

Platycodin D sensitizes head and neck squamous cell carcinoma to cisplatin by inducing autophagy arrest

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Abstract. Head and neck squamous cell carcinoma (HNSCC) is one of the most common malignancies worldwide. Cisplatin, a widely used chemotherapeutic agent, exerts its anticancer effect by inducing DNA damage, and inhibiting transcription and replication. However, its repeated administration can lead to severe side effects and the development of drug resistance, thereby limiting its clinical efficacy. Thus, novel combination strategies are urgently needed to enhance the anticancer effects of cisplatin. The present study aimed to investigate the potential of Platycodin D (PD), a natural saponin extracted from *Platycodon grandiflorus*, to enhance cisplatin sensitivity in HNSCC cells. The results demonstrated that combination treatment with PD and cisplatin synergistically reduced cell viability and markedly suppressed colony formation compared with either agent alone. Furthermore, the combination treatment markedly increased intracellular reactive oxygen species (ROS) levels, and markedly downregulated the expression levels of antioxidant genes, including heme oxygenase-1, NAD(P)H quinone dehydrogenase 1, superoxide dismutase 1 and sulfiredoxin 1. Additionally, the combination treatment excessively activated autophagy, whereas PD inhibited autophagic flux, as determined by LC3A/B and p62 accumulation following Bafilomycin A1 treatment, ultimately promoting apoptosis. These findings suggested that PD may serve as a potential cisplatin sensitizer by enhancing ROS accumulation

and disrupting autophagy, thereby presenting a promising combination therapeutic strategy for the treatment of HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the seventh most common malignancy worldwide and accounts for >90% of all head and neck cancers. According to the GLOBOCAN 2020 estimates, ~890,000 new cases of HNSCC are diagnosed annually worldwide (1-3). HNSCC arises from the epithelial mucosa of the larynx, oropharynx, nasopharynx and oral cavity (4). The incidence of HNSCC is primarily associated with tobacco smoking, alcohol consumption and human papillomavirus infection (5). Additionally, the use of smokeless tobacco products and genetic susceptibility are significant risk factors contributing to its development (6). The first-line treatment for HNSCC includes surgery and radiotherapy, often supplemented with chemotherapy. Among chemotherapeutic agents, cisplatin is the most commonly used platinum-based drug (7,8). Cisplatin forms covalent bonds with guanine residues in the DNA, leading to the crosslinking of the double-stranded helix. This process inhibits DNA replication, promotes the accumulation of DNA damage and ultimately induces apoptosis, establishing cisplatin as an essential chemotherapeutic agent for HNSCC treatment. However, prolonged cisplatin administration can lead to the development of resistance mechanisms, necessitating combination therapy with other chemotherapeutic agents or immunotherapies (9,10). Despite the availability of established treatment strategies, patients with HNSCC continue to have a poor prognosis, with a 5-year overall survival rate of ~40-50% worldwide, as documented in studies published in 2025 (11,12). Therefore, novel therapeutic strategies are required to improve the poor prognosis of HNSCC. Although substantial progress has been made in anticancer research, conventional chemotherapeutic agents remain associated with significant adverse effects, including toxicity to normal cells and the development of drug resistance (13). There is growing evidence demonstrating the importance of natural product-derived compounds with

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potent anticancer activity and minimal side effects. Extensive research has been conducted to investigate the molecular mechanisms underlying their effects, including the inhibition of cancer cell proliferation and angiogenesis. Furthermore, these studies have demonstrated the induction of cancer cell apoptosis (14,15).

Platycodon grandiflorus (PG), which is widely used in traditional oriental medicine, is known for its therapeutic properties in the treatment of respiratory diseases, including asthma, bronchitis and tonsillitis. Additionally, PG exhibits immunostimulatory, anti-inflammatory, anti-obesity and anti-atherosclerotic activities (16,17). Platycodin D (PD), a triterpenoid saponin abundant in the root of PG, is the major active natural compound responsible for these diverse pharmacological effects (18,19). Notably, numerous studies have demonstrated that PD possesses anticancer properties, including the inhibition of cell proliferation, and the regulation of oxidative stress and autophagy. Furthermore, PD disrupts autophagic flux, thereby exacerbating cellular stress and promoting cancer cell death (18-20). However, the anticancer effects of PD on HNSCC have not yet been adequately investigated.

Autophagy is a highly conserved cellular degradation and recycling process that is essential for maintaining cellular homeostasis and enabling adaptation to environmental stressors, such as nutrient deprivation, oxidative stress and hypoxia. This process involves the sequestration of damaged organelles and misfolded proteins within autophagosomes, which subsequently fuse with lysosomes for degradation (20,21). Autophagy functions as a cytoprotective mechanism by eliminating unnecessary or damaged cellular components and thereby promoting cell survival. However, excessive activation or inhibition of autophagy can lead to autophagy-mediated cell death (22,23). Due to this dual role, autophagy has been extensively investigated as a therapeutic target in cancer (23-25). The regulation of autophagy is primarily mediated by autophagy-related genes and key regulatory proteins, such as LC3, which undergo lipidation to facilitate autophagosome formation. Following autophagosome formation, fusion with lysosomes enables the degradation of autophagic cargo (26). In the context of cancer therapy, autophagy may either promote cell survival through protective autophagy mechanisms or enhance treatment efficacy by inducing autophagy-dependent cell death, further underscoring its complex role in tumor biology (27,28). Oxidative stress, predominantly mediated by reactive oxygen species (ROS), is known as a key regulator of autophagy (29). Understanding the interaction between ROS, autophagy and cell death is crucial for improving anticancer treatment strategies. Based on these considerations, it was hypothesized that cisplatin-induced ROS accumulation may initially activate autophagy as a cellular stress response, whereas the known autophagic flux-blocking activity of PD could further exacerbate cellular stress by impairing autophagic degradation. Furthermore, the combined effect of excessive ROS production and autophagic flux inhibition was proposed to synergistically promote cytotoxic stress and cell death in HNSCC cells.

Therefore, the present study aimed to investigate whether PD enhanced cisplatin chemosensitivity in HNSCC cells and to elucidate the underlying mechanism, with a particular focus on ROS accumulation and autophagic flux regulation.

Materials and methods

Reagents. PD powder was purchased from MilliporeSigma and dissolved in DMSO at a stock concentration of 20 mM. Cisplatin was purchased from MilliporeSigma and dissolved according to the manufacturer's instructions.

