Biochimica et Biophysica Acta 1850 (2015) 401–410

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

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

Tae Hyung Kim [a,1], Hyuk Soon Kim [b,1], Yoon Jong Kang [a], Sungpil Yoon [c], Jaewon Lee [d], Wahn Soo Choi [b], Jee H. Jung [d], Hyung Sik Kim [a,*]

[a] Division of Toxicology, School of Pharmacy, Sungkyunkwan University, 2066, Seobu-ro, Jangan-gu, Suwon, Gyeonggi-do 440-746, Republic of Korea
[b] School of Medicine, Konkuk University, Chungju 380-701, Republic of Korea
[c] Research Institute, National Cancer Center, 809 Madu 1-dong, Ilsan-gu, Goyang-si, Gyeonggi-do 411-764, Republic of Korea
[d] College of Pharmacy, Pusan National University, San 30, Jangjeon-dong, Geumjeong-gu, Busan 609-735, Republic of Korea

ARTICLE INFO

Article history:
Received 11 July 2014
Received in revised form 16 October 2014
Accepted 6 November 2014
Available online 12 November 2014

Keywords:
Breast cancer
Doxorubicin
Drug-resistant
Autophagy
SIRT1

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

* Corresponding author at: School of Pharmacy, Sungkyunkwan University, 2066, Seobu-ro, Jangan-gu, Suwon, Gyeonggi-do, 440-746, Republic of Korea. Tel.: +82 31 290 7789; fax: +82 31 292 8800.
E-mail address: hkims@skku.edu (H.S. Kim).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbagen.2014.11.007
0304-4165/

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.

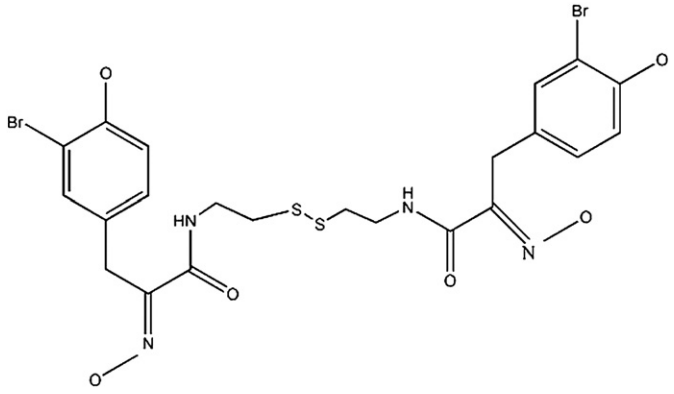

Fig. 1. The structure of Psammaplin A (PsA). PsA is a natural bromotyrosine derivative and a symmetrical conjugate of cystamine, from the *Psammaplysilla* sponge.

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

and emission at 520 nm. SIRT1 activity was expressed as relative fluorescence units.

2.7. siRNA transfection

SIRT1 siRNA and non-silencing siRNA (Silencer Negative Control) were obtained from Bioneer Corporation in Korea. Transfection complexes were prepared in Opti-MEM Reduced Serum Medium (GIBCO, 31985-070) by mixing Lipofectamine RNAiMAX (Invitrogen, 13778-075) and 10 nM of each siRNA. MCF-7/adr cells (1×10^5 cells/well) were plated in a 6-well format, and transfection complexes were added. The cells were incubated for 48 h before gene knockdown was assessed.

2.8. Immunofluorescence

Cells (5×10^4 cells/well) were incubated in a 6-well format overnight in culture medium and fixed by incubation for 20 min at room temperature in freshly prepared 4% formaldehyde (Sigma, P6148) in PBS. The cells were washed three times with PBS and permeabilized by incubation for 20 min with 0.5% Triton X-100 (Sigma, T8787) in PBS. The cells were rinsed briefly in PBS and blocked with 4% BSA (Sigma, A4503) for 1 h. The primary antibody was added for 60 min, and the cells were then treated with a secondary Alexa fluor 488 or Alexa fluor 568 goat anti-mouse or anti-rabbit antibody for 30 min in the dark. The nuclei were visualized using PI (Sigma, P4864) or 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, D21490). The cells were examined using a confocal laser-scanning microscope (Olympus, FV10i).

2.9. Reverse transcription PCR

Total RNA was isolated from MCF-7/adr cells using Trizol reagent (Invitrogen, 15596-026). The reverse transcription reaction was performed using 2 μg of total RNA, reverse transcribed into cDNA using reverse transcriptase (Invitrogen, 18064-014) in the presence of oligo dT (Invitrogen, 18418-012), and amplified by reverse transcription polymerase chain reaction (RT-PCR) with the following primers: silent mating type information regulation 2 homolog 1 (*SIRT1* 232 bp) (F) 5′-CTAG GTGCCCAGCTGATGAA-3′, (R) 5′-TGCAGATGAGGCAAAGGTTC-3′; and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH* 110 bp) (F) 5′-ATCGACCACTACCTGGGCAA-3′, (R) 5′-AGGATAACGCAGGCGATGT-3′. The PCR products were analyzed by electrophoresis on 2% agarose (BIO-RAD, 161-3101) gels with ethidium bromide (Sigma, E1385) staining and photographed using a High Performance DNA Image Visualizer™ (SeouLin Bioscience, SL-20).

2.10. Western blotting

MCF-7 and MCF-7/adr cells were cultured in normal medium, containing 10% FBS, or starvation medium, containing 1% FBS. The cells were then washed twice in PBS, suspended in a lysis buffer (50 mM Tris (pH8), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 100 μg/ml phenylmethylsulphonyl fluoride (PMSF), 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 μg/ml leupeptin), and placed on ice for 30 min. The supernatant was collected after centrifugation at 15,000 *g* for 15 min at 4 °C. Equal amounts of lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 8-15% gels and transferred to polyvinylidene difluoride membranes (Millipore, IPVH 00010) in buffer containing 25 mM Tris–HCl pH 8.5, 192 mM glycine, and 20% methanol. The membranes were blocked with 5% skim milk and probed sequentially with specific antibodies (dilution range: 1:1000) for protein detection. The blots were developed using enhanced chemiluminescence (Millipore, WBLUR0100).

2.11. Immunoprecipitation

Cells were lysed using a radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM PMSF, and 1 mM EDTA. Lysates were incubated with rotation for 12 h at 4 °C with 2 μg of a Bcl-2 or Beclin 1 antibody and 20 μl of Protein A/G PLUS-beads (Santa Cruz, sc-2003). The immunoprecipitated proteins were washed four times, resuspended in SDS sample buffer, separated by SDS-PAGE on 12% gels and detected by immunoblotting as described in Section 4.10.

2.12. Acridine orange staining

MCF-7/adr cells were cultured in 6-well plates and treated with PsA for 6 h. The cells were stained with acridine orange (1 μg/ml) for 15 min and washed with PBS. The cells were harvested, and acidic vesicular organelles (AVOs) were detected using a fluorescence-activated cell sorter (FACS) (Accuri C6 flow cytometer; BD Biosciences). The data were analyzed using the Accuri C6 software.

