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석 사 학 위 논 문

HSP90 Specific Inhibitor, PSY-2-12, Ameliorates Cellular Senescence via Activating Autophagy: a Poteintial Senolytic Drug Against Acute Kidney Injury

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이 논문을 석사학위 논문으로 제출함

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1. Introduction

Cellular senescence (CS), first discovered in 1961 by Hayflick and Moorhead, is the process of a cell permanently stopping division and entering a state of growth arrest without completely dying. This occurs in response to various stresses, both intrinsic and extrinsic, including DNA damage, mitochondrial dysfunction, and oxidative stresses and ER stresses (1,2). During senescence, cells even change morphology and exhibit a characteristic secretome termed the senescence-associated secretory phenotype (SASP) that reinforces the CS of senescent cells in an autocrine manner and induces the senescence of surrounding cells in a paracrine manner (3,4). Recently, there has been a focus on senotherapeutics aimed at addressing CS in the context of aging research. These therapeutics are broadly classified into two categories: senomorphic and senolytic drugs. The key difference between these two categories is that senolytics selectively target and eliminate senescent cells (5).

Cisplatin (CP) is an efficacious chemotherapeutic drug for various cancer treatment, but its usage is limited due to adverse effects, particularly nephrotoxicity (6,7). Under specific conditions, CP induced tubular cellular damage and senescence due to the repair phase (8). These mechanisms are believed to represent the key detrimental processes driving the progression from acute kidney injury (AKI), characterized by a reduction in kidney function, possibility of primary harmful processes of chronic kidney disease (9,10). This study utilizes CP to create a model of CS and AKI to evaluate the potential senolytic effect.

Heat shock protein 90 (HSP90) is a molecular chaperone, main

function is remodeling misfolded proteins, as well as ligand binding under various conditions, whether physiological or pathological (11). Since HSP90 has hundreds of substrate proteins, it regulates a myriad of cellular processes: proliferation, survival, cell cycle, hormone signaling, and others. Understandably, HSP90 protein function is well known to be involved in many diseases, including infectious diseases and neurodegenerative diseases (12,13). Additionally, HSP90 is a promising target for treating various diseases, especially in cancer therapy, as numerous studies have shown the effectiveness of HSP90 inhibitors in chemotherapy (14,15). The unexpected role of HSP90 inhibitors as a new class of senolytics has been identified, showing significant senolytic activity in a mouse model of a human progeroid syndrome (16).

In this study, PSY-2-12 (PSY), an HSP90 specific inhibitor, is proposed to ameliorate or delay the progression of AKI through its senolytic activity. The anti-senescence effect of PSY demonstrated at a non-toxic concentration significantly attenuated CS in both CP-induced senescent kidney proximal tubular cells and angiotensin II (AngII)-induced senescent human umbilical vein endothelial cells . Furthermore, the senolytic effect of PSY was validated in a mouse model of CP-induced AKI.

2. Materials and Methods

2.1 Synthesis of PSY:

The PSY compound, an HSP90-specific inhibitor, was developed by Professor Young Ho Seo from the College of Pharmacy, Keimyung University. Following the serial reaction, N-methyl benzylamine was synthesized through the reductive amination of benzaldehyde using methyl amine and sodium borohydride, achieving a yield of 55%. Concurrently, commercially available 2,4-dihydroxybenzoic acid was esterified, resulting in an ester with an 86% yield. Next, a 5'-chloro substituent derivative was obtained by treating the ester with sulfonyl chloride in dichloromethane, yielding 76%. This was followed by hydrolysis with lithium hydroxide, converting the ester functional group into a carboxylic acid with a yield of 94%. Finally, the targeted HSP90-specific inhibitor PSY was synthesized through an amide coupling reaction, resulting in a yield of 32%.

2.2 Cell culture:

Human proximal tubular HK-2 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640, WELGENE, Gyeongsan, Korea) with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic from Gibco (Grand Island, NY, USA) at 37 °C, 5% CO₂. Human umbilical vein endothelial cells (HUVECs) were purchased from Sigma-Aldrich (Seoul, Korea) and were cultured in 0.4%

gelatin-coating plates with endothelial cell growth medium-2 (ECGM-2, Lonza, Bend, OR, USA) containing EGMTM-2 Single Quots[™] Supplements #CC-4176 (Lonza, Bend, OR, USA). For the senescence study model, HK-2 cells pre-treated with PSY or geldanamycin (GA) (MedChemExpress, Monmouth Junction, NJ, USA) at non-cytotoxic concentrations and then treated with cisplatin (CP)-induces senescent HK-2 and Angiotensin-II (AngII)-induces senescent HUVECs. Both CP and AngII were purchased from Sigma-Aldrich (Seoul, Korea)

2.3 Animals study:

Male C57BL/6J mice (8 weeks old, weighing 23 ± 1.0 g) were maintained according to the institutional guidelines of Keimyung University, School of Medicine. All animal studies were carried out according to the approved experimental protocol (KM-2022-19R1). Mice were randomly grouped (5 mice per group) and adapted for 1 week in individually ventilated cages (25 ± 2 °C, $50 \pm 10\%$ relative humidity, and a 12-hour light-dark rhythm). PSY was prepared freshly in 5% DMSO-corn oil from Sigma-Aldrich (Seoul, Korea) with a final concentration at 20 mg/kg, and then daily injected intraperitoneally (i.p.) for 7 days in a 25 mg/kg CP-induced AKI model. After sampling, blood collected from coronary veins was used to determine renal function status, the kidney was embedded in paraffin for immunohistochemistry.

2.4 Senescence associated- β -galactosidase (SA- β -Gal) staining:

SA- β -Gal staining solution was prepared by diluting 4% X-Gal (Sigma-Aldrich, Seoul, Korea) in β -Gal buffer with a ratio of 1:40. Cells were seeded into 6 well plates (1.5×10^4 cells/well), washed twice with 1X phosphate-buffered saline (PBS) (pH=7.4), and fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature (RT). Cells were then stained in SA- β -Gal staining solution (pH 6.0) for 16 hours at 37 °C. Cryo-section of kidney tissue (10 μ m thick), was embedded in OCT, and fixed in 4% PFA for 10 minutes, followed by washing 3 times in PBS and overnight incubating at 37 °C in SA- β -Gal staining solution (pH 4.5). Positive SA- β -Gal (blue) staining was detected by light microscopy (DM750, Leica, Wetzlar, Germany). and counted by ImageJ (National Institutes of Health, Bethesda, MD, USA) for quantification.

2.5 Cell viability:

Cell viability was assessed using the Cell Counting Kit 8 (CCK-8, Dogindo, Nagasaki, Japan) following the manufacturer's instructions. For cytotoxicity analysis, cells were seeded 5×10^3 cell/well. Additionally, growth curve analysis was performed in 96-well plates (2×10^3 cells/well), which were treated with CP, GA, or PSY for the specified time points.

2.6 Western immunoassay:

Proteins were extracted from cell cultures and frozen renal tissue using radioimmunoprecipitation (RIPA) lysis buffer containing 1% protease inhibitor cocktail (100X) and 0.2 nM phenylmethylsulfonyl fluoride (PMSF). The protein concentration was measured using a BCA kit assay (Thermo Fisher Scientific, Boston, MA, USA). Following lysis, 30 µg of protein was loaded onto a SDS-PAGE gel, separated by electrophoresis, and then transferred to a nitrocellulose membrane (GE Healthcare, Little Chalfont, UK). The membrane was blocked for 30 minutes with 5% skim milk in 1X TBS-T and then incubated overnight at 4 °C with anti-HSP90α, anti-HSP70, anti-p53, anti-p21, anti-p62, anti-beclin-1, anti-LC3I/II (1:1,000 dilution) from Cell Signaling Technology company (Danvers, MA, USA) and anti-p16, anti-lamin-b1 (1:1,000 dilution) from Abchem (Cambridge, UK), followed by horseradish peroxidase-conjugate secondary antibody (1:5,000 dilution, Cell Signalling Technology, MA, USA) incubation. For the frozen renal tissue, 15 µg of protein was loaded onto an SDS-PAGE gel. Detection of protein bands were applied with the Super Signal West Pico or Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Boston, MA, USA) and observed under the Bio-Rad ChemiDoc™ MP Imaging System (Hercules, CA, USA).