Cell culture. The human HNSCC cell line HSC3 was purchased from the Japanese Collection of Research Bioresources Cell Bank. The FaDu cell line was obtained from the American Type Culture Collection. The HaCaT cell line was kindly provided by Dr Jin-Woo Lee (Kyung Hee Medical Center, Seoul, South Korea) and the original supplier of the cell line was Cell Lines Service GmbH. All cell lines were maintained at 37°C in a humidified incubator containing 5% CO₂. HSC3 cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; HyClone; Cytiva) and FaDu cells were cultured in Minimum Essential Medium (MEM; HyClone; Cytiva), both supplemented with 10% heat-inactivated FBS (HyClone; Cytiva) and 1% penicillin-streptomycin (Corning, Inc.). HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; Cytiva) supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin. When cell confluency reached ~80% in a 100-mm dish, cells were washed with Dulbecco's PBS (DPBS; pH 7.0-7.6) and detached using trypsin-EDTA for subculturing. Cell line authentication was conducted by short tandem repeat profiling using the Korean Cell Line Bank (Korean Cell Line Research Foundation) within the past year. During the initial thawing of cell cultures, all cell lines were treated with a mycoplasma removal agent (cat. no. 093050044; MP Biomedicals, LLC) at 37°C for 24 h in a humidified incubator to eliminate potential mycoplasma contamination.

Cell viability assay. HaCaT, HSC3 and FaDu cells were seeded into 96-well plates at a density of 3×10^3 cells/well and allowed to attach by incubating them at 37°C for 24 h. Subsequently, HSC3 and FaDu cells were treated with PD (0, 5, 10 and 15 μ M) and cisplatin (0, 5 and 10 μ M) at 37°C for 48 h, either as a single treatment or in combination, whereas HaCaT cells were treated with PD alone (0, 0.5, 1, 3, 5 and 10 μ M) at 37°C for 48 h. Control cells were treated with an equivalent volume of vehicle (DMSO) under the same experimental conditions. Thereafter, cell viability was assessed using a WST-8-based cell viability assay according to the manufacturer's protocol (EZ-Cytox; DoGenBio), and 10 μ l of the reagent was added to each well. After incubation for 1 h, the formation of chromogenic formazan was quantified by measuring the absorbance at 450 nm using a SPARK® multimode microplate reader (Tecan Group, Ltd.). Cell viability (%) was calculated as [mean optical density (OD) of the sample/mean OD of the control] x100%.

Colony formation assay. HSC3 and FaDu cells were seeded into 6-well plates at a density of 300 cells per well and maintained at 37°C. After 24 h of incubation, the cells were treated at 37°C with 5 μ M PD and 5 μ M cisplatin at 48-h intervals. HSC3 and FaDu cells were divided into four groups: Control, PD-treated, cisplatin-treated and combination-treated. HSC3 cells were cultured for 7 days and FaDu cells were cultured for 10 days. The colonies that developed from surviving cells were

stained with crystal violet solution (0.5% crystal violet in 50% methanol) at room temperature for 3 min without prior fixation. Colonies were defined as clusters containing ≥ 50 cells. The number of colonies was counted manually.

Reverse transcription-quantitative PCR (RT-qPCR). HSC3 and FaDu cells were seeded into 6-well plates at densities of 1×10^5 and 3×10^5 cells/well, respectively. After 24 h, the cells were treated with 10 μM PD or cisplatin, either as a single treatment or in combination, at 37°C for 12 h in HSC3 cells and for 3 h in FaDu cells. Total RNA was extracted using RiboEx™ (GeneAll Biotechnology Co., Ltd.), according to the manufacturer's protocol. A NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.) was used to assess the RNA concentration and quality. Extracted RNA was reverse transcribed into cDNA using a Tetro cDNA Synthesis Kit (Meridian Bioscience, Inc.). Reverse transcription was performed at 45°C for 30 min, followed by enzyme inactivation at 85°C for 5 min, according to the manufacturer's instructions. RT-qPCR was performed using SYBR Green Premix Ex (Meridian Bioscience, Inc.) with the following thermocycling conditions: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing at 60°C for 30 sec. The reaction was evaluated using melting curve analysis. Each experiment was performed in triplicate using independent biological replicates, and the data were analyzed by relative quantitation using the $2^{-\Delta\Delta C_q}$ method and normalized to β -actin (30). The following primer sequences were used: Heme oxygenase-1 (HO-1) forward, 5'-CCAGGCAGAGAATGCTGAGTTC-3' and reverse, 5'-AAGACTGGGCTCTCCTTGTTGC-3'; NAD(P)H quinone dehydrogenase 1 (NQO1) forward, 5'-CCTGCCATTCTGAAAGGCTGGT-3' and reverse, 5'-GTGGTGATGGAAAGCACTGCCT-3'; superoxide dismutase 1 (SOD1) forward, 5'-CTCACTCTCAGGAGACCATTGC-3' and reverse, 5'-CCACAAGCCAAA CGACTTCCAG-3'; sulfiredoxin 1 (SRXN1) forward, 5'-GCA GAGCCTCGTGGACACGAT-3' and reverse, 5'-ATGGTC TCTCGCTGCAGTTGCT-3'; and β -actin forward, 5'-CAT GTACGTTGCTATCCAGGC-3' and reverse, 5'-CTCCTT AATGTCACGCACGAT-3'. The means of three independent biological replicates were calculated. The fold change was calculated by dividing the mean expression of the treated groups by that of the untreated control group. Statistical analysis of the differences among cells treated with PD, cisplatin or their combination was performed using one-way ANOVA followed by Tukey's post hoc test.

ROS assay. HSC3 and FaDu cells were seeded into 6-well plates at densities of 1×10^5 and 3×10^5 cells/well, respectively. After 24 h of incubation, the cells were pretreated with 100 nM rapamycin (37094; MilliporeSigma) at 37°C for 2 h as indicated, followed by treatment with 10 μM PD and cisplatin, either alone or in combination at 37°C for the designated time points.

Based on preliminary time-course experiments conducted to determine appropriate detection time points, intracellular ROS levels were assessed at multiple time points (3, 6, 12, 24 and 48 h) following PD and cisplatin treatment. These pilot experiments indicated that ROS accumulation peaked at ~12 h in HSC3 cells and at 3 h in FaDu cells (data not shown).