2.13. Tumor xenograft model

Six-week-old female nude mice (BALB-c nu/nu) (Japan SLC, Inc.) weighing approximately 20 g were housed in filtered-air laminar-flow cabinets at a controlled temperature (22 ± 2 °C) with a 12 h light/dark cycle, and handled using aseptic procedures. The institutional animal care committee of Pusan National University approved the experimental procedure. MCF-7/adr cells (5×10^5 cells/0.1 ml) in serum-free medium containing 50% Matrigel (BD, 354234) were injected subcutaneously. Prior to drug administration, the mice were randomized to four groups (n = 4): Group 1 was the control group; Group 2 was treated with Dox (4 mg/kg); Group 3 was treated with PsA (1 mg/kg); and Group 4 was treated with PsA (5 mg/kg). Dox was injected intraperitoneally (i.p.) once per week, and PsA was injected i.p. twice per week. The mice were treated for 4 weeks. Tumor volume (V) was calculated using the following standard formula: $V\ (\mathrm{mm}^3) = 0.52(ab^2)$, where a is the length and b is the width of tumor. Body weights were recorded before dosing and at termination. On day 28, the mice were killed by carbon dioxide asphyxiation.

2.14. Statistical analysis

Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Dunn's test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Drug-resistant cells over-express SIRT proteins

In both the wild-type MCF-7 cell line and MCF-7/adr cells, SIRT expression levels increased significantly after cultured in starvation media. It is worth noting that SIRT expression was significantly higher in MCF-7/adr cells in both conditions (i.e., normal and starvation) (Fig. 2).

3.2. The effect of PsA on cell viability is more potent in MCF-7/adr cells

The MCF-7/adr cells were treated with increasing concentrations of PsA, and the effect on cell viability was assessed by enzymatic reduction of MTT. As shown in Fig. 3, the viability of MCF-7/adr cells decreased significantly after treatment with PsA in a concentration-dependent manner (IC_{50} 2.13 μM). The PsA induced less apparent toxicity in wild-type MCF-7 cells in terms of viability (IC_{50} 4.03 μM).

In flow cytometry analysis, MCF-7 cells underwent G1 arrest at 5 μM PsA (Fig. 4A). However, the MCF-7/adr cells exhibited a concentration-

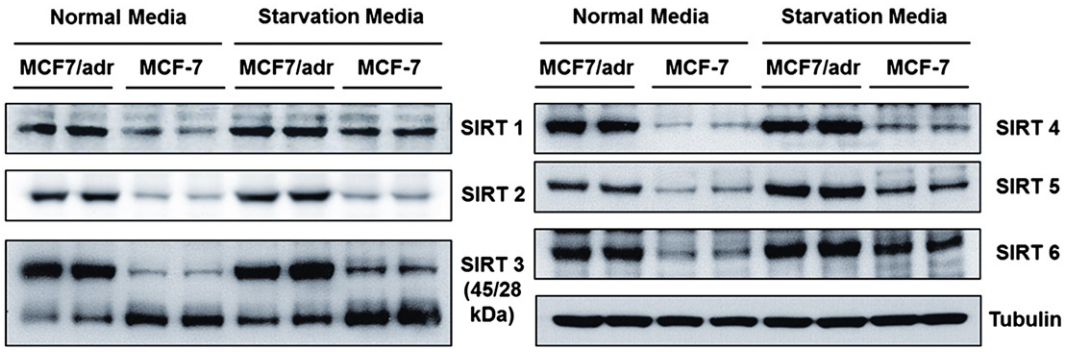

Fig. 2. SIRT1-6 protein levels were detected by Western blotting in MCF-7 and MCF-7/adr cells. Starvation media indicates nutrient-depleted treated cells, as described in Materials and methods. Tubulin was used as an internal loading control.

dependent increase in G1 phase. In addition, after treatment with 5 μM PsA, MCF-7/adr cells accumulated predominantly in G2/M phase (Fig. 4B). These results indicate a more potent growth inhibitory effect of PsA treatment in MCF-7/adr cells than in MCF-7 cells.

3.3. PsA induces acetyl-protein accumulation and reduces nuclear SIRT1 levels

As we showed previously, PsA induces cell cycle arrest and apoptosis through inhibition of HDAC [31]. We extended these findings here by evaluating histone acetylation using specific antibodies against acetylated histones H3 (AcH3) and H4 (AcH4). Levels of AcH3 and AcH4 in MCF-7/adr cells increased markedly after PsA treatment (Fig. 5A). As shown previously, MCF-7/adr cells maintain the expression of SIRT proteins as a group. Thus, we hypothesized that the predominant effects of PsA on MCF-7/adr cells involves these proteins. Interestingly, PsA treatment did not affect the expression level of SIRT proteins except SIRT1 (Fig. 5B). In PsA-treated cells, the expression of SIRT1 mRNA and protein both decreased in a concentration-dependent manner (Fig. 5C,D). To test the correlation of SIRT1 expression with SIRT1 activity, an activity assay was performed in the presence of the well-known SIRT inhibitors such as nicotinamide, sirtinol and salermide. PsA significantly inhibited SIRT1 activity at lower concentrations (1–10 μM) compared to other inhibitors (Fig. 5E). These results suggest that PsA specifically inhibit SIRT1 in MCF-7/adr cells.

3.4. Acetylated p53 accumulates in PsA-treated MCF-7/adr cells

To further characterize the anticancer activity of PsA, we tested the effects of PsA on expression of the tumor suppressor protein p53 since the function of SIRT1 in cancer biology is closely connected to p53 [13]. Relative amounts of acetylated p53 increased after PsA treatment, which is consistent with the SIRT1 expression data obtained in this study (Fig. 6A). Our immunoblotting data showed, however, that p53 protein expression decreased in a concentration-dependent manner after PsA treatment. Further immunofluorescence experiments revealed that a marked increase in acetyl-p53 levels occurs only in the nucleus and not in the cytosol (Fig. 6B). To analyze the interrelationship of p53 activation and SIRT1 inhibition in PsA-treated cells, a p53 luciferase activity assay and a small-interfering RNA (siRNA) experiment were performed. As shown in Fig. 6C, SIRT1 inhibited p53 luciferase activity. However, in the presence of PsA and SIRT1 together, the p53 luciferase activity returned to control levels, indicating that PsA influenced both p53 expression and activity levels (Fig. 6C). In addition, overall SIRT1 expression was significantly reduced in SIRT1 siRNA-treated cells compared to negative control cells. Consistent with previously reported

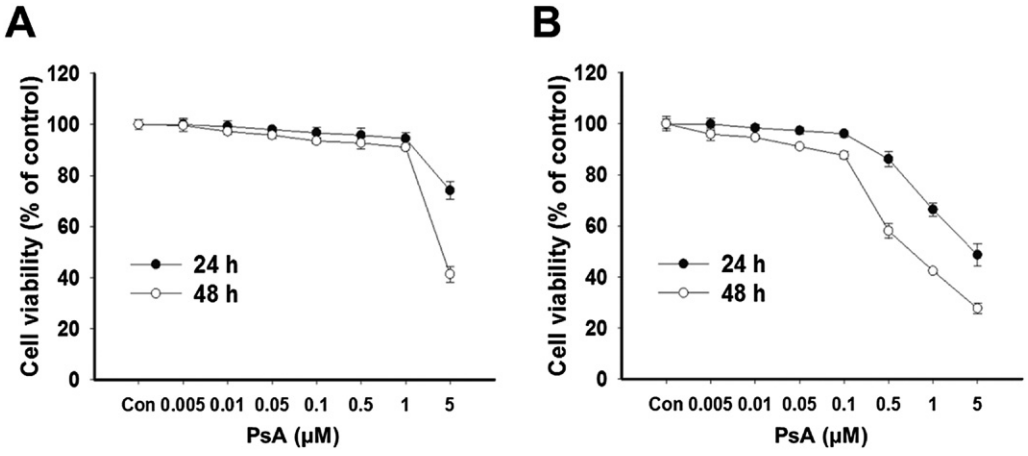

Fig. 3. The effect of PsA on the proliferation of MCF-7 and MCF-7/adr cells. MCF-7 (A) and MCF-7/adr (B) cells were treated with PsA at the indicated concentrations for 24 h or 48 h. Cell viability was measured using the MTT assay. The percentage of viable cells was calculated as percentage to control cells. Control cells were incubated with vehicle alone. The data are expressed as the mean ± SD of three independent experiments.