2.7 Immunofluorescence staining (IF):

Cells were seeded into a 24-well plate (5×10^4 cells/well) and fixed with 4% PFA overnight at 4 °C. They were then permeabilized with 0.1% Triton X-100 in 1X PBS, blocked for 30 minutes with 1% bovine serum albumin (BSA) in PBS-Tween at RT, and incubated

with anti-lamin b1 (1:1,000 dilution, Abchem, Cambridge, UK) or anti-LC3B (1:500 dilution, Cell Signalling Technology, Danvers, MA, USA) overnight at 4 °C. Afterwards, the cells were incubated with Alexa fluor 488-conjugated goat anti-rabbit IgG (1:800 dilution, Thermo Scientific, Boston, MA, USA) for 1 hour at RT. The slides were rinsed twice in 1X PBS and stained with 0.5 µg/mL 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, Waltham, CA, USA) for 10-15 minutes. The mounted slides were then analyzed using the Carl Zeiss LSM5 EXCITER fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

2.8 Quantitative real-time polymerase reaction (PCR):

Trizol (Invitrogen, Waltham, CA, USA) using for RNA extraction from cells and tissues and then reverse transcribed into cDNA according to the manufacturer's protocol. The PCR reaction mixture consisted of SYBR Green PCR Master Mix (TOYOBO, Osaka, Japan), diethyl pyrocarbonate (DEPC) water (Sigma-Aldrich, Seoul, Korea), 10 pmol/µL forward and reverse primers (sequences are shown in Table 1), and template cDNA. The Real time-PCR reaction was performed using a LightCycler® 480 (Roche, Mannheim, Germany) instrument.

2.9 Cell cycle analysis:

Flow cytometry was used to assess the distribution of the cell cycle. HK-2 cells were fixed in ice-cold 70% ethanol-PBS at 4 °C overnight. Then, the fixed cells were washed twice in cold 1X PBS

and stained with a mixture consisting of 0.1 mg/mL propidium iodide (PI) and 0.2 mg/mL ribonuclease A (Thermo Fisher Scientific, Boston, MA, USA), followed by incubation for 15 minutes at 37 °C. The cell cycle was then analyzed by a FACS Canto II flow cytometer (BD, Franklin Lakes, NJ, USA).

2.10 Immunohistochemistry (IHC):

IHC was performed on 4 µm paraffin sections. Paraffin slides were deparaffinized and rehydrated in serially diluted xylene-ethanol solutions and antigen-retrieved in citrate buffer (pH 6.0). The slides were then blocked for 1 hour with 5% goat serum at RT and incubated with anti-KIM-1 (1:500 dilution), anti-p21 (1:400 dilution) and anti-p16 (1:400 dilution) from Cell Signaling Technology company (Danvers, MA, USA) overnight at 4 °C, followed by incubation with anti-rabbit IgG (1:200 dilution, Cell Signalling Technology, MA, USA) at RT for 1 hour. The positive signal was generated using a diaminobenzidine chromogen (DAB) substrate kit (Vector Laboratories, Burlingame, CA, USA), and counterstained with hematoxylin (Dako, Glostrup, Denmark). The stained slide was mounted and observed under light microscopy (DM750, Leica, Wetzlar, Germany).

2.11 Renal function assessment:

The renal functional assessment was conducted by measuring the levels of urea, creatinine, and kidney injury molecule-1 (KIM-1) in the plasma. Urea and creatinine levels were measured using a

Colorimetric Urea Assay Kit and a Creatinine Assay Kit (Abchem, Cambridge, UK). The level of KIM-1 was determined using a TIM-1 ELISA Kit (Abchem, Cambridge, UK)

2.12 siRNA transfection:

One day before transfection, cells were seeded at 50% confluency and then incubated with 30 nM siRNA in 1.2 μ L/mL of Oligofectamine (Invitrogen, Carlsbad, CA, USA) in Opti-MEM for 48 hours. The si-negative control (NC), (Forward: 5'-ACGUGACACGUUCGGA GAA(UU) -3'; Reverse: 5'-UUCUCCGAACGUGUCACGU-3') which does not target any endogenous transcripts, as a control group. The variants of BECN1-specific siRNAs (Forward: 5'- CCGACUUGU UCCUUACGG -3'; Reverse: 5'- UCCGUAAGGAACAAGUCG -3') (Bioneer Inc, Daejeon, Korea).

2.13 Statistical analysis:

All data were obtained from at least three independent experiments with triplicate sets. Data were analyzed using GraphPad Prism 8 (GraphPad, Boston, MA, USA) and presented as the mean \pm SD. Image intensity was quantified using the ImageJ program. Statistical analyses were performed using an unpaired two-tailed Student's *t*-test, with significance set at $p < 0.05$.

Table 1. Details of Primer Pair Sequences

Species	Name	Sequences of primers
Human	IL-1 α	Forward : 5' - TGATCAGTACCTCACGGCTG - 3'
		Reverse : 5' - TGGTCTTCATCTTGGGCAGT - 3'
	IL-1 β	Forward : 5' - CTGTCCTGCGTGTTGAAAGA - 3'
		Reverse : 5' - TTGGGTAATTTTGGGATCT - 3'
	IL-6	Forward : 5' - TACCCAGGAGGAGAAGATTC - 3'
		Reverse : 5' - GGTTGTTTTCTGCCAGTGCC - 3'
	IL-8	Forward : 5' - TTCAGCGCCAGCAGAGCAC - 3'
		Reverse : 5' - AGCACTCCTTGGA AAACTG - 3'
	β -actin	Forward : 5' - GATTCCTATGTGGGCGAGGA - 3'
		Reverse : 5' - AGGTCTAACAACATGATCT - 3'
Mouse	IL-1 α	Forward : 5' - AGCAGCCTTATTTCCGGGAGT - 3'
		Reverse : 5' - ATCATATGTCCGGGTGGCTG - 3'
	IL-1 β	Forward : 5' - GCCCATCCTCTGTGACTCAT - 3'
		Reverse : 5' - AGGCCACAGGTATTTTGTCG - 3'
	IL-6	Forward : 5' - TCCCACGATTTCAGAGAA - 3'
		Reverse : 5' - CCTTCTTGGGACTGATGGTG - 3'
	GAPDH	Forward : 5' - ACTCCCACTCCACGGCAAA - 3'
		Reverse : 5' - TCTCCATGGTGGTGAAGACA - 3'

IL-1 α : Interleukin 1-alpha; IL-1 β : Interleukin 1-beta; IL-6:
 Interleukin 6; IL-8: Interleukin 8.

3. Results

3.1 PSY attenuates cellular senescence in CP-induced senescent in HK-2 cells:

Since the synthesis of HSP90 inhibitors, PSY and GA, are well-known as chemotherapeutic drugs, a cytotoxic assay was performed to evaluate the non-cytotoxic conditions for application in 5 μ M CP-induced senescent HK-2 (Figure 1A&B). Following the experimental scheme (Figure 2A). Then cells were pre-treated with 0.5 and 1.0 μ M PSY or 0.5 μ M GA for 4 hours, and then treated with optimized concentration CP for 24 hours.