After incubation, the culture medium was removed and 20 μM 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA; cat. no. ab113851; Abcam) was added to each well to measure ROS levels. The cells were then incubated at 37°C for 30 min, washed twice with DPBS, detached using trypsin-EDTA and resuspended in DPBS, diluted 1:2 with DPBS, and adjusted to a final volume of 1 ml prior to analysis. Flow cytometry analysis was performed using a FACSCalibur flow cytometer (BD Biosciences) with CellQuest software (version 6.0; BD Biosciences).

Morphological analysis. HSC3 and FaDu cells were seeded at 5×10^4 and 1×10^5 cells, respectively. After 24 h of incubation, cells were treated with PD (10 μM), cisplatin (10 μM), or the combination at 37°C for 48 h. Cell morphology was then examined under a light microscope to evaluate vesicular structure accumulation.

Confocal microscopy. HSC3 and FaDu cells were seeded into 8-well plates at a density of 5×10^3 cells/well. After 24 h of incubation at 37°C, the cells were treated with 10 μM PD or 10 μM cisplatin as a single treatment, or in combination, at 37°C. After 48 h of treatment, the cells were fixed with 4% paraformaldehyde at room temperature for 15 min and washed three times with DPBS. Permeabilization was then performed using 0.1% Triton X-100 diluted in DPBS, followed by blocking with 5% BSA (cat. no. A0100-010; GenDEPOT, LLC) at room temperature for 30 min. The cells were incubated overnight at 4°C with primary antibodies against LC3B (1:1,000; cat. no. 2775S; Cell Signaling Technology, Inc.) and sequestosome 1 (SQSTM1)/p62 (1:1,000; cat. no. 5114S; Cell Signaling Technology, Inc.). After incubation, the cells were washed twice with DPBS. Secondary antibodies [Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) cat. no. ab150077; Abcam] were applied at a dilution of 1:500 in BSA at room temperature for 40 min, followed by three washes with PBS. The cells were then stained with DAPI (Roche Diagnostics) at room temperature for 5 min. Cells were observed at a magnification of x200 using a confocal fluorescence microscope (Zeiss AG) in a dark room. Fluorescence intensity analysis and puncta quantification were performed using ZEN software (version 700; Zeiss AG), which is integrated with the confocal imaging system.

Western blotting. Prior to western blotting, HNSCC cells were treated with 10 μM PD and 10 μM cisplatin at 37°C for 48 h. When indicated, Bafilomycin A1 (BafA1; 10 nM; cat. no. S1413; Selleck Chemicals), a lysosomal inhibitor used as a positive control for late-stage autophagy inhibition, was added 1 h prior to cell harvest. Lysates from HNSCC cells were homogenized in RIPA buffer [1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5) and 2 mM ethylenediaminetetraacetic acid (pH 8.0)] purchased from Biosesang containing a protease inhibitor cocktail. The protein concentration was then quantified using a Pierce Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Equal amounts of protein (10 μg per lane) were mixed with loading dye (5X SDS-polyacrylamide gel electrophoresis loading buffer; Invitrogen Biotechnology, Inc.). After boiling for

10 min, cell protein samples were added to each lane and separated on a 10 or 15% SDS-polyacrylamide gel. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (MilliporeSigma) and blocked at room temperature for 1 h in 5% skimmed milk and Tris-buffered saline with 0.1% Tween-20. These membranes were then incubated with appropriate primary antibodies overnight at 4°C. The following antibodies were used: Anti-LC3A/B (1:1,000; cat. no. 12741S; Cell Signaling Technology, Inc.), anti-SQSTM1/p62 (1:1,000; cat. no. 5114S; Cell Signaling Technology, Inc.), anti-poly(ADP-ribose) polymerase (PARP; 1:1,000; cat. no. 9542S; Cell Signaling Technology, Inc.), anti-caspase 3 (1:1,000; cat. no. 9662S; Cell Signaling Technology, Inc.) and anti- β -actin (1:5,000; cat. no. 47778; Santa Cruz Biotechnology, Inc.). The membranes were then washed with TBST containing 0.1% Tween-20 and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Anti-rabbit IgG (cat. no. 7074S; Cell Signaling Technology, Inc.) and anti-mouse IgG (cat. no. 7076S; Cell Signaling Technology, Inc.) were used at dilutions of 1:5,000 and 1:10,000, respectively. The protein-antibody complexes were detected using enhanced chemiluminescence (cat. no. RPN2232; Cytiva) according to the manufacturer's protocol. Band intensities were quantified by densitometric analysis using ImageJ software (version 1.54; National Institutes of Health) and normalized to β -actin.

Flow cytometry. For apoptosis analysis, HSC3 and FaDu cells were treated with PD (10 μ M), cisplatin (10 μ M), or their combination for 48 h at 37°C. Cells were washed with PBS, trypsinized and centrifuged at 1,000 $\times$ g for 5 min at 4°C. The cell pellets were washed with cold PBS and centrifuged again under the same conditions. Cells were resuspended in 1 $\times$ binding buffer and stained with annexin V-FITC for 15 min at room temperature in the dark, followed by the addition of propidium iodide (PI) immediately prior to flow cytometric analysis under cold conditions. Annexin V-FITC/PI staining was performed according to the manufacturer's protocol using an apoptosis detection kit (Biobud; cat. no. LS-02-100; <https://www.biobud.com/>). Apoptotic cells were measured using a FACSCalibur cell analyzer (BD Biosciences) and data were analyzed using CellQuest software (version 6.0; BD Biosciences), and the apoptotic rate was calculated as the sum of early apoptotic (annexin V-positive/PI-negative), late apoptotic (annexin V-positive/PI-positive), and necrotic (annexin V-negative/PI-positive) cell populations.

Statistical analysis. All data are presented as the mean $\pm$ standard deviation (SD) from at least three independent experiments. The 50% inhibitory concentration (IC₅₀) was calculated from dose-response curves using GraphPad Prism software (version 5.01; Dotmatics). To evaluate the synergistic effect between PD and cisplatin, CompuSyn software (ComboSyn, Inc.; version not specified by the manufacturer) was used to calculate the combination index (CI). A CI value <1 indicated synergy, CI=1 represented an additive effect and CI>1 suggested antagonism. Statistical analysis for multiple-group comparisons was performed using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Synergistic effect of PD and cisplatin on HNSCC cell viability. To evaluate the cytotoxic and synergistic effects of PD and cisplatin on HNSCC cells, viability was assessed in HSC3 and FaDu cells following treatment with various concentrations of PD (5, 10 and 15 μ M) or cisplatin (5 and 10 μ M), either alone or in combination, for 48 h. The combined treatment with PD and cisplatin induced cell death in a dose-dependent manner (Fig. 1A).