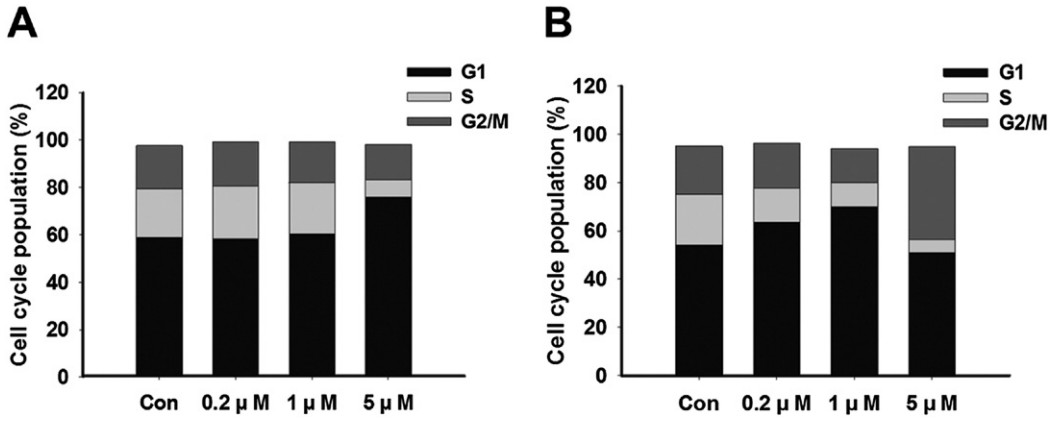

Fig. 4. The effect of PsA on cell cycle distribution in MCF-7 and MCF-7/adr cells. MCF-7 (A) and MCF-7/adr (B) cells were treated with PsA at the indicated concentrations for 24 h. The cells were stained with propidium iodide (PI) and analyzed by flow cytometry to determine the cell distribution at each phase of the cell cycle. The data are representative of three independent experiments.

data, we found that SIRT1 gene silencing significantly increased levels of acetylated p53 (Fig. 6D). These findings offered further indication that the PsA treatment down-regulates SIRT1, which may explain the observed increase in p53 acetylation.

3.5. PsA induces autophagic cell death

Mechanisms of autophagic cell death and potential involvement of p53 were investigated in MCF-7/adr cells using acridine orange staining to assist in visualization of the autophagosomes. Histograms of fluorescence intensities from the control and PsA-treated groups showed increased autophagic activity (i.e., intracellular formation of acidic vacuoles) (Fig. 7A; control fluorescence intensities are shown in black and those from PsA-treated cells are shown in red). Immunoblotting analysis revealed specific increases in expression levels of the microtubule-associated protein 1 light chain 3 (LC3), a marker of autophagy [32], and Beclin 1, a protein that participates in double-membrane autophagosome formation [33]. We also monitored the expression level of p62, an important LC3 interactor under autophagy stimulation [34,35]. We found that the loss of p62 following PsA treatment. The concentration-dependent changes in autophagy-related proteins (Atgs) also suggested that autophagy is initiated in response to PsA (Fig. 7B). The binding interaction between Beclin 1 and Bcl-2, which inhibits autophagy [36,37], was shown by co-immunoprecipitation to proceed as expected in the presence of PsA. However PsA has no effect on Beclin 1/Bcl-2 interaction (Fig. 7C). We further examined the influence of 3-MA in PsA-induced increase of LC3 in MCF-7/adr cells. The results showed that the levels of LC3 in 3-MA co-treated cells were lower than that of the cells treated with PsA alone (Fig. 7D). To determine whether induction of autophagy was due to SIRT1 inhibition pathway, MCF-7/adr cells were treated with either control siRNA or siRNA directed against SIRT1. Following SIRT1 knockdown, LC3 expression level was decreased (Fig. 7E). Both cells transfected with a SIRT1 siRNA and treated with 1 mM of 3-MA showed mild effect following PsA treatment (Fig. 7F). These findings suggested that SIRT1 is more likely to influence autophagy through interaction with other molecules specifically involved in p53 signaling.

3.6. DRAM activation is essential in PsA-induced autophagic cell death

Damage-regulated autophagy modulator (DRAM), a putative component of p53-mediated autophagy, is a gene frequently found to be dysregulated in human cancers [22]. In cultured MCF-7/adr cells, we found that DRAM expression, and concurrently p21 expression, increased significantly following treatment with PsA (Fig. 7G). Immunofluorescence analysis confirmed this effect of PsA on DRAM (Fig. 7H). These results are consistent with involvement of two p53-dependent components, p21 and DRAM, in PsA-induced autophagic cell death.

3.7. The effect of PsA on growth of MCF-7/adr cell tumors in a xenograft model

To evaluate effects of PsA in vivo, nude mice were inoculated with MCF-7/adr cells and treated with Dox(4 mg/kg, i.p.) or PsA (5 mg/kg, i.p.) for 4 weeks. While Dox failed to inhibit tumor growth significantly compared to the vehicle-treated control group, PsA treatment reduced tumor volume by 70% relative to the control group (Fig. 8A), and tumor weights were reduced by approximately 60% (Fig. 8B). Any significant adverse effects were not observed by PsA treatment. Consistent with the inhibitory action of PsA on SIRT1 expression in vitro, the levels of SIRT1 and to a lesser extent, p53 protein were significantly lower in tumors from PsA-treated animals compared to the control group. The DRAM and acetylated p53 proteins were slightly elevated in PsA-treated tumors (Fig. 8C). Taken together, these data showed that the inhibitory effects of PsA on tumor cell proliferation and the down-regulation of SIRT1 expression in vitro correlated with in vivo anti-cancer effects.

4. Discussion

An association between SIRT1 expression and tumorigenesis is widely accepted, based on SIRT1 over-expression and/or activation in a variety of human cancers, and on the capacity of SIRT1 to block apoptosis while promoting cell proliferation and angiogenesis [38,39]. In particular, SIRT1 participates in oncogenic signaling in breast cancer cells [40], and drug-resistant breast cancer cells express higher levels than their parent cells [40]. SIRT1 over-expression leads to deacetylation of multiple proteins, including the tumor suppressor proteins p53, p73 and Ku70 [41–43]. Thus, agents that specifically inhibit SIRT1 may potentially be applied in treating cancer.