To determine the anti-senescent effect of PSY, SA- β -Gal activity and other senescent markers were measured. Consistent with positive control GA treatment, results showed that PSY treatment at non-cytotoxic concentrations (0.5 and 1.0 μ M) significantly decreased SA- β -Gal staining in CP-induced senescence in HK-2 cells (Figure 2B). Additionally, there was an increase in the level of lamin B1, a nuclear molecule known to decrease in senescent cells (17), and reduces senescence-associated marker (p53, p21 and p16) (Figure 2C). Since PSY is an HSP90 inhibitor, its general function is to delay the activity of HSP90 rather than degrade protein expression. HSP70 is known for its co-chaperone activity with HSP90 (18,19) and its expression increased in both GA and PSY treatments, indicating that the HSP90 inhibitor treatment was effective (Figure 2C). Cell cycle analysis in CP-induced senescent HK-2 cells showed that PSY shortened the G1/G0 phases compared

to the prolonged G1/G0 phase induced by CP (Figure 2D). Additionally, GA and PSY also increased cell proliferation at non-cytotoxic concentrations (Figure 2E). These results collectively indicate that PSY, at non-cytotoxic concentration, exerts an anti-senescent effect in HK-2 cells.

3.2 PSY downregulates senescence-associated secretory phenotype (SASP) genes:

Since the senolytic drug not only reduced CS but also reduced SASP genes, also termed as senescence-messaging secretome (5), is a phenotype that senescent cells develop to act on neighboring prosenescent cell to become senescent. Then Real time-PCR result of interleukin (IL)-1 α , IL-1 β , IL-6 and IL-8 shown almost genes decreasing significantly in GA or PSY treatment, except IL-1 β and IL-8, in CP-induced senescence on HK-2 cells (Figure 3A-D).

3.3 PSY ameliorates cellular senescence in AngII-induced senescent in HUVEC cells:

Next, primary cell HUVEC was used to validate and confirm the anti-senescence effect of PSY. Following the experimental scheme (Figure 4A), 2.0 μ M angiotensin-II (AngII) was used to induce senescence at 24 hours, then treated with 1.0 μ M PSY or 0.5 μ M GA at the next 24 hours. The results showed that PSY markedly diminished SA- β -Gal activity (Figure 4B) and also reversed AngII-induced changes in senescence markers (decreased lamin B1

and increased p53, p21 levels) (Figure 4C). Lastly, GA and PSY down-regulated SASP genes, which were significantly different compared to AngII-induced senescent HUVECs (Figure 4D). Overall, these findings suggest that PSY acts as a senolytic agent, similar to GA in HUVECs.

3.4 PSY attenuates CS via regulating beclin-1 mediated autophagy:

Following a previous study that established a relationship between HSP90 inhibitors and autophagy (20,21). PSY or GA were pre-treated in a CP-induced senescence model in HK-2 and determined autophagy markers (p65, beclin-1, and LC3 II/I) by western blot and under immunofluorescent imaging. PSY treatment dramatically increased beclin-1 and the ratio of LC3 II/I, indicating a transient increase in exercise-induced autophagy (22) (Figure 5A&B). Moreover, positive LC3B detected from confocal fluorescence microscopy (Figure 5C) were consistent with the western blot results, showing that autophagy was activated during PSY treatment.

Next, to validate whether autophagy reduced CS in HK-2, 20 nM bafilomycin A1 (Baf), an autophagy inhibitor, was used in combination treatment with PSY or GA. Results showed that the autophagy inhibitor delayed the anti-senescent effect of PSY, with no change in SA- β -Gal activity (Figure 6A) and a rapid decrease in positive lamin B1 in combination with Baf and PSY or GA (Figure 6B). After evaluating the relationship between autophagy and the reduction of CS, beclin-1 was knocked down using siRNA (Figure

7A), and determined senescence-associated markers. Upon knockdown of beclin-1, the results showed decreased the protein level of lamin B1 and increased p21 (Figure 7B), and also the SA- β -Gal activity was also similar to that of CP-induced senescent HK-2 (Figure 7C), clarifying that the anti-senescent effect of PSY was blocked. The results confirm again that the knockdown of beclin-1 did not attenuate CP-induced senescent HK-2. It is possible to conclude that PSY reduced CP-induced cellular senescence via beclin-1-mediated autophagy (Figure 7D).

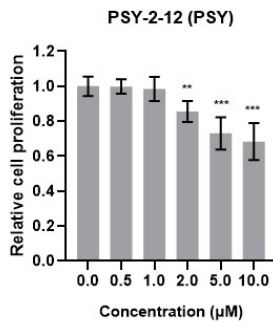
3.5 PSY ameliorates CP-induced AKI *in vivo* via suppressing renal senescence:

To investigate whether the *in vitro* results in the current study are recapitulated *in vivo*, 20 mg/kg of PSY was administered i.p. daily in a CP-induced AKI mouse model (Figure 8A). Renal function markers, serum of urea and creatinine, increased due to CP injection, while PSY led to a reduction in the levels of urea and creatinine (Figure 8B), and also decreased the level of KIM-1 in plasma (Figure 8C) and positive KIM-1 in renal sections (Figure 8D), a specific kidney injury marker well-known as kidney injury molecule-1 (23). These results show that PSY ameliorates CP-induced AKI.

Based on the assessment of SA- β -Gal activity, senescence markers (p21, p16), and SASP genes in the renal section, the results demonstrated a significant increase in samples injected with CP, indicating that CP-induced AKI enhances renal senescence. Furthermore, the results suggest that PSY, which exhibits a

senolytic effect *in vitro*, also demonstrates this effect *in vivo*, as evidenced by a reduction in SA- β -Gal activity. (Figure 8E), increased lamin B1, and senescence-induced markers (p21, p16) in the renal section (Figures 8F&G). Lastly, treatment with PSY decreased mRNA expression of SASP in CP-induced AKI (Figure 8H). Finally, these results coordinate with PSY suppressing renal senescence and protection from CP-induced AKI (Figure 9).

A



B

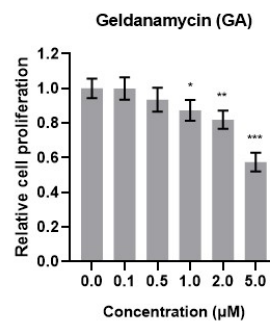
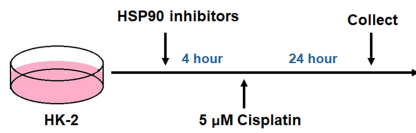
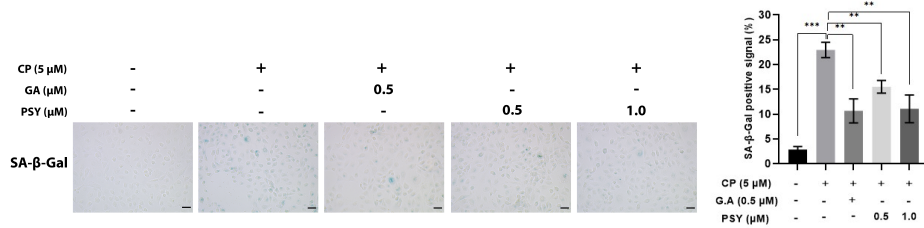


Figure 1. Assessment of the drugs cytotoxicity on HK-2 cells. HK-2 cells were treatment over 24 hour period, cell viability was evaluated via the CCK-8 assay. (A) PSY treatment in serial concentration. (B) GA treatment in serial concentration. Experiment were performed at least three times independently. Data present as mean \pm SD (n = 5). Significant difference are indicated: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. CCK-8: Cell Counting Kit-8; PSY: PSY-2-12; GA: geldanamycin.