CI analysis revealed that all tested combinations exhibited CI values <1, confirming a synergistic cytotoxic effect of PD and cisplatin in both HSC3 and FaDu cells (Fig. 1B). These results suggested that, despite their limited cytotoxic effects when used as single treatments, the combination of PD and cisplatin markedly enhanced cellular sensitivity to treatment, supporting a sensitizing role of PD toward cisplatin.

In addition, to evaluate the potential cytotoxic effects of PD on normal cells, HaCaT human keratinocytes were treated with PD and cell viability was assessed. PD treatment did not markedly reduce cell viability at concentrations ranging between 0 and 10 μ M, indicating minimal cytotoxic effects in normal cells (Fig. S1).

Inhibition of colony formation in HNSCC cells by PD and cisplatin combination treatment. To assess long-term clonogenic survival beyond short-term viability, colony formation assays were conducted in HSC3 and FaDu cells treated with PD and cisplatin as a single treatment or in combination. HSC3 and FaDu cells were treated with PD and cisplatin (5 μ M each) for 7-10 days. The results showed that while PD and cisplatin alone reduced colony formation, the extent of inhibition was substantially greater with combined treatment using PD and cisplatin in both HSC3 and FaDu cells (Fig. 2A and B). This reduction in colony formation was statistically significant ($P < 0.05$), indicating a strong synergistic effect of the combined treatment. Furthermore, the combination treatment resulted in fewer and smaller colonies compared with single-drug treatments and the control group. These findings indicated that PD increased the sensitivity of HNSCC cells to cisplatin and contributed to the suppression of colony formation. Collectively, the results suggested that combined treatment with PD and cisplatin markedly impairs the long-term clonogenic survival of HNSCC cells, indicating sustained inhibition of tumor cell proliferative potential beyond short-term cytotoxic effects.

ROS-associated cytotoxic effect of PD and cisplatin combination treatment. Several studies have reported that cisplatin induces DNA damage in cancer cells, leading to an increase in intracellular ROS levels (31-33). Additionally, numerous studies have demonstrated that PD also promotes ROS accumulation (34,35). Considering these studies, the present study aimed to determine whether the suppression of cell proliferation and induction of cell death observed following PD and cisplatin combination treatment in HNSCC cells were associated with increased ROS levels.

HO-1, NQO1, SOD1 and SRXN1 are well-known antioxidant enzymes involved in maintaining cellular redox homeostasis (36,37). To evaluate the effect of PD and cisplatin on the expression of these antioxidant genes, the present study

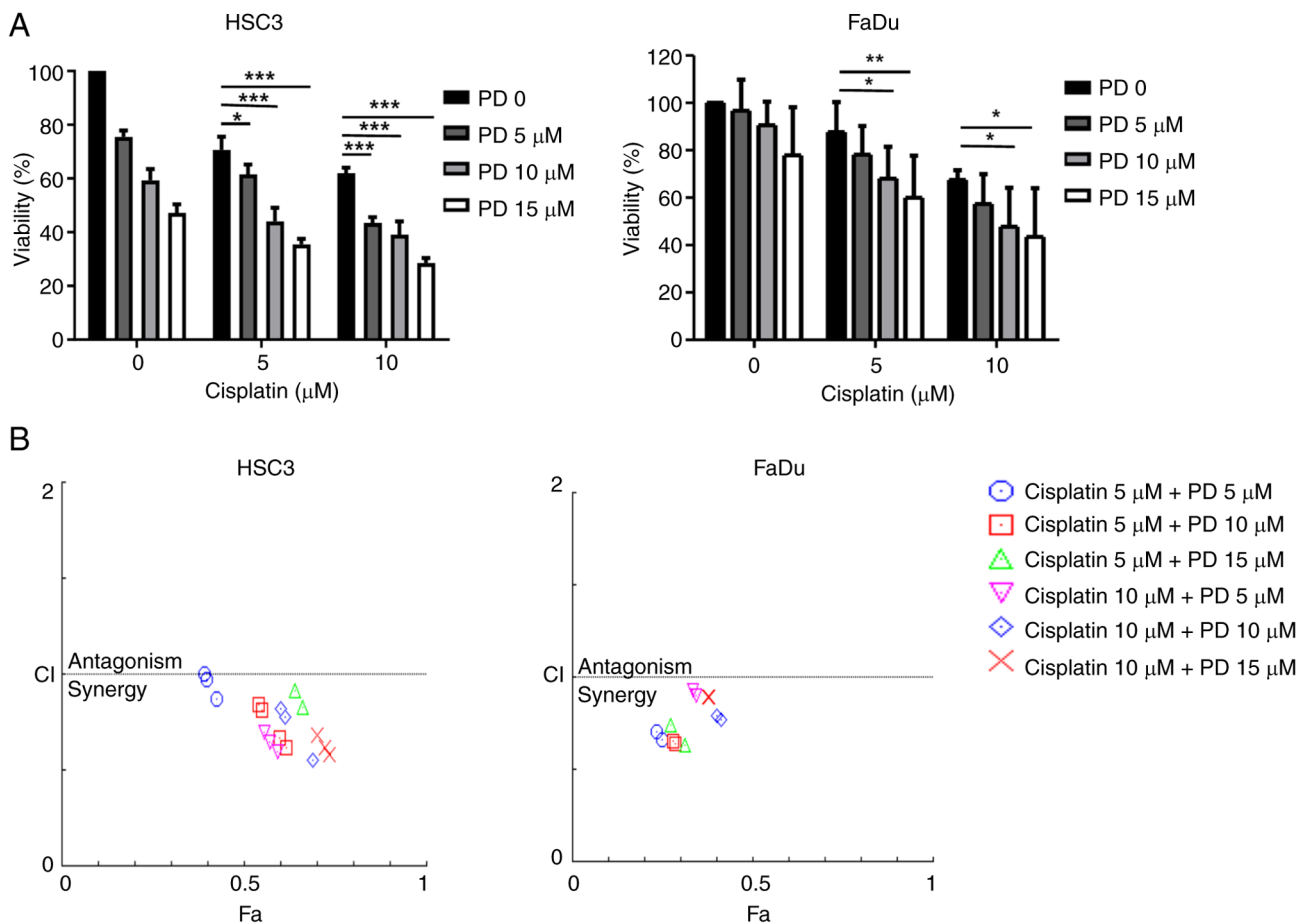


Figure 1. PD and cisplatin combination treatment synergistically reduces viability in head and neck squamous cell carcinoma cells. (A) HSC3 and FaDu cells were treated with PD (5, 10 or 15 μM), cisplatin (5 or 10 μM) or combination treatment for 48 h. Cell viability was assessed using an MTT assay. Statistical significance was determined by one-way ANOVA. (B) CI values were calculated, with a CI <1 indicating a synergistic effect. Fa (fraction affected) represents the fraction of cells affected by drug treatment and was calculated based on cell viability data. Data are presented as the mean $\pm$ SD ($n \geq 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. PD, Platycodin D; CI, combination index; Fa, fraction affected.