Although PsA was originally reported to act as an HDAC inhibitor, the mechanisms underlying this activity are not clearly defined. Of the known SIRT1 inhibitors, only EX-527, which has an IC_{50} value of 0.38 μM, is in clinical trials. Reported IC_{50} values for sirtinol and salermide are 37.6 μM and 76.2 μM, respectively [44]. Despite greater apparent potency, EX-527 fails to invoke the anti-proliferative effect of the other SIRT1 inhibitors unless combined with other cytotoxic and genotoxic agents [45,46]. Based on fluorimetric assay of SIRT1

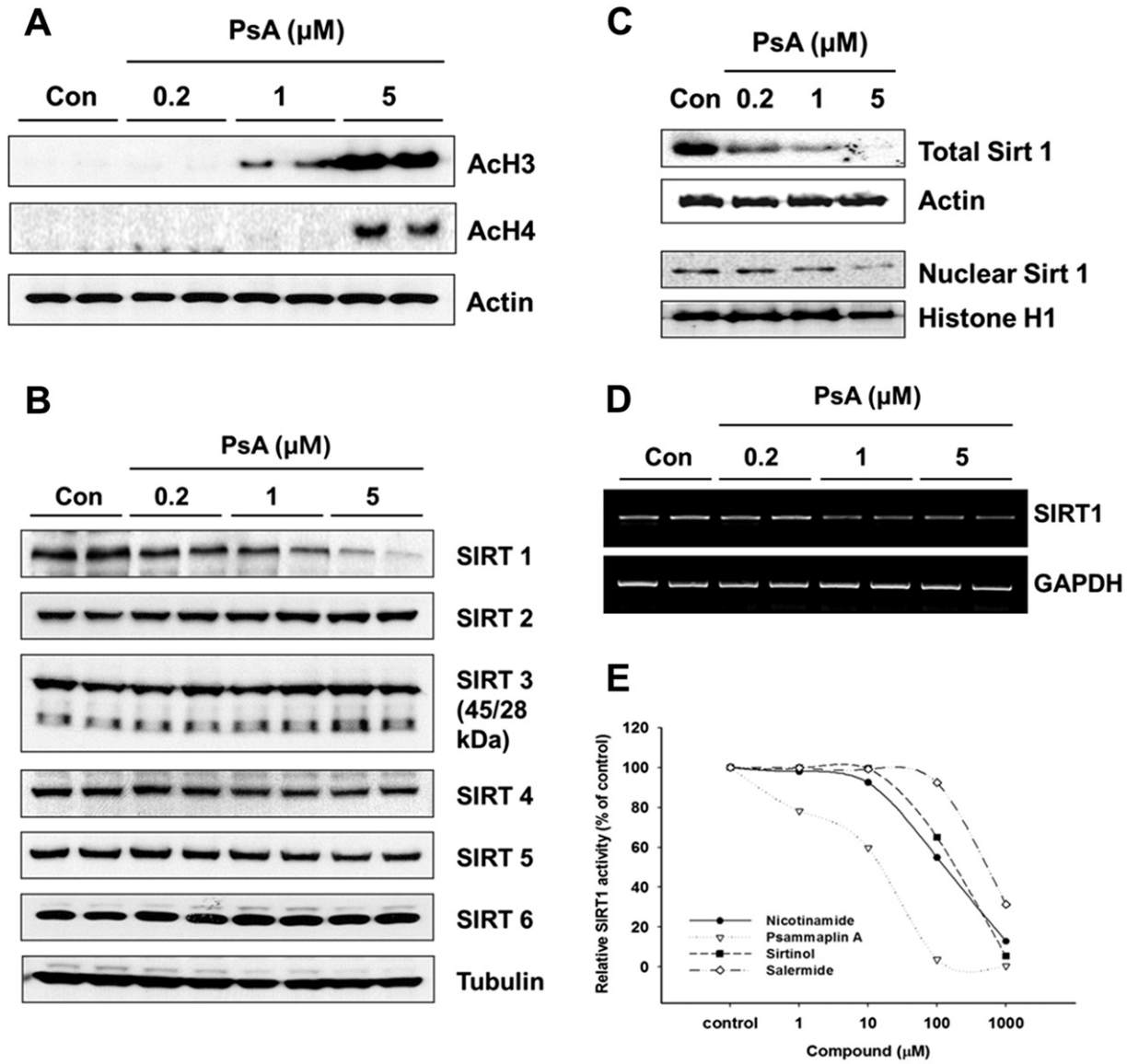

Fig. 5. (A) AcH3 and AcH4 protein levels were determined by Western blotting in MCF-7/adr cells. The cells were treated with the indicated concentrations of PsA for 24 h. Actin was used as an internal loading control. (B) MCF-7/adr cells were treated with PsA for 24 h. Expression of the SIRT proteins was determined by Western blotting. Tubulin was used as an internal loading control. (C) Expression of the SIRT1 protein was detected following PsA treatment in MCF-7/adr cells. Total cell lysates and nuclear cell lysates were prepared and analyzed on Western blots. Actin and Histone H1 were used as internal loading controls. (D) MCF-7/adr cells were treated with PsA for 24 h. Total RNA was isolated as described in Materials and methods, and mRNA expression levels were determined by RT-PCR. GAPDH was used as a housekeeping control gene. The data are representative of three independent experiments. (E) SIRT1 activity was measured using a fluorimetric assay. Relative activities of the experimental groups are shown, as compared to the control group. Nicotinamide, sirtinol and salermide were used as SIRT1 inhibitor controls.

deacetylation activity, the inhibitory effect of PsA may be higher than that of other SIRT1 inhibitors, and thus PsA may find application in treating SIRT1 over-expressing cancers.

Accordingly, we tested the effect of PsA on Dox-resistant human breast cancer cells. It is noteworthy that the cytotoxic effect of PsA is particularly strong in MCF-7/adr cells compared to other cancer cells, including wild-type MCF-7 cells. However, no previous study has tested the effect of a SIRT1 inhibitor on the response to chemotherapy in MCF-7/adr cells. In this study, PsA treatment reduced expression of both SIRT1 protein and mRNA. We also showed for the first time an association between SIRT1 expression and p53 acetylation status in chemotherapy-resistant cancer cells. In agreement with previous studies, we found high levels of SIRT1 expression in drug-resistant MCF-7/adr cells comparable to SIRT1 levels in wild type MCF-7 cells. In vitro, the reduction in SIRT1 expression induced by PsA treatment significantly increased chemosensitivity of MCF-7/adr cells. This is consistent with a previous study showing a significant association between SIRT1 overexpression and resistance to chemotherapeutical agents [47]. Silencing of SIRT may also enhance sensitivity to radiation and radiation-induced apoptosis in glioma CD133-positive cells [48]. In addition, SIRT1 knockout cells show greater susceptibility to cell death induction following ionizing radiation and cisplatin treatment [9]. Thus data consistently support targeting of SIRT1 in the development new cancer chemotherapy.

The SIRT1 and p53 proteins are interconnected in cancer biology in that SIRT1 specifically acetylates p53 at Lys382. In cells treated with PsA, the level of p53 diminished and the level of acetyl-p53 increased markedly. In some conditions, SIRT1 inhibition may induce simultaneous increases in p53 and acetylated p53 levels [49–51]. In our study, however, SIRT1 inhibition led to a decrease in cytoplasmic levels of a