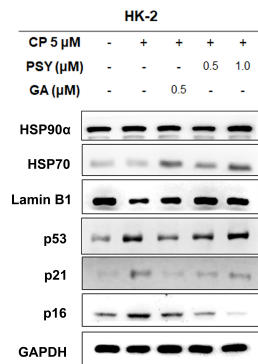
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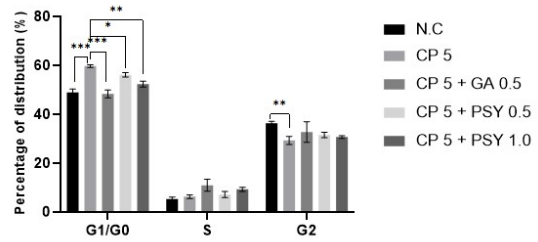
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C



D



E

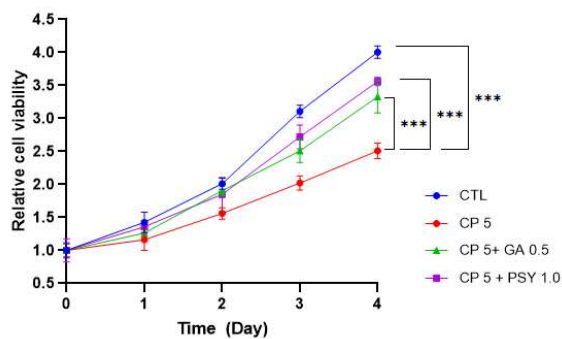


Figure 2. Effect of PSY on anti-senescence in CP-treated HK-2 cells. HK-2 cells were pre-treatment PSY or GA in CP-induced cellular senescence model. (A) Illustration of experiment schematic. (B) SA- β -Gal staining in HK-2 cell. Result performed via light microscope (original magnification x 20; scale bar 100 μ m). Quantification signal by counting the percentage of positive cells (blue) per image. (C) Western blot analysis of senescence marker (p53, p21 and p16) as well as lamin b1, HSP90 α and HSP70 expression in HK-2 cells. (D) Cell cycle analysis by flow cytometer. (E) Cell proliferation assay performed daily using CCK-8. HK-2 pre-treated 0.5 μ M GA or 1.0 μ M PSY on CP-induces senescent at 24 hour. Experiments were performed at least three times independently. Data present as mean \pm SD (n = 3). Significant difference are indicated: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. HSP90: heat shock protein 90; SA- β -Gal: senescence-associated β -galactosidase; p53: tumour protein 53; p21: cyclin-dependent kinase inhibitor 1A; p16: cyclin-dependent kinase inhibitor 2A; CCK-8: Cell Counting Kit-8; CP: cisplatin; PSY: PSY-2-12; GA: geldanamycin.

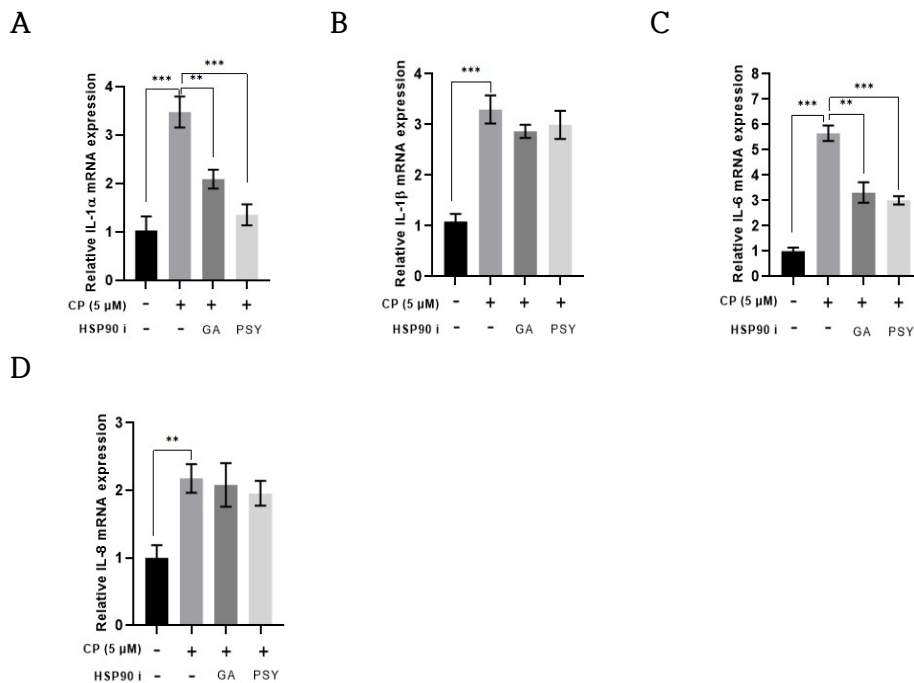
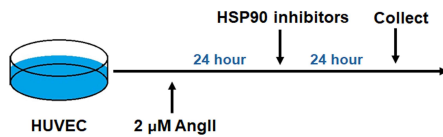
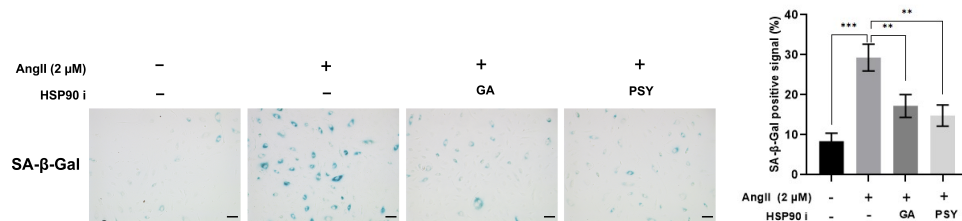


Figure 3. Real time-PCR analysis of SASP genes on CP-induced cellular senescence. HK-2 cells were pre-treated 1.0 μM PSY or 0.5 μM GA in 5 μM CP-induced cellular senescence with same experimental scheme. (A) Relative mRNA expression of IL-1α. (B) Relative mRNA expression of IL-1β. (C) Relative mRNA expression of IL-6. (D) Relative mRNA expression of IL-8. mRNA expression normalized with β-actin. Experiments were performed at least three times independently. Data present as mean ± SD (n = 3). Significant difference are indicated: **: $p < 0.01$, ***: $p < 0.001$. SASP: senescence-associated secretory phenotype; IL-1α: Interleukin 1-alpha; IL-1β: Interleukin 1-beta; IL-6: Interleukin 6; IL-8: Interleukin 8; CP: cisplatin; PSY: PSY-2-12; GA: geldanamycin.

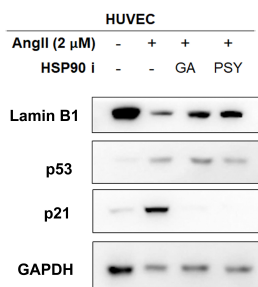
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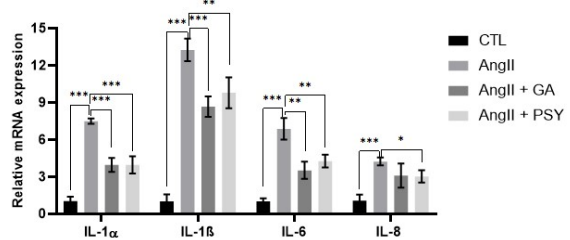
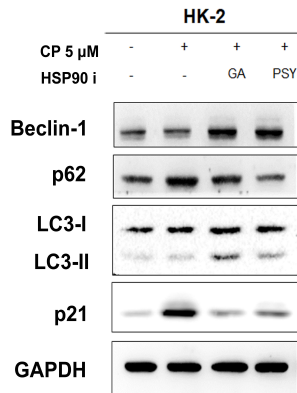