conducted RT-qPCR in HSC3 and FaDu cells following treatment with either drug alone or in combination. The expression levels of HO-1, NQO1, SOD1 and SRXN1 showed variable changes following single treatment, with some genes exhibiting increased or decreased expression compared with the vehicle control, and no consistent pattern was observed. By contrast, combination treatment with PD and cisplatin resulted in a significant decrease in the expression of all four antioxidant genes compared with either single treatment alone in both HSC3 and FaDu cells (Fig. 3A and B), indicating a pronounced impairment of the cellular antioxidant defense system.

To further assess the regulation of ROS levels by PD and cisplatin, DCF-DA staining followed by flow cytometry was performed in HSC3 and FaDu cells treated with each drug (10 μM), as a single treatment or in combination. No significant differences in ROS levels were observed between the vehicle control and single treatment groups. By contrast, ROS levels were significantly increased in the combination treatment group compared with those in the individual drug treatment groups (Fig. 4A and B). Collectively, these results indicated that PD and cisplatin synergistically enhanced ROS accumulation in HNSCC cells, potentially contributing to their synergistic cytotoxic effects. The significant downregulation of antioxidant genes

along with increased ROS levels demonstrated a potential mechanism by which combination treatment exacerbated oxidative stress, ultimately leading to enhanced cell death and impaired proliferation.

Induction of autophagy arrest following combination treatment with PD and cisplatin in HNSCC cells. Previous experiments have shown that ROS serve a crucial role in cellular stress responses and the regulation of autophagy (38). To analyze the effect of PD and cisplatin combination treatment on cellular phenotype modifications, HSC3 and FaDu cells were treated for 48 h, followed by microscopy to observe cell morphology modifications. An excessive increase in vesicular structures was observed in cells treated with PD alone or in combination with cisplatin (Fig. 5). To identify the type of these vesicles, additional experiments were conducted (Fig. 5).

Since PD has already been reported to be associated with autophagy (39), the present study considered the possibility that the increased ROS induced by PD and cisplatin combination treatment could influence the autophagy process. ROS can activate the early signaling pathways of autophagy; however, excessive ROS accumulation can impair autophagic flux, resulting in autophagy arrest (38). To determine whether ROS accumulation induced by combined PD and cisplatin treatment

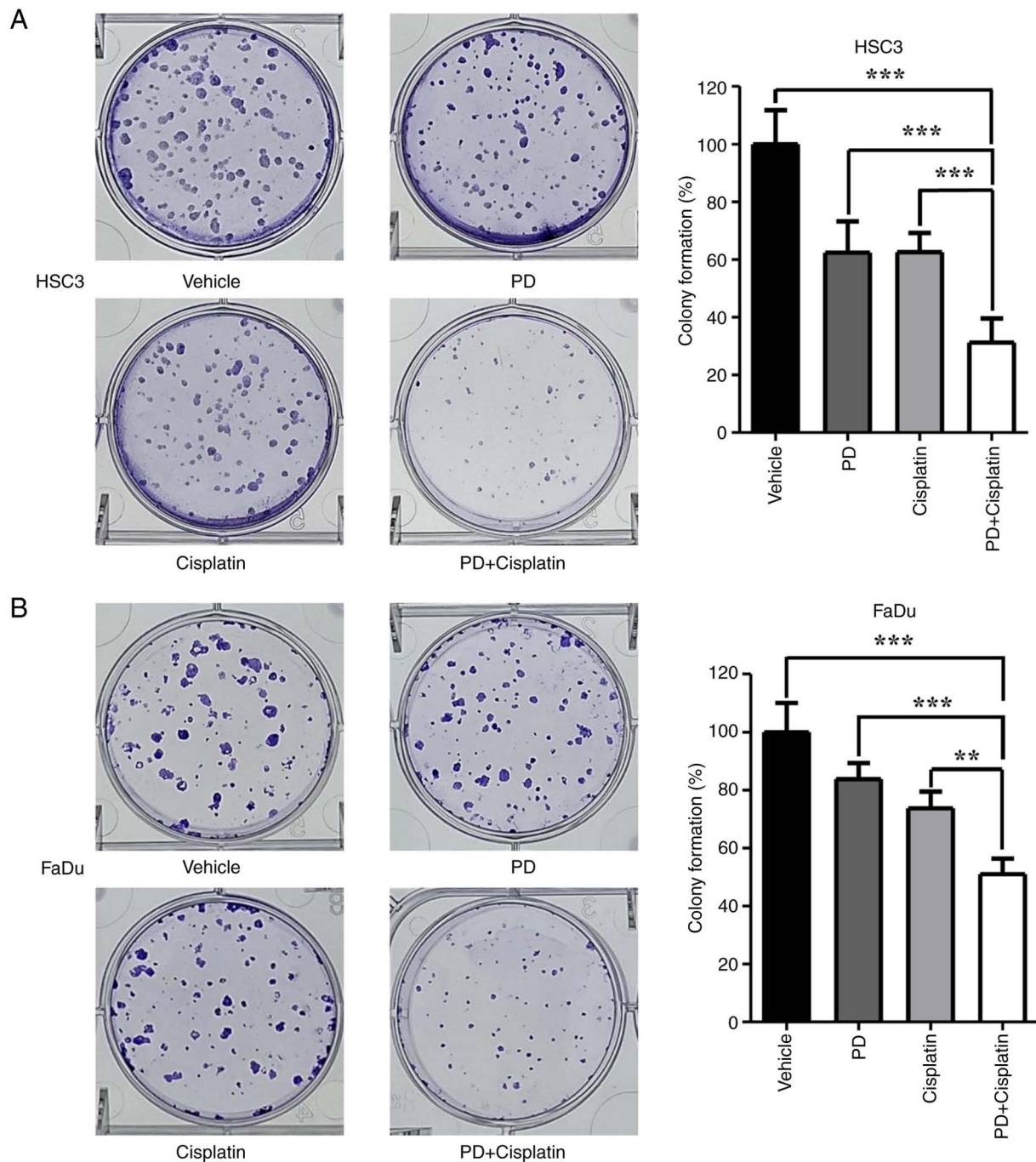


Figure 2. Combination of PD and cisplatin markedly suppresses colony formation in head and neck squamous cell carcinoma cells. (A) HSC3 and (B) FaDu cells were seeded at 300 cells per well and treated with PD (5 μ M), cisplatin (5 μ M) or combination treatment for 7-10 days. The quantitative graph represents the number of colonies. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. Data are presented as the mean $\pm$ SD (n=3). **P<0.01, ***P<0.001. PD, Platycodin D.

disrupts autophagic flux progression, leading to flux arrest, the present study analyzed changes in key autophagy markers.