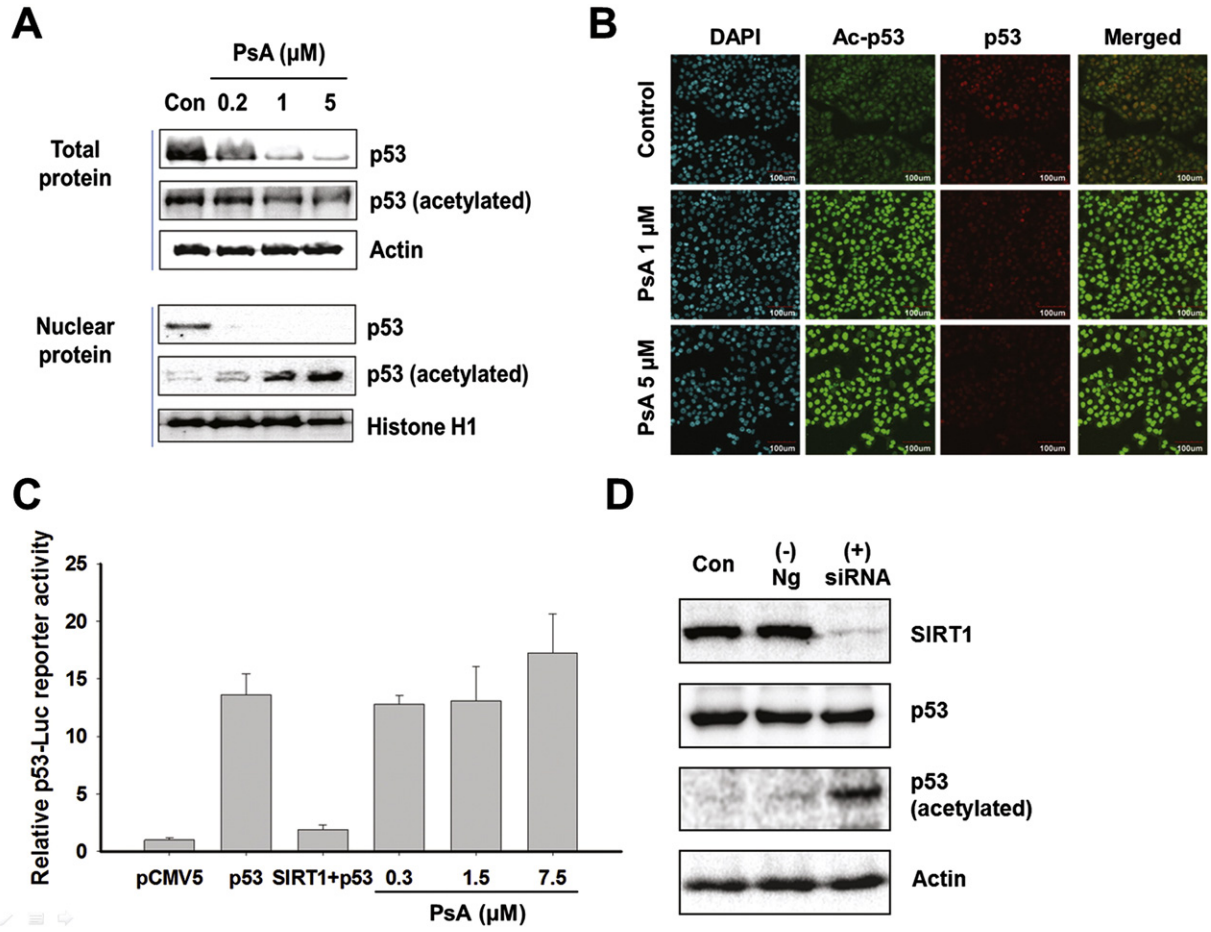

Fig. 6. (A) p53 and acetylated p53 were detected following PsA treatment in MCF-7/adr cells. Total cell lysates and nuclear cell lysates were prepared and analyzed on Western blots. Actin and histone H1 were used as internal loading controls. (B) Representative images of p53 and acetylated p53 staining in MCF-7/adr cells. The cells were treated with PsA at the indicated concentrations for 24 h and counterstained with 4′6-diamidino-2-phenylindole (DAPI). (C) Transcriptional activity of p53 was determined using the p53-responsive luciferase reporter, p53-Luc. Relative luciferase activities were calculated by normalizing the promoter-driven firefly luciferase activity to hRenilla luciferase activity. The data shown are representative of three independent experiments. (D) Cells were treated with non-targeting siRNA or SIRT1-specific siRNA duplexes. All siRNA were used at 100 nM. Con, indicates untreated control; Ng, non-targeting siRNA transfection; siRNA, SIRT1 siRNA transfection. 24 h after transfection, levels of SIRT1, p53 and acetylated p53 proteins in cells treated with the indicated siRNA were determined by Western blotting. Actin was used as an internal loading control.

mutant type of p53, while nuclear levels of the acetyl-p53 increased. We now report a novel mechanism by which SIRT1 might induce autophagy by potentiating transcriptional effects of a mutant type of p53.

Autophagy is a cellular mechanism for degrading intracellular constituents in lysosomes in response to starvation or metabolic stress [52–54], and numerous pathways may converge in its regulation. The tumor suppressor protein p53 is involved in autophagy reportedly as an inhibitor [19,55]. However, p53 is known to participate in autophagy induction by at least two different mechanisms [20,56]. First, p53 may activate AMPK and subsequently inhibit the downstream target mTOR, which is a suppressor of autophagy [57]. Second, p53 may induce autophagy through mediators such as Sestrin2 [21,58], Bax, p53-upregulated modulator of apoptosis (PUMA) [59], and DRAM, a p53 target gene that promotes autophagosome accumulation and triggers autophagy [23].

In the present study, p53 activation had no effect on the physical interaction between Beclin 1 and Bcl-2, indicating that autophagic cell death induced by PsA treatment may depend on another p53-related pathway. Increased DRAM expression was detected by Western blot and immunocytochemical analysis. These results suggest that DRAM activation is required to trigger autophagic cell death following PsA treatment. The anti-tumorigenic effects of PsA were also demonstrated using a xenograft model, in which tumor growth was significantly retarded in PsA-treated groups.

SIRT1 activation enhances autophagy by promoting maturation of autophagosome, and SIRT1 depletion leads to dysfunctional autophagic degradation [60–63]; and it is commonly accepted that these processes also occur in cancer cells. However, the relationship between SIRT1 and autophagy in cancer remains unclear. In this study, we examined the relationship of PsA-induced SIRT1 inhibition and autophagic cell death.

The PsA-induced autophagic cell death appears to depend on p53 activation; and now we have shown that the down-regulation of SIRT1 by PsA is significantly associated with the activation of autophagy. Further study of mechanisms underlying this association may contribute to development of anticancer agents that induce autophagy through SIRT1 inhibition and may be particularly effective against breast cancers that over-express SIRT1.

Acknowledgements

This work was supported by National Research Foundation of Korea (NRF) grants funded by the Korean Government (2013R1A1A2008940).