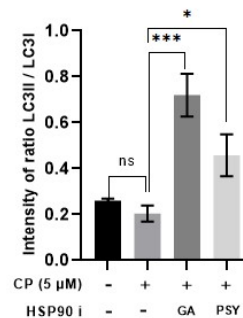
Figure 4. Effect of PSY on anti-senescence in AngII-treatment on HUVECs. Primary HUVECs were treated with 1.0 μM PSY or 0.5 μM GA after treated with 2 μM AngII-induced cellular senescence model. (A) Illustration of experiment schematic. (B) SA-β-Gal staining in HUVECs. Result performed via light microscope (original magnification x 20; scale bar 100 μm). Quantification signal by counting the percentage of positive cells (blue) per image. (C) Western blot analysis of senescence markers (p53 and

p21) as well as lamin b1 expression in HUVECs. (D) Real time-PCR analysis performed in HUVECs. mRNA of SASP genes expression were normalized with β -actin. Experiments were performed at least three times independently. Data present as mean \pm SD (n = 3). Significant difference are indicated: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. AngII: angiotensin-II; SA- β -Gal: senescence-associated β -galactosidase; p53: tumour protein 53; p21: cyclin-dependent kinase inhibitor 1A; IL-1 α : Interleukin 1-alpha; IL-1 β : Interleukin 1-beta; IL-6: Interleukin 6; IL-8: Interleukin 8; PSY: PSY-2-12; GA: geldanamycin. HUVECs: human umbilical vein endothelial cells.

A



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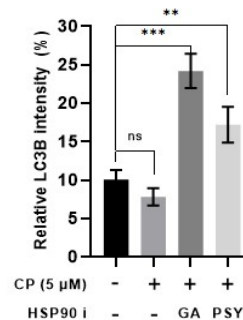
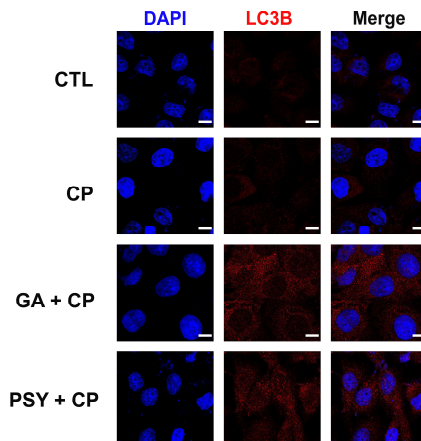
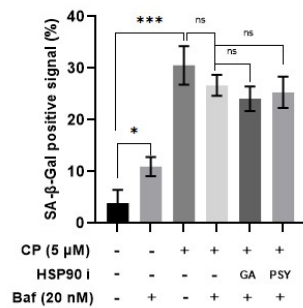
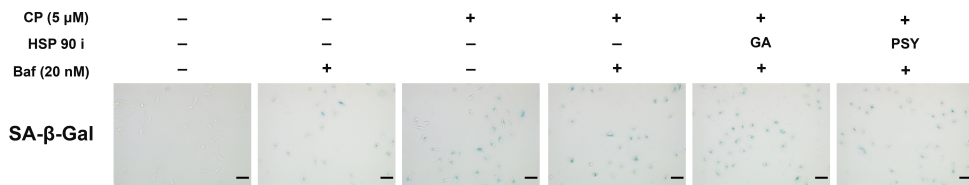


Figure 5. Analysis of autophagy expression in PSY reduces CP-induced senescent HK-2 cells. HK-2 cells were pre-treated with 1.0 μ M PSY or 0.5 μ M GA in 5 μ M CP-induced cellular senescence at 24 hour. (A) Western blot analysis of autophagy markers (p62 beclin-1, and LC3I/II), as well as p21 protein expression. (B) Quantification of western blot results of LC3II over LC3I by using ImageJ software. (C) Positive LC3B (scale bar 20

μm) was detected via confocal fluorescent microscopy and quantify intensity by ImageJ software. Experiments were performed at least three times independently. Data present as mean ± SD (n = 3). Significant difference are indicated: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant. p62: sequestosome 1; LC3: Microtubule-associated protein 1A/1B-light chain 3; p21: cyclin-dependent kinase inhibitor 1A; CP: cisplatin; PSY: PSY-2-12; GA: geldanamycin; DAPI: 4',6-diamidino-2-phenylindole.

A



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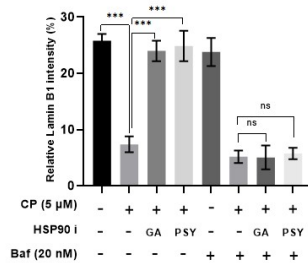
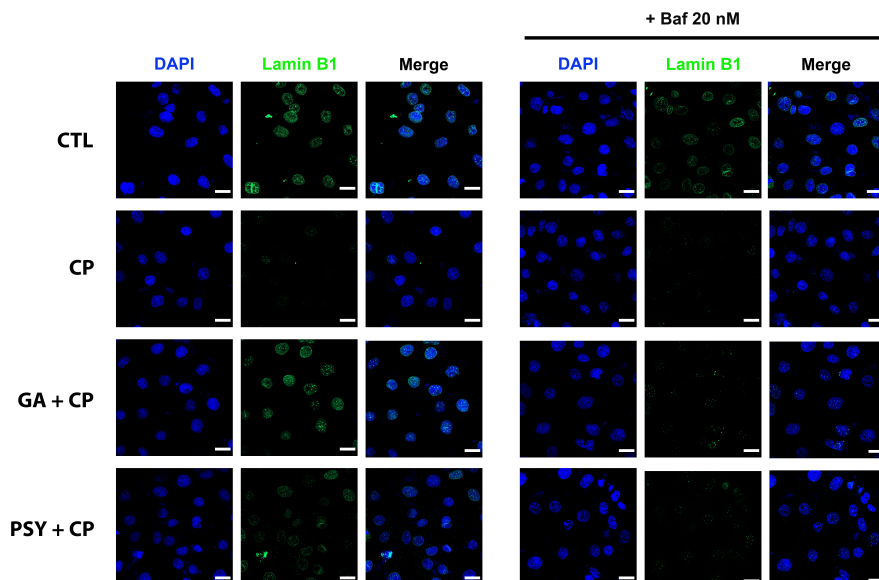
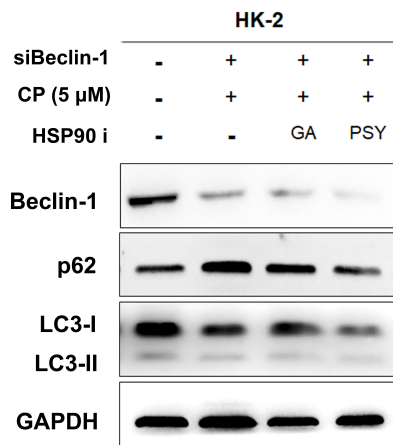
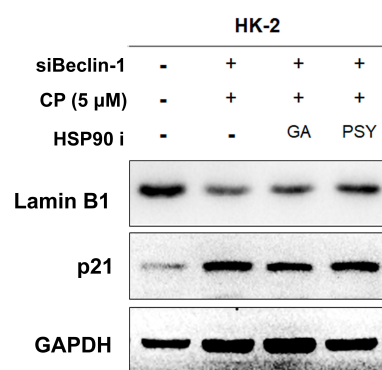


Figure 6. Combination treatment of autophagy inhibitor reduces effect of PSY on senescent HK-2 cells. HK-2 cells were pre-treated with 1.0 μ M PSY or 0.5 μ M GA, then combination with or without 20 nM Baf in 5 μ M CP-induced cellular senescence at 24 hour. (A) SA- β -Gal staining in HK-2 cell. Result performed via light microscope (original magnification x 20; scale bar 100 μ m). Quantification signal by counting the percentage of positive cells (blue) per image. (B) Positive lamin B1 (scale bar 10 μ m) was detected via confocal fluorescent microscopy and quantify intensity by ImageJ software. Experiments were performed at least three times independently. Data present as mean \pm SD (n = 3). Significant difference are indicated: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant. SA- β -Gal: senescence-associated β -galactosidase; Baf: Bafilomycin A1; DAPI: 4',6-diamidino-2-phenylindole; CP: cisplatin; PSY: PSY-2-12; GA: geldanamycin.