During the initiation of autophagy, the cytosolic form of LC3 (LC3-I) is lipidated through conjugation with phosphatidylethanolamine to generate LC3-II, which associates with the autophagosomal membrane and is essential for autophagosome formation. In the later stages, p62 functions as an adaptor protein that mediates the sequestration of ubiquitinated proteins into autophagosomes and is subsequently degraded upon fusion of autophagosomes with lysosomes (40). Therefore, LC3 and p62 serve as major markers for evaluating

autophagy. HSC3 and FaDu cells were treated with 10 μ M PD and cisplatin for 48 h, followed by staining with LC3B and p62 antibodies and visualization using confocal microscopy. The results revealed that PD treatment significantly increased LC3B levels in both cell lines, with a further and more pronounced increase observed following combined treatment with cisplatin (Fig. 6A and C). Furthermore, p62 expression was significantly increased by PD treatment and was markedly further elevated in the combination treatment group compared with the single treatment groups (Fig. 6B and D). The increase in p62 expression suggested excessive accumulation of

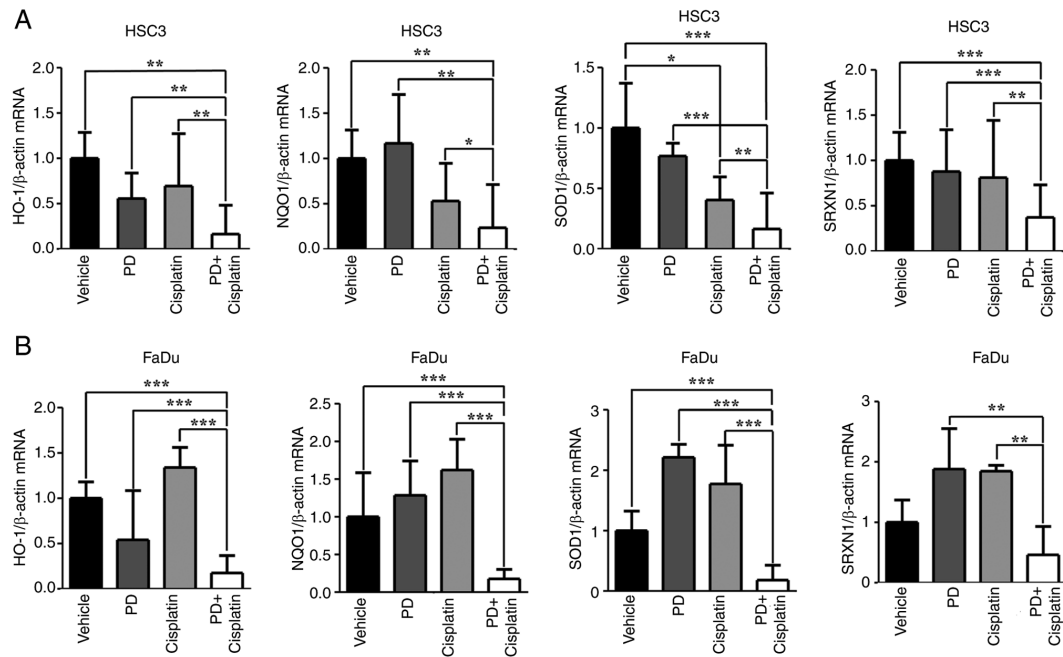


Figure 3. Combination of PD and cisplatin downregulates antioxidant gene expression in head and neck squamous cell carcinoma cells. (A) HSC3 and (B) FaDu cells were treated with PD (10 μ M), cisplatin (10 μ M) or combination treatment for 12 h (HSC3 cells) or 3 h (FaDu cells). mRNA expression levels of antioxidant genes (HO-1, NQO1, SOD1 and SRXN1) were measured by reverse transcription-quantitative PCR and normalized to β -actin expression. Combination treatment markedly reduced the expression of all four genes compared with single treatments. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. * P <0.05, ** P <0.01, *** P <0.001. PD, Platycodin D; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone dehydrogenase 1; SOD1, superoxide dismutase 1; SRXN1, sulfiredoxin 1.

