purchased from Sigma Aldrich. The final concentrations were as indicated in each experiment. The chemicals were prepared immediately before drug treatments, and the final concentration of DMSO never exceeded 0.1% (vol/vol).

Primary antibodies used were anti-SIRT1 (ab75435), anti-SIRT2 (ab75436), anti-SIRT5 (ab13697), anti-SIRT6 (ab62739), anti-acetylated K382 p53 (ab75754) and anti-p62 (ab56416) from Abcam; anti-SIRT3 (5490), anti-actin (4970), anti-Acetyl Histone H3 (9677), anti-acetyl Histone H4 (2591), anti-LC3B (3868), anti-Atg3 (3415), anti-Atg5 (8540), anti-Atg7 (2631), anti-Beclin1 (3495) and anti-Atg12 (4180) from Cell Signaling Technology; and anti-SIRT4 (sc-135053), anti-Histone H1 (sc-8030), anti-tubulin (sc-8035), anti-p53 (sc-126), anti-p21 (sc-6246), anti-Bcl2 (sc-7382), anti-BECN1 (sc-48341) and anti-DRAM (sc-98654) from Santa Cruz Biotechnology. The secondary antibodies used were goat anti-rabbit (7074) and goat anti-mouse (7076) from Cell Signaling Technology; and Alexa Fluor 488 goat anti-mouse and anti-rabbit (A11001 and A11008) and Alexa Fluor 568 goat anti-mouse and anti-rabbit (A11004 and A11011) from Invitrogen.

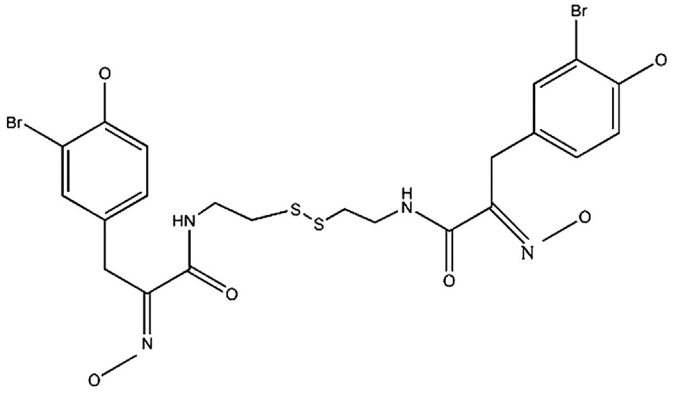


**Fig. 1.** The structure of Psammaplin A (PsA). PsA is a natural bromotyrosine derivative and a symmetrical conjugate of cystamine, from the *Psammaplysilla* sponge.

### 2.2. Cell lines and culture conditions

Dox-resistant MCF-7 (MCF-7/adr) human breast cancer cells were kindly provided by Professor Keon Wook Kang from Seoul National University. MCF-7/adr cells were developed as previously described [30]. The cells were maintained as monolayers in 5% $CO_2$ at 37 °C, in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, 12800-058) containing 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, SH30919.03) and 1% penicillin/streptomycin (GIBCO, 15240-062). When the cells were 80% confluent, they were sub-cultured to fresh medium. The cultures were incubated for 24 h before the experimental treatments.

### 2.3. Cytotoxicity assay

Cell viability was determined using the microculture tetrazolium (MTT) assay. Cultures were initiated in 96-well plates at a density of 3000 cells per well. At the end of the treatment period, 15 μl of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide in PBS) (Sigma, M5655) was added to each well. The plates were incubated for an additional 4 h in the dark at 37 °C. The supernatants were aspirated, and formazan crystals were solubilized in 100 μl DMSO at 37 °C for 10 min with agitation. The absorbance of each well was measured at 540 nm with a microplate reader (Molecular Devices, VersaMax).

### 2.4. Flow cytometry analysis

MCF-7 and MCF-7/adr cells were treated with PsA at various concentrations for 24 h. Attached cells were then collected, washed in 1% bovine serum albumin (BSA) (Sigma, A4503), and fixed in 95% ethanol containing 0.5% Tween 20 (GenDEPOT, T9100-010) for 30 min at −20 °C. After washing with BSA, cells were stained with a mixture of 10 μg/ml propidium iodide (PI) (Sigma, P4864) and 100 μg/ml Ribonuclease A (Sigma, R4642) in PBS for 20 min at room temperature in the dark. The cell cycle distribution of each sample was then analyzed using a flow cytometer (BD Biosciences, Accuri C6).

### 2.5. Reporter gene assay

The SIRT1 over-expressing plasmid and the p53-Luc reporter plasmid were kindly donated by Dr. KY Lee from Chonnam National University. Reporter gene activity was determined using a dual-luciferase reporter assay system (Promega). MCF-7/adr cells that were plated in 12-well plates were transiently transfected with the p53-luc reporter plasmid/phRL-SV plasmid with or without the SIRT1 over-expressing plasmid. The cells were then incubated in culture medium without serum for 18 h, and the firefly and hRenilla luciferase activities in the cell lysates were measured using an LB941 luminometer (Berthold Technologies).

### 2.6. SIRT1 activity assay

SIRT1 activity was determined using the Sensolyte 520 FRET SIRT1 Fluorimetric Assay Kit (Anaspec, 72155) according to the manufacturer's instructions. Briefly, cells were incubated at 37 °C with recombinant human SIRT1 in the presence of PsA, sirtinol, salermide, or nicotinamide. The reaction was initiated by addition of the SIRT1 FRET substrate. After 1 h, developer was added, and the mixture was incubated for an additional 10 min at room temperature. Fluorescence was measured using a fluorimetric reader with excitation at 490 nm