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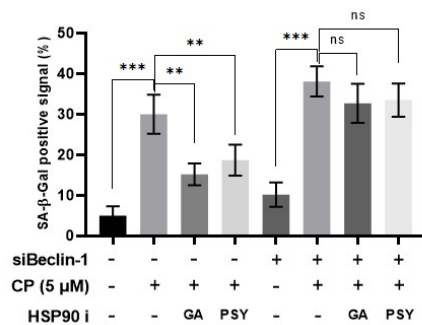
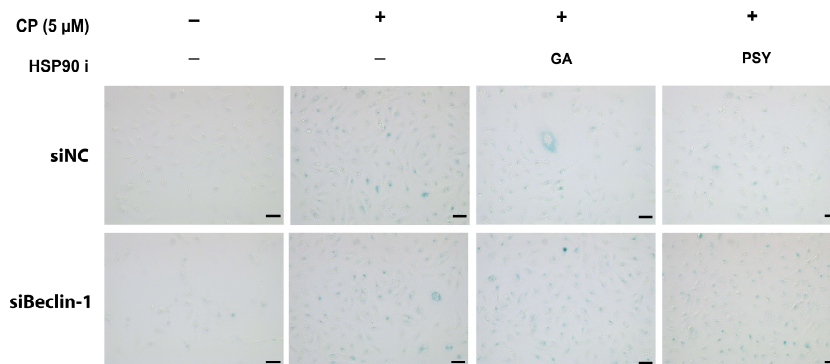
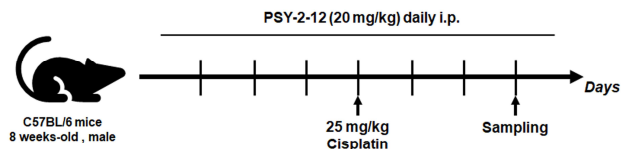
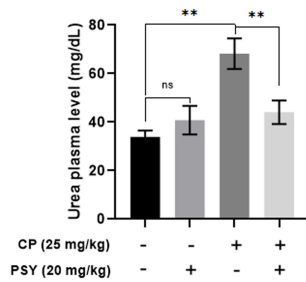


Figure 7. Knockdown beclin-1 delays anti-senescence effect of PSY on CP-induced senescent HK-2 cells. HK-2 cells were transfected with siBeclin-1 or si-NC for 48 hour, then pre-treated with 1.0 μ M PSY or 0.5 μ M GA in 5 μ M CP-induced cellular senescence at 24 hour. (A) Western blot analysis of autophagy markers (p62 beclin-1, and LC3I/II). (B) Western blot analysis of lamin b1 and p21 protein expression. (C) SA- β -Gal staining in HK-2 cells. Result performed via light microscope (original magnification x 20; scale bar 100 μ m). Quantification signal by counting the percentage of positive cells (blue) per image. Experiments were performed at least three times independently. Data present as mean \pm SD (n = 3). Significant difference are indicated: **: $p < 0.01$, ***: $p < 0.001$, ns: not significant. SA- β -Gal: senescence-associated β -galactosidase; p62: sequestosome 1; LC3: Microtubule-associated protein 1A/1B-light chain 3; siBeclin-1: siRNA of beclin-1; siNC: siRNA of negative control; CP: cisplatin; PSY: PSY-2-12; GA: geldanamycin.

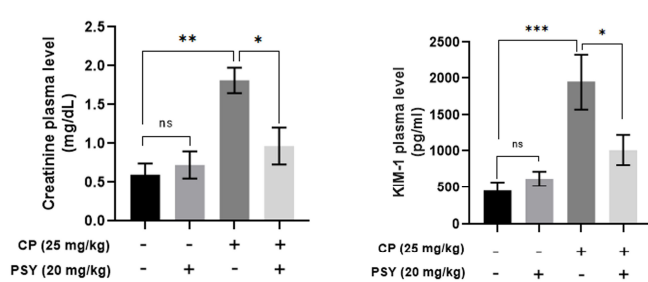
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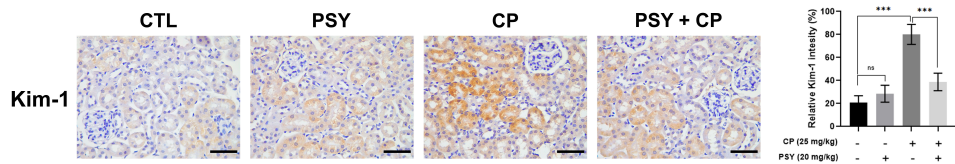
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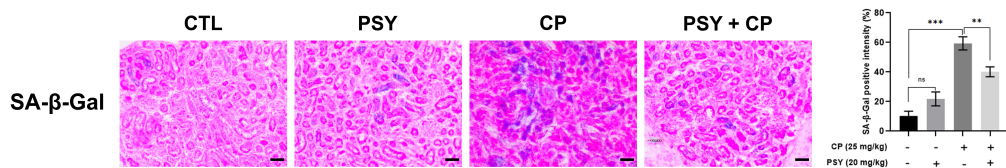
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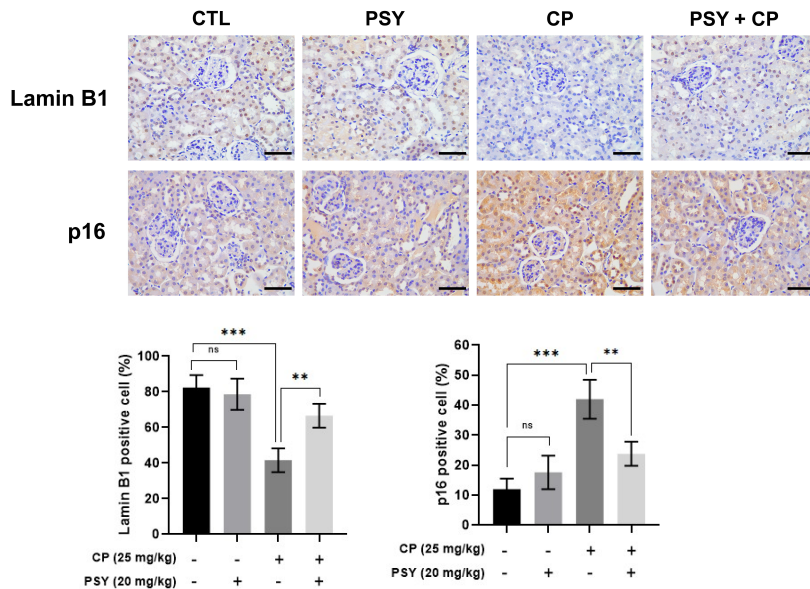