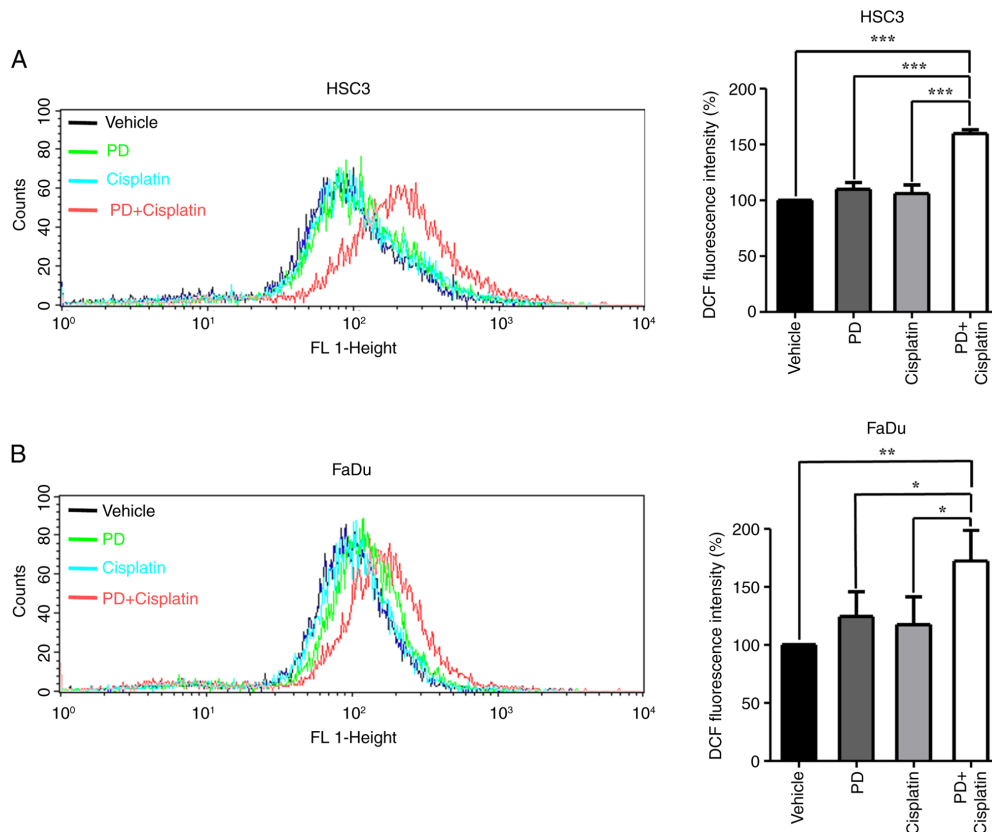


Figure 4. Combination of PD and cisplatin synergistically increases ROS levels in head and neck squamous cell carcinoma cells. (A) HSC3 and (B) FaDu cells were treated with PD (10 μ M), cisplatin (10 μ M) or combination treatment, and intracellular ROS levels were assessed by DCF-DA staining. Combination treatment markedly increased ROS levels compared with single treatments. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. In the graphs, vehicle, PD, cisplatin and combination treatment groups are represented by black, green, blue and red lines, respectively. FL indicates fluorescence intensity measured by flow cytometry and reflects intracellular ROS levels. * P <0.05, ** P <0.01, *** P <0.001. PD, Platycodin D; ROS, reactive oxygen species; DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate.

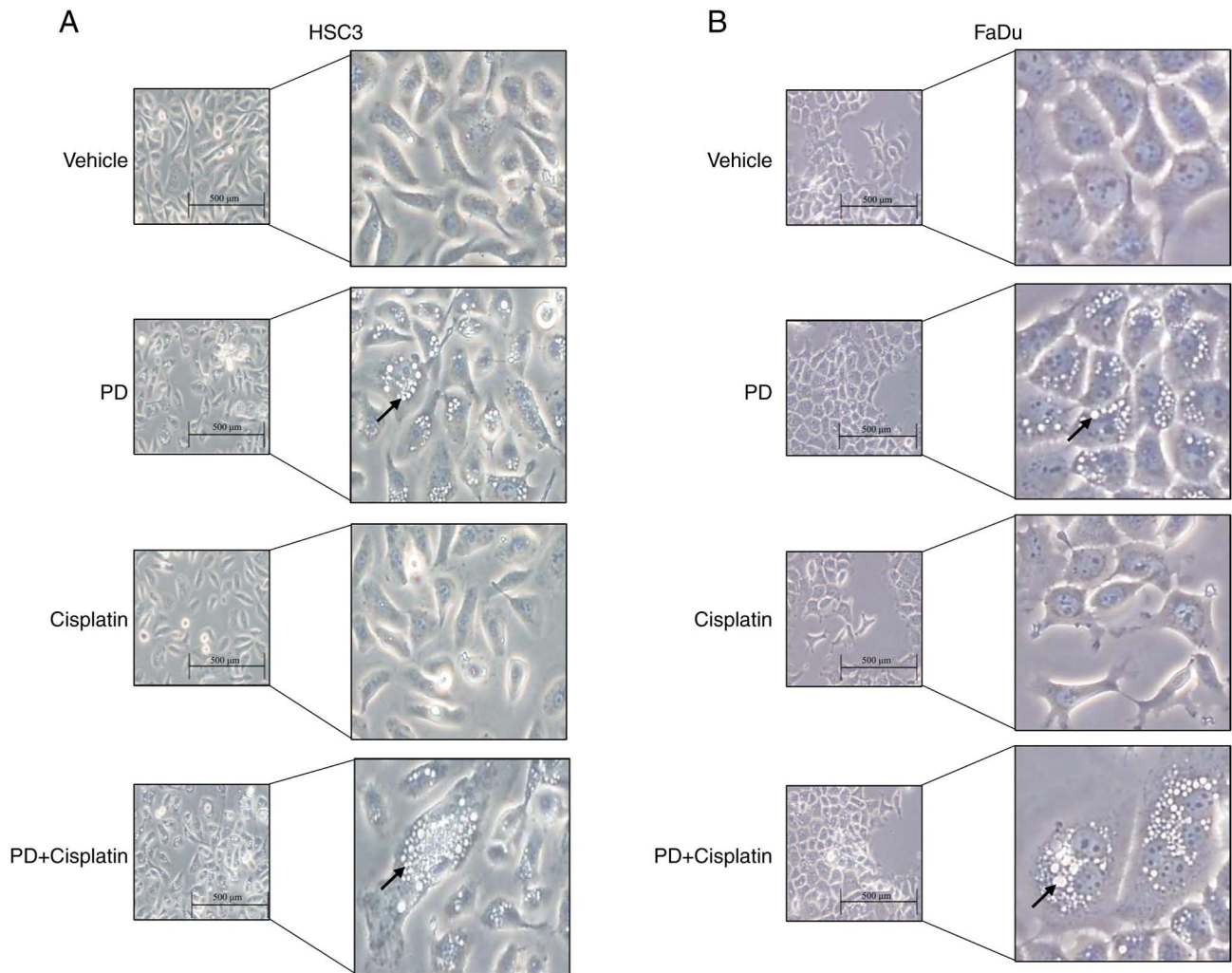


Figure 5. PD and cisplatin combination treatment induces morphological changes and vesicle accumulation. (A) HSC3 and (B) FaDu cells were treated with PD (10 μM), cisplatin (10 μM) or combination treatment for 48 h. Cell morphology was observed under a light microscope to evaluate vesicular structure accumulation. The images on the right show enlarged views of the boxed regions in the images on the left. Increased vesicular structures were observed in cells treated with PD alone or in combination with cisplatin and arrows indicate representative vesicular structures. Scale bar, 500 μm . PD, Platycodin D.

ubiquitinated proteins, indicating that combination treatment may disrupt the degradation process of autophagosomes.