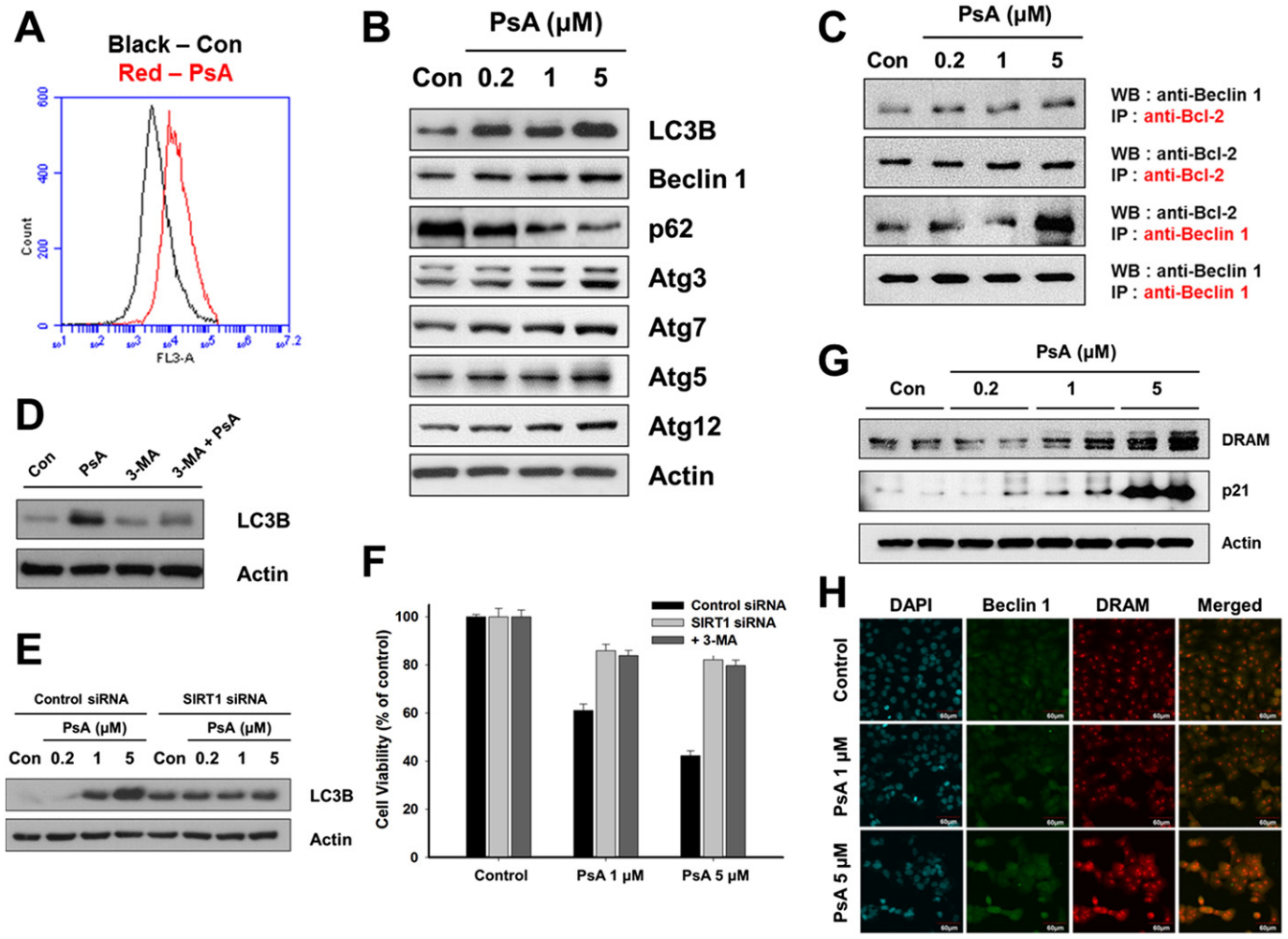

Fig. 7. (A) Acridine orange staining was used as an internal marker of lysosomal acidification. The cells were treated with 5 μM of PsA for 6 h followed by incubation with acridine orange (1 μg/ml) for 15 min at 37 °C. Histograms show fluorescence intensities from the control and PsA-treated cells that were assessed using a flow cytometer. The data shown are representative of three independent experiments. (B) The cells were harvested and analyzed on Western blots using antibodies specific for the following proteins: LC3B, Beclin 1, p62, Atg3, Atg7, Atg12 and Atg5. Actin was used as an internal loading control. (C) Co-immunoprecipitation, using anti-Beclin 1 or anti-Bcl-2 antibodies, from the lysates of MCF-7/adr cells. The lysates were analyzed by immunoblot (WB) assay using anti-Beclin 1 and anti-Bcl-2 antibodies, to detect proteins. (D) The cells were treated with 5 μM PsA or 1 mM 3-methyladenine (3-MA), either alone or in combination. After 24 h treatment, expression level of LC3B was detected. Actin was used as an internal loading control. (E) Expression level of LC3B was detected following PsA treatment in SIRT1 knocked down MCF-7/adr cells. (F) Cell viability of MCF-7/adr cells transfected with a non-targeting siRNA or with a siRNA against SIRT1 or treated with 1 mM of 3-MA. Cells were treated with a combination of PsA for 24 h and the cell viability was measured using the MTT assay. Cell viability (%) was expressed as a percentage compared to the control cells. (G) The cells were treated with indicated concentrations of PsA for 24 h, and the relative levels of DRAM and p21 proteins were determined by Western blot analysis. Representative bands from three independent experiments are shown. (H) Representative images of Beclin 1 and DRAM staining in MCF-7/adr cells. The cells were treated with PsA at the indicated concentrations for 24 h and then counterstained with DAPI.

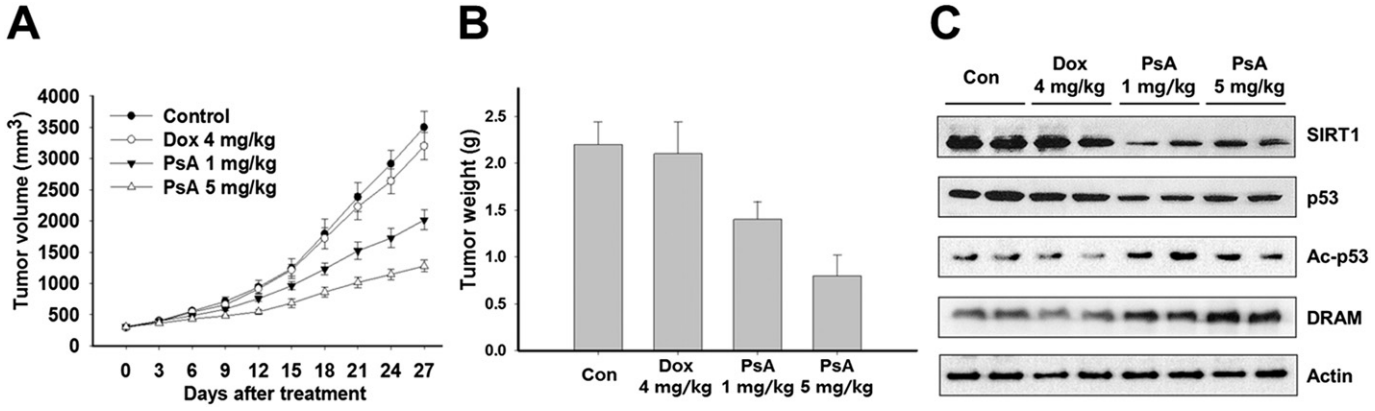

Fig. 8. The effects of PsA and Dox on MCF-7/adr tumor growth in nude mice. Mice with established tumors were randomized into four groups, and Dox (i.p.) or PsA (i.p.) were administered to the tumor-bearing mice. (A) Mean tumor volumes ± SD for each treatment group is indicated. (B) Each bar represents the mean tumor weight. (C) SIRT1, p53, acetylated p53 and DRAM were detected in tumors using Western blot analysis. Representative bands from one of three independent experiments are shown.

References

[1] A. Brunet, L.B. Sweeney, J.F. Sturgill, K.F. Chua, P.L. Greer, Y. Lin, H. Tran, S.E. Ross, R. Mostoslavsky, H.Y. Cohen, L.S. Hu, H.L. Cheng, M.P. Jedrychowski, S.P. Gygi, D.A. Sinclair, F.W. Alt, M.E. Greenberg, Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase, Science 303 (2004) 2011–2015.

[2] M.E. Giannakou, L. Partridge, The interaction between FOXO and SIRT1: tipping the balance towards survival, Trends Cell Biol. 14 (2004) 408–412.

[3] S. Michan, D. Sinclair, Sirtuins in mammals: insights into their biological function, Biochem. J. 404 (2007) 1–13.

[4] H. Vaziri, S.K. Dessain, E. Ng Eaton, S.I. Imai, R.A. Frye, T.K. Pandita, L. Guarente, R.A. Weinberg, hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase, Cell 107 (2001) 149–159.

[5] A.J. Olaharski, J. Rine, B.L. Marshall, J. Babiarz, L. Zhang, E. Verdin, M.T. Smith, The flavoring agent dihydrocoumarin reverses epigenetic silencing and inhibits sirtuin deacetylases, PLoS Genet. 1 (2005) e77.