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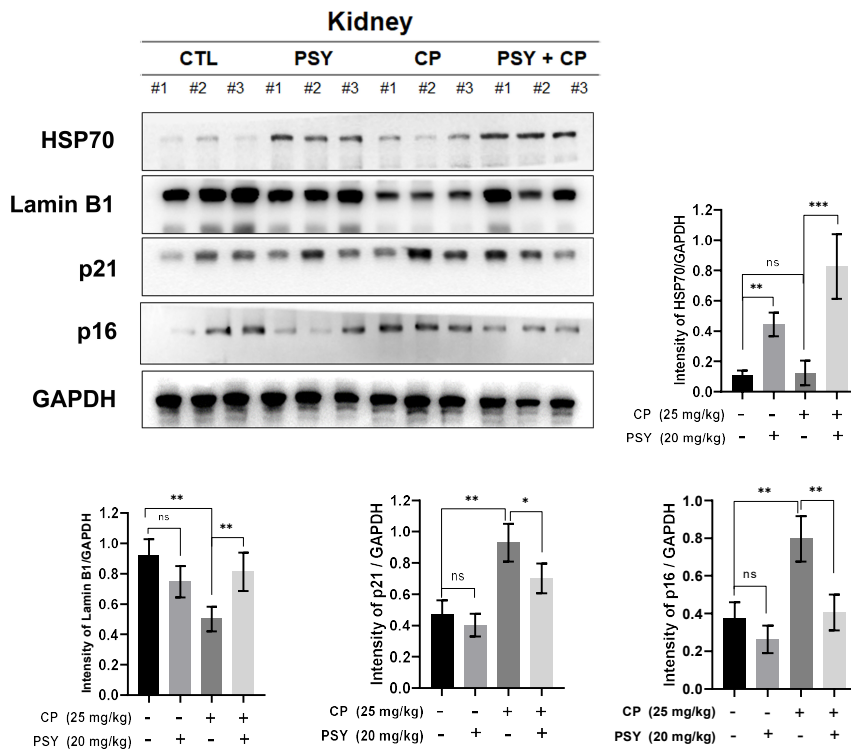


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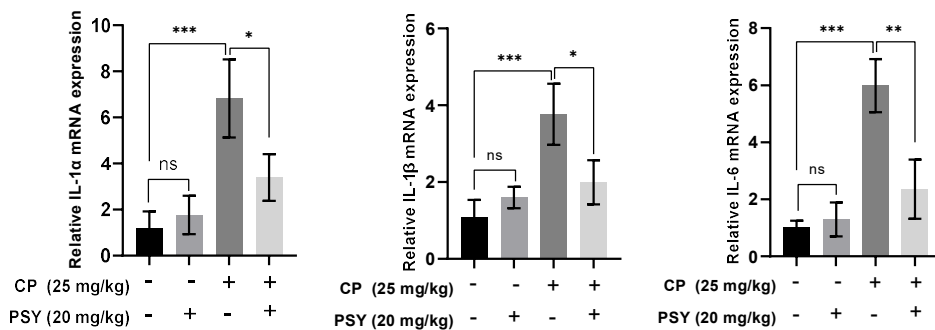


Figure 8. Effect of PSY in CP-induced AKI mice model. (A) Illustration of *in vivo* experiment schematic used CP-induced AKI in co-treated with PSY. (B) Renal function was determined through level urea and creatinine of plasma samples. (C) KIM-1 plasma level was evaluated by using an ELISA kit. (D) IHC result performed KIM-1 positive signal was obtained in the kidney section. (E) SA- β -Gal staining in kidney section. Result performed via light microscope (original magnification x20; scale bar 50 μ m). Quantification signal by counting the percentage of positive cells (blue) per image. (F) IHC result was performed lamin b1 and p16 positive signal was obtained in the kidney section (scale bar 50 μ m). (G) Western blot analysis of senescence markers (p21, p16) as well as lamin b1 and HSP70 protein expression in kidney section. (H) Real time-PCR analysis performed in kidney section. mRNA of SASP genes (IL-1 α , IL-1 β and IL-6) expression were normalized

with GAPDH. Data present as mean \pm SD (n = 3). Significant difference are indicated: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant. SASP: senescence-associated secretory phenotype; SA- β -Gal: senescence-associated β -galactosidase; KIM-1: kidney injury molecule-1; p21: cyclin-dependent kinase inhibitor 1A; p16: cyclin-dependent kinase inhibitor 2A; IL-1 α : Interleukin 1-alpha; IL-1 β : Interleukin 1-beta; IL-6: Interleukin 6; IL-8: Interleukin 8; CP: cisplatin; PSY: PSY-2-12;

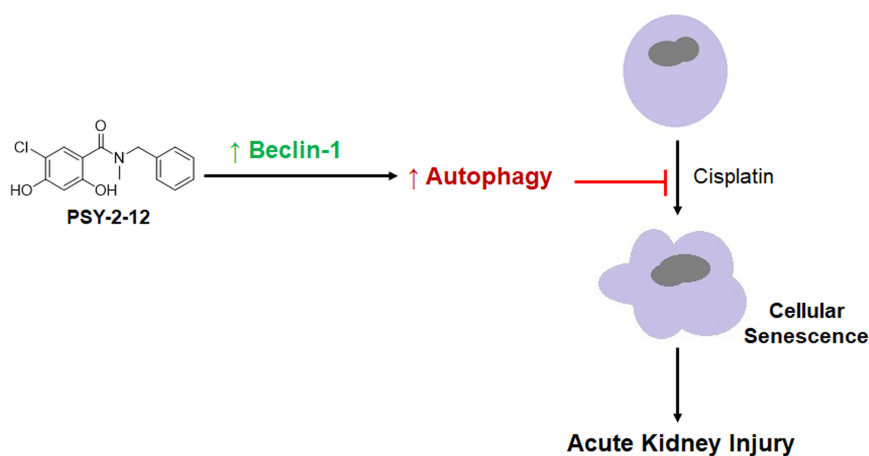


Figure 9. Schematic illustration of the main results. PSY-2-12 activates autophagy by increasing levels of protein beclin-1, which results in a reduction of CP-induced cellular senescence. CP: cisplatin.

4. Discussion

This study shows the potential usage of PSY as a senolytic drug by showing PSY reduced cellular senescence, as evidenced by decreased SA- β -Gal activity, increased lamin B1 expression, and decreased senescent markers, including p16 and p21, as well as downregulated SASP genes in CP-induced senescent HK-2 cells and AngII-induced senescence in HUVECs as opposed to control senescent cells. In addition, PSY could stimulate the autophagy pathway, which elucidated mechanistic details of LC3-II and beclin-1 increased and p62 decreased. Inhibition of autophagy and knockdown of beclin-1 reversed the PSY-induced anti-senescent effect. These results collectively demonstrate that PSY exerts senolytic effects via the beclin-1 mediated autophagic pathway. Finally, PSY could be a promising therapeutic modality as a senolytic drug for the treatment of AKI by proving the anti-senescent effect of PSY in a CP-induced AKI *in vivo* mice model.

Although HSP90 was initially recognized as a promising target for cancer treatment, the development of numerous inhibitors has not led to any FDA approvals. This lack of approval is mainly due to the toxicity of these drugs, which restricts their use, along with compensatory reactions that activate cytoprotective pathways (24). A promising new application for HSP90 inhibitors as repurposed drugs has recently been discovered (16). Researchers demonstrate that HSP90 inhibitors, including GA and 17-DMAG, are senolytic drugs at concentrations as low as 1.0 μ M in various cell types, including

urine mesenchymal stem cells and human fibroblasts when senescence was induced by mechanisms such as oxidative stress, DNA damage, and telomere shortening. In the current study, HK-2 tubular cells were utilized to evaluate the potential therapeutic use of PSY as a repurposed senolytic drug for treating AKI. Additionally, HUVECs were included to determine whether the anti-senescent effect of PSY is specific to certain cell types. PSY at non-cytotoxic concentrations (0.5 and 1.0 μM) significantly reduced cellular senescence in CP-senescent HK-2 cells and AngII-induced HUVECs. These findings align with previous research using GA as an HSP90 inhibitor (16).

Emerging evidence indicates that cellular senescence plays a role in the pathogenesis of various kidney diseases, including AKI, chronic kidney disease, kidney cancer, and transplantation. Cellular senescence occurs in response to stressful stimuli, such as DNA damage, oncogene activation, loss of tumour suppressor genes, and epigenetic changes (25,26). CP-induced AKI murine model to evaluate the senolytic effect of the PSY drug, based on previous studies demonstrating that CP induces kidney senescence primarily through its DNA damaging effects (27). Additionally, CP-induced kidney senescence plays a critical role in the progression of chronic kidney disease following AKI (28,29). *In vivo*, the effects of PSY differed markedly from those of GA. Daily i.p injections of GA at doses of 5 and 10 mg/kg for three days were lethal to mice even before CP treatment, while daily injections of PSY at a concentration of 20 mg/kg for seven days were safe and demonstrated protective effects against CP-induced AKI. As result shown PSY attenuates renal senescence in CP-induced AKI mice.

Regarding the effect of HSP90 inhibitors on autophagy, prior research has demonstrated that HSP90 inhibitors, including ganetespib, AUY922, and GA, reduce autophagy, thereby contributing to chemoresistance in non-small cell lung cancer (30). Another study showed that HSP90 forms a complex with Atg1/Ulk1 and suppresses the kinase activity of Atg1/Ulk1 leading to inhibition of autophagy initiation and dissociation of the HSP90: Atg1/Ulk1 complex activates Atg1/Ulk1 to initiate autophagy (31). Conversely, autophagy is a recognized protective mechanism activated in renal tubular cells to remove damaged organelles and toxic protein aggregates. It is upregulated in response to stress conditions that may result in AKI, including cellular starvation, hypoxia, and nutrient deprivation (32,33). This suggests that autophagy may play a role in alleviating CP-induced cellular senescence. Additionally, the results of the current study support the conclusion that the inhibition of HSP90 activates autophagy. The conflicting findings regarding the effects of HSP90 inhibition may arise from the diverse array of HSP90 client proteins involved in various cellular processes, as well as the complex interactions between HSP90 and its client proteins, which encompass feedback and compensatory mechanisms. Specifically, we speculate that effect of HSP90 inhibitors could be concentration-dependent based on the findings that high concentration of HSP90 inhibitor, such as GA at 25 μ M, inhibits autophagy (30), while low concentration of HSP90 inhibitors activated autophagy, such as PSY (0.5 and 1.0 μ M) and GA (0.5 μ M) in this study.

In conclusion, the findings of the current study indicate that PSY, an HSP90 inhibitor, has the potential to be repositioned as a novel

therapeutic agent for the treatment of AKI. This potential is attributed to its senolytic effects against cisplatin (CP)-induced senescence in proximal tubular cells, as well as its influence on CP-induced AKI. Further research is necessary to ascertain whether the anti-senescent effects of PSY are specific to particular cell types or species. Additionally, investigation of the mechanisms of PSY that underlying its anti-senescence effects would significantly enhance the validation and confirmation.

5. Summary

The aim of this study is to clarify the senolytic effect of PSY, specifically HSP90 inhibitor, on both CP-induced HK-2 senescence and AngII-induced cellular senescence in HUVECs. Since pre-treatment with PSY, the results showed reduced SA- β -Gal activity, suppressed senescence protein markers (p52, p21, and p16), and down-regulated SASP genes in the senescence *in vitro* model. In addition, the autophagy inducer proteins (beclin-1, LC3II/I) increased during PSY treatment, acting as a role of the mechanical pathway. The reverse experiment was performed with combination treatment with the autophagy inhibitor Baf and knockdown of beclin-1 to validate again the mechanism of PSY in reducing cellular senescence in CP-induced senescence in HK-2. Finally, PSY was able to be applied in an *in vivo* study, with a CP-induced AKI model as well as increased renal senescence. As a result, treatment with PSY led to recovery of kidney status, with plasma levels of urea, creatinine, and KIM-1 decreasing in CP-injected samples. Additionally, PSY treatment also suppressed renal senescence, due to decreases in SA- β -Gal activity, SASP genes, and senescence markers (p21 and p16) in renal tissue.

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HSP90 Specific Inhibitor, PSY-2-12, Ameliorates Cellular Senescence via Activating Autophagy: a Potential Senolytic Drug Against Acute Kidney Injury

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(Abstract)

An unexpected senolytic function of heat shock protein 90 (HSP90) inhibitors, drugs that have long been developed as cancer therapeutic reagents, has recently been unveiled. Thus, an HSP90-specific inhibitor, PSY-2-12 (PSY), was explored for its potential therapeutic usage as a senolytic agent. Here, PSY, at a non-toxic concentration, reduced cellular senescence in both cisplatin (CP)-induced senescent kidney proximal tubular cells and angiotensin-II-induced senescent human umbilical vein endothelial cells. The senolytic effect of PSY was confirmed in a CP-induced acute kidney injury (AKI) mice model. PSY treatment attenuated CP-induced kidney injury, as evidenced by decreased urea, creatinine, and kidney injury molecule-1 levels, and ameliorated

CP-induced senescence, as evidenced by decreased SA- β -Gal staining and increased lamin B1 and p16 levels compared with control. Mechanistically, PSY exerts a senolytic effect via beclin-1-mediated stimulation of autophagy. As a result, PSY increased beclin-1 expression in CP-treated proximal tubular cells and autophagic flux. Inhibition of autophagy reversed the senolytic effect of PSY in CP-induced senescent proximal tubular cells. Knockdown of beclin-1 also reversed the senolytic effect of PSY in CP-induced senescent proximal tubular cells. In summary, this study suggests that PSY, an HSP90-specific inhibitor, could be a promising senolytic therapeutic agent for the treatment of AKI.

HSP90 Specific Inhibitor, PSY-2-12, Ameliorates Cellular Senescence via Activating Autophagy: a Potential Senolytic Drug Against Acute Kidney Injury

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(지도교수 하 은 영)

(초록)

열충격단백질 90(HSP90) 억제제는 오랜 기간 암 치료제로 개발된 대표적인 약물 중 하나이다. 최근 열충격단백질 90(HSP90) 억제제의 항노화 기능이 밝혀졌다. 따라서, 본 연구는 HSP90 특이 억제제인 PSY-2-12 (PSY)의 항노화 제제로서의 잠재적 치료 활용 가능성을 탐색하였다. PSY는 세포 독성을 나타내지 않는 농도에서 세포 노화 감소 효과를 나타내었으며, 이러한 세포 노화 감소 효과는 cisplatin (CP)으로 노화가 유도된 신장 근위세뇨관 세포와 안지오텐신-II로 노화가 유도된 인체 제대 정맥 내피 세포 모두에서 확인되었다. CP로 유도된 급성 신장 손상(AKI) 마우스 모델에서 PSY의 항노화 효과도 확인하였다. PSY 치료는 CP로 유도된 신장 손상을 감소시켰으며, 이는 요소, 크레아티닌, 신장 손상 분자(kidney injury molecule-1)의 수치 감소로 나타났다. 또한, PSY 치료는 CP로 유도된 세포 노화를 개선하였으며,

이는 SA- β -Gal 염색 감소 및 대조군에 비해 증가된 lamin B1 과 p16 발현으로 확인되었다. 기전적으로는, PSY는 beclin-1 매개 자가포식 활성을 통해 항노화효과를 발휘한다는 것을 발견하였다. 결과적으로, PSY는 CP에 의해 노화가 유도된 근위세뇨관 세포에서 beclin-1 발현을 증가시키고 자가포식 활성을 촉진한다. 자가포식을 억제하면 CP로 유도된 노화 근위세뇨관 세포에서 PSY의 노화효과가 나타나지 않았다. 또한 CP로 유도된 노화 근위세뇨관 세포에서 beclin-1의 발현을 감소시키면 PSY의 노화효과가 나타나지 않았다. 요약하자면, 본 연구는 HSP90 특이 억제제인 PSY가 항노화 작용을 통해 AKI 치료제로 활용될 수 있음을 제안한다.

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☐ 논문 및 저서

None