[6] L. Guarente, Sir2 links chromatin silencing, metabolism, and aging, Genes Dev. 14 (2000) 1021–1026.

[7] S. Nemoto, M.M. Fergusson, T. Finkel, Nutrient availability regulates SIRT1 through a forkhead-dependent pathway, Science 306 (2004) 2105–2108.

[8] M.F. Fraga, R. Agrelo, M. Esteller, Cross-talk between aging and cancer: the epigenetic language, Ann. N. Y. Acad. Sci. 1100 (2007) 60–74.

[9] X.J. Liang, T. Finkel, D.W. Shen, J.J. Yin, A. Aszalos, M.M. Gottesman, SIRT1 contributes in part to cisplatin resistance in cancer cells by altering mitochondrial metabolism, Mol. Cancer Res. 6 (2008) 1499–1506.

[10] C.L. Brooks, W. Gu, How does SIRT1 affect metabolism, senescence and cancer? Nat. Rev. Cancer 9 (2009) 123–128.

[11] J.P. Kruse, W. Gu, Modes of p53 regulation, Cell 137 (2009) 609–622.

[12] J. Luo, A.Y. Nikolaev, S. Imai, D. Chen, F. Su, A. Shiloh, L. Guarente, W. Gu, Negative control of p53 by Sir2alpha promotes cell survival under stress, Cell 107 (2001) 137–148.

[13] K.H. Vousden, D.P. Lane, p53 in health and disease, Nat. Rev. Mol. Cell Biol. 8 (2007) 275–283.

[14] A.J. Levine, M. Oren, The first 30 years of p53: growing ever more complex, Nat. Rev. Cancer 9 (2009) 749–758.

[15] M. Hollstein, D. Sidransky, B. Vogelstein, C.C. Harris, p53 mutations in human cancers, Science 253 (1991) 49–53.

[16] P. Hainaut, M. Hollstein, p53 and human cancer: the first ten thousand mutations, Adv. Cancer Res. 77 (2000) 81–137.

[17] R. Scherz-Shouval, H. Weidberg, C. Gonen, S. Wilder, Z. Elazar, M. Oren, p53-dependent regulation of autophagy protein LC3 supports cancer cell survival under prolonged starvation, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 18511–18516.

[18] E. Morselli, E. Tasdemir, M.C. Maiuri, L. Galluzzi, O. Kepp, A. Criollo, J.M. Vicencio, T. Soussi, G. Kroemer, Mutant p53 protein localized in the cytoplasm inhibits autophagy, Cell Cycle 7 (2008) 3056–3061.

[19] E. Tasdemir, M. Chiara Maiuri, E. Morselli, A. Criollo, M. D'Amelio, M. Djavaheri-Mergny, F. Cecconi, N. Tavernarakis, G. Kroemer, A dual role of p53 in the control of autophagy, Autophagy 4 (2008) 810–814.

[20] X. Sui, L. Jin, X. Huang, S. Geng, C. He, X. Hu, p53 signaling and autophagy in cancer: a revolutionary strategy could be developed for cancer treatment, Autophagy 7 (2011) 565–571.

[21] M.C. Maiuri, S.A. Malik, E. Morselli, O. Kepp, A. Criollo, P.L. Mouchel, R. Carnuccio, G. Kroemer, Stimulation of autophagy by the p53 target gene Sestrin2, Cell Cycle 8 (2009) 1571–1576.

[22] D. Crighton, S. Wilkinson, J. O'Prey, N. Syed, P. Smith, P.R. Harrison, M. Gasco, O. Garrone, T. Crook, K.M. Ryan, DRAM, a p53-induced modulator of autophagy, is critical for apoptosis, Cell 126 (2006) 121–134.

[23] D. Crighton, S. Wilkinson, K.M. Ryan, DRAM links autophagy to p53 and programmed cell death, Autophagy 3 (2007) 72–74.

[24] J. Tan, S. Cang, Y. Ma, R.L. Petrillo, D. Liu, Novel histone deacetylase inhibitors in clinical trials as anti-cancer agents, J. Hematol. Oncol. 3 (2010) 5.

[25] L. Stimson, V. Wood, O. Khan, S. Fotheringham, N.B. La Thangue, HDAC inhibitor-based therapies and haematological malignancy, Ann. Oncol. 20 (2009) 1293–1302.

[26] J.H. Jung, C.J. Sim, C.O. Lee, Cytotoxic compounds from a two-sponge association, J. Nat. Prod. 58 (1995) 1722–1726.

[27] D. Kim, I.S. Lee, J.H. Jung, C.O. Lee, S.U. Choi, Psammaplin A, a natural phenolic compound, has inhibitory effect on human topoisomerase II and is cytotoxic to cancer cells, Anticancer Res. 19 (1999) 4085–4090.

[28] J.N. Tabudravu, V.G. Eijsink, G.W. Gooday, M. Jaspars, D. Komander, M. Legg, B. Synstad, D.M. van Aalten, Psammaplin A, a chitinase inhibitor isolated from the Fijian marine sponge *Aplysinella rhax*, Bioorg. Med. Chem. 10 (2002) 1123–1128.

[29] I.C. Pina, J.T. Gautschi, G.Y. Wang, M.L. Sanders, F.J. Schmitz, D. France, S. Cornell-Kennon, L.C. Sambucetti, S.W. Remiszewski, L.B. Perez, K.W. Bair, P. Crews, Psammaplins from the sponge *Pseudoceratina purpurea*: inhibition of both histone deacetylase and DNA methyltransferase, J. Org. Chem. 68 (2003) 3866–3873.

[30] J.A. Kim, K.B. Cho, M.R. Kim, B.C. Park, S.K. Kim, M.Y. Lee, K.W. Kang, Decreased production of vascular endothelial growth factor in adriamycin-resistant breast cancer cells, Cancer Lett. 268 (2008) 225–232.

[31] M.Y. Ahn, J.H. Jung, Y.J. Na, H.S. Kim, A natural histone deacetylase inhibitor, Psammaplin A, induces cell cycle arrest and apoptosis in human endometrial cancer cells, Gynecol. Oncol. 108 (2008) 27–33.

[32] Y. Kabeya, N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi, T. Yoshimori, LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing, EMBO J. 19 (2000) 5720–5728.

[33] X.H. Liang, S. Jackson, M. Seaman, K. Brown, B. Kempkes, H. Hibshoosh, B. Levine, Induction of autophagy and inhibition of tumorigenesis by beclin 1, Nature 402 (1999) 672–676.

[34] G. Bjorkoy, T. Lamark, A. Brech, H. Outzen, M. Perander, A. Overvatn, H. Stenmark, T. Johansen, p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death, J. Cell Biol. 171 (2005) 603–614.

[35] R. Mathew, C.M. Karp, B. Beaudoin, N. Vuong, G. Chen, H.Y. Chen, K. Bray, A. Reddy, G. Bhanot, C. Gelinas, R.S. Dipaola, V. Karantza-Wadsworth, E. White, Autophagy suppresses tumorigenesis through elimination of p62, Cell 137 (2009) 1062–1075.

[36] Y. Cao, D.J. Klionsky, Physiological functions of Atg6/Beclin 1: a unique autophagy-related protein, Cell Res. 17 (2007) 839–849.

[37] S. Pattingre, A. Tassa, X. Qu, R. Garuti, X.H. Liang, N. Mizushima, M. Packer, M.D. Schneider, B. Levine, Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy, Cell 122 (2005) 927–939.

[38] W.Y. Chen, D.H. Wang, R.C. Yen, J. Luo, W. Gu, S.B. Baylin, Tumor suppressor HIC1 directly regulates SIRT1 to modulate p53-dependent DNA-damage responses, Cell 123 (2005) 437–448.

[39] D.M. Huffman, W.E. Grizzle, M.M. Bamman, J.S. Kim, I.A. Eltoum, A. Elgavish, T.R. Nagy, SIRT1 is significantly elevated in mouse and human prostate cancer, Cancer Res. 67 (2007) 6612–6618.

[40] S. Elangovan, S. Ramachandran, N. Venkatesan, S. Ananth, J.P. Gnana-Prakasam, P.M. Martin, D.D. Browning, P.V. Schoenlein, P.D. Prasad, V. Ganapathy, M. Thangaraju, SIRT1 is essential for oncogenic signaling by estrogen/estrogen receptor alpha in breast cancer, Cancer Res. 71 (2011) 6654–6664.

[41] S. Lavu, O. Boss, P.J. Elliott, P.D. Lambert, Sirtuins—novel therapeutic targets to treat age-associated diseases, Nat. Rev. Drug Discov. 7 (2008) 841–853.

[42] H.Y. Cohen, S. Lavu, K.J. Bitterman, B. Hekking, T.A. Imahiyerobo, C. Miller, R. Frye, H. Ploegh, B.M. Kessler, D.A. Sinclair, Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis, Mol. Cell 13 (2004) 627–638.

[43] J.M. Dai, Z.Y. Wang, D.C. Sun, R.X. Lin, S.Q. Wang, SIRT1 interacts with p73 and suppresses p73-dependent transcriptional activity, J. Cell. Physiol. 210 (2007) 161–166.

[44] B. Peck, C.Y. Chen, K.K. Ho, P. Di Fruscia, S.S. Myatt, R.C. Coombes, M.J. Fuchter, C.D. Hsiao, E.W. Lam, SIRT inhibitors induce cell death and p53 acetylation through targeting both SIRT1 and SIRT2, Mol. Cancer Ther. 9 (2010) 844–855.

[45] V.M. Nayagam, X. Wang, Y.C. Tan, A. Poulsen, K.C. Goh, T. Ng, H. Wang, H.Y. Song, B. Ni, M. Entzeroth, W. Stunkel, SIRT1 modulating compounds from high-throughput screening as anti-inflammatory and insulin-sensitizing agents, J. Biomol. Screen. 11 (2006) 959–967.

[46] B. Heltweg, T. Gatbonton, A.D. Schuler, J. Posakony, H. Li, S. Goehle, R. Kollipara, R.A. Depinho, Y. Gu, J.A. Simon, A. Bedalov, Antitumor activity of a small-molecule inhibitor of human silent information regulator 2 enzymes, Cancer Res. 66 (2006) 4368–4377.

[47] F. Chu, P.M. Chou, X. Zheng, B.L. Mirkin, A. Rebbaa, Control of multidrug resistance gene mdr1 and cancer resistance to chemotherapy by the longevity gene sirt1, Cancer Res. 65 (2005) 10183–10187.

[48] C.J. Chang, C.C. Hsu, M.C. Yung, K.Y. Chen, C. Tzao, W.F. Wu, H.Y. Chou, Y.Y. Lee, K.H. Lu, S.H. Chiou, H.I. Ma, Enhanced radiosensitivity and radiation-induced apoptosis in glioma CD133-positive cells by knockdown of SirT1 expression, Biochem. Biophys. Res. Commun. 380 (2009) 236–242.

[49] A.M. Kalle, A. Mallika, J. Badiger, Alinakhi, P. Talukdar, Sachchidanand, Inhibition of SIRT1 by a small molecule induces apoptosis in breast cancer cells, Biochem. Biophys. Res. Commun. 401 (2010) 13–19.

[50] E. Lara, A. Mai, V. Calvanese, L. Altucci, P. Lopez-Nieva, M.L. Martinez-Chantar, M. Varela-Rey, D. Rotili, A. Nebbioso, S. Ropero, G. Montoya, J. Oyarzabal, S. Velasco, M. Serrano, M. Witt, A. Villar-Garea, A. Imhof, J.M. Mato, M. Esteller, M.F. Fraga, Salermide, a Sirtuin inhibitor with a strong cancer-specific proapoptotic effect, Oncogene 28 (2009) 781–791.

[51] I.W. Jolma, X.Y. Ni, L. Rensing, P. Ruoff, Harmonic oscillations in homeostatic controllers: dynamics of the p53 regulatory system, Biophys. J. 98 (2010) 743–752.

[52] Z. Xie, D.J. Klionsky, Autophagosome formation: core machinery and adaptations, Nat. Cell Biol. 9 (2007) 1102–1109.

[53] N. Mizushima, B. Levine, A.M. Cuervo, D.J. Klionsky, Autophagy fights disease through cellular self-digestion, Nature 451 (2008) 1069–1075.

[54] B. Levine, D.J. Klionsky, Development by self-digestion: molecular mechanisms and biological functions of autophagy, Dev. Cell 6 (2004) 463–477.

[55] E. Tasdemir, M.C. Maiuri, L. Galluzzi, I. Vitale, M. Djavaheri-Mergny, M. D'Amelio, A. Criollo, E. Morselli, C. Zhu, F. Harper, U. Nannmark, C. Samara, P. Pinton, J.M. Vicencio, R. Carnuccio, U.M. Moll, F. Madeo, P. Paterlini-Brechot, R. Rizzuto, G. Szabadkai, G. Pierron, K. Blomgren, N. Tavernarakis, P. Codogno, F. Cecconi, G. Kroemer, Regulation of autophagy by cytoplasmic p53, Nat. Cell Biol. 10 (2008) 676–687.

[56] M.C. Maiuri, L. Galluzzi, E. Morselli, O. Kepp, S.A. Malik, G. Kroemer, Autophagy regulation by p53, Curr. Opin. Cell Biol. 22 (2010) 181–185.

[57] N. Chen, V. Karantza-Wadsworth, Role and regulation of autophagy in cancer, Biochim. Biophys. Acta 1793 (2009) 1516–1523.

[58] M. D'Amelio, F. Cecconi, A novel player in the p53-mediated autophagy: Sestrin2, Cell Cycle 8 (2009) 1467.

[59] K.S. Yee, S. Wilkinson, J. James, K.M. Ryan, K.H. Vousden, PUMA- and Bax-induced autophagy contributes to apoptosis, Cell Death Differ. 16 (2009) 1135–1145.

[60] M.J. Powell, M.C. Casimiro, C. Cordon-Cardo, X. He, W.S. Yeow, C. Wang, P.A. McCue, M.W. McBurney, R.G. Pestell, Disruption of a Sirt1-dependent autophagy checkpoint

in the prostate results in prostatic intraepithelial neoplasia lesion formation, Cancer Res. 71 (2011) 964–975.
[61] N. Hariharan, Y. Maejima, J. Nakae, J. Paik, R.A. Depinho, J. Sadoshima, Deacetylation of FoxO by Sirt1 plays an essential role in mediating starvation-induced autophagy in cardiac myocytes, Circ. Res. 107 (2010) 1470–1482.
[62] A. Salminen, K. Kaarniranta, SIRT1: regulation of longevity via autophagy, Cell. Signal. 21 (2009) 1356–1360.
[63] I.H. Lee, L. Cao, R. Mostoslavsky, D.B. Lombard, J. Liu, N.E. Bruns, M. Tsokos, F.W. Alt, T. Finkel, A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 3374–3379.