To confirm these findings, western blot analysis was performed, which showed results consistent with the results of confocal microscopy. PD treatment induced the accumulation of LC3B, whereas combination treatment with cisplatin resulted in LC3B accumulation accompanied by increased p62 levels compared with the single treatment groups (Fig. 7A and B). The accumulation of LC3B can result from either enhanced autophagy initiation or impaired degradation in the later autophagy stages, while p62 accumulation occurs when the fusion of autophagosomes with lysosomes is blocked, preventing degradation (41,42). Given the marked increase in p62 expression observed in the present study, cells were treated with the lysosomal inhibitor Bafilomycin A1, a well-established positive control for late-stage autophagy inhibition (43), to further validate this interpretation. Bafilomycin A1 treatment resulted in marked accumulation of both LC3B and p62, mimicking the expression patterns observed in PD and cisplatin co-treated cells (Fig. 7A and B). Collectively, these findings suggested that combination

treatment with PD and cisplatin induced autophagy arrest by inhibiting autophagosome degradation at the late stage of autophagy, thereby contributing to the suppression of HNSCC cell proliferation.

The present study examined whether autophagy modulation serves a causal role in ROS accumulation induced by PD and cisplatin. HSC3 and FaDu cells were pretreated with the autophagy activator rapamycin prior to PD and cisplatin treatment, followed by DCF-DA staining and flow cytometry. Consistent with previous observations, treatment with PD or cisplatin alone resulted in a slight increase in ROS levels, whereas combination treatment markedly enhanced ROS accumulation. Notably, rapamycin pretreatment markedly reduced ROS levels induced by PD and cisplatin co-treatment (Fig. S2A). This reduction in ROS upon autophagy activation suggested that autophagic flux inhibition contributed to excessive oxidative stress under combination treatment conditions. These findings indicated that impaired autophagy served a functional role in ROS accumulation induced by PD and cisplatin, supporting a mechanistic association between autophagic flux arrest and enhanced oxidative stress in HNSCC cells.

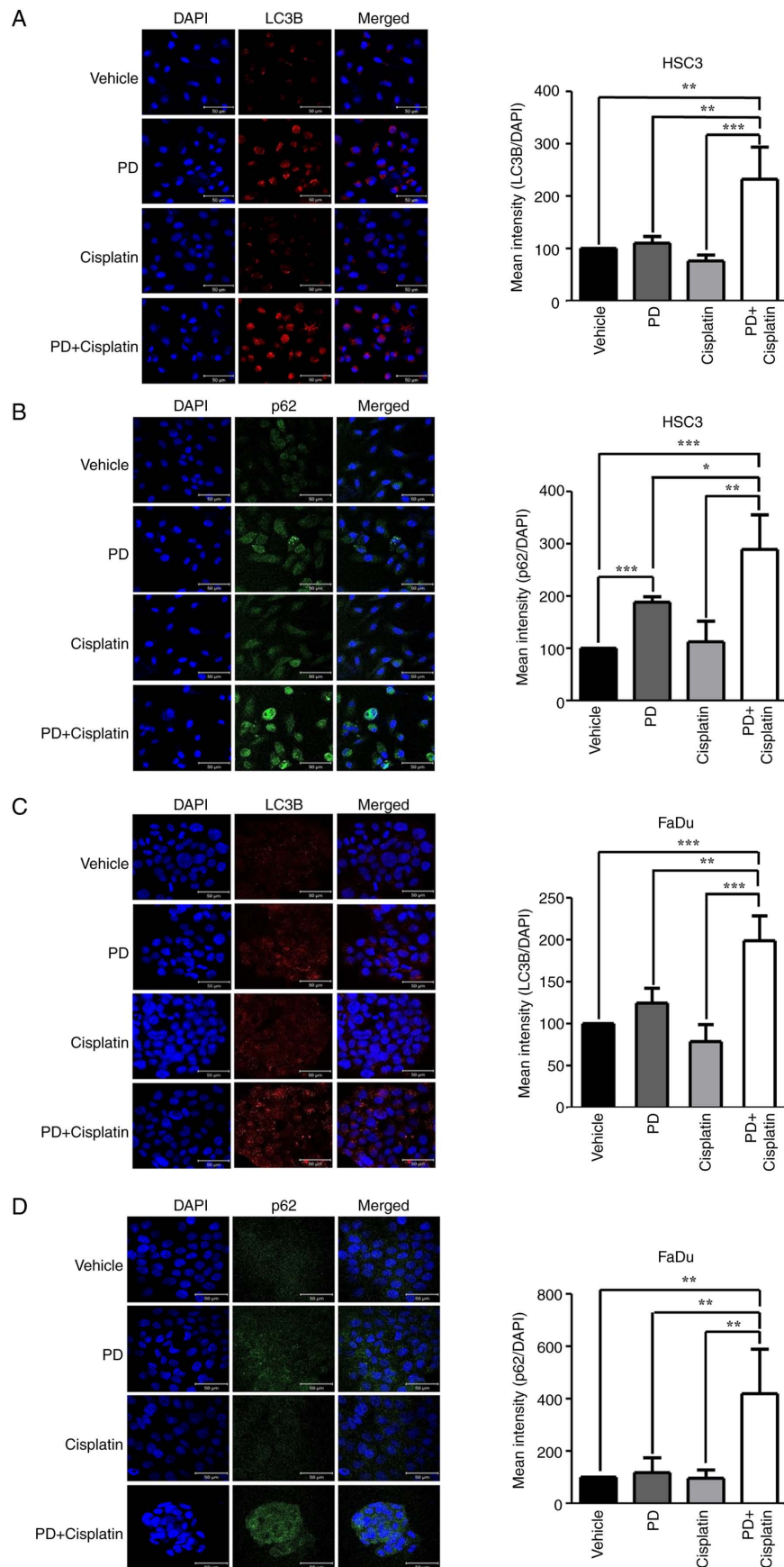


Figure 6. PD and cisplatin combination treatment increases autophagy marker levels in head and neck squamous cell carcinoma cells. (A) HSC3 and (C) FaDu cells were treated with PD (10 μ M), cisplatin (10 μ M) or combination treatment for 48 h. Cells were stained with LC3B antibody and analyzed using confocal microscopy. (B) In HSC3 cells, p62 immunofluorescence was increased following combination treatment compared with single treatments. (D) In FaDu cells, p62 staining similarly showed higher p62 levels in the combination treatment group than in the single treatment groups. Nuclei were stained with DAPI (blue), LC3B was stained red and p62 was stained green. Fluorescence intensity and puncta quantification were analyzed using ZEN software (Zeiss AG). Scale bar, 50 μ m. Data are presented as the mean $\pm$ SD (n=3). *P<0.05, **P<0.01, ***P<0.001. PD, Platycodin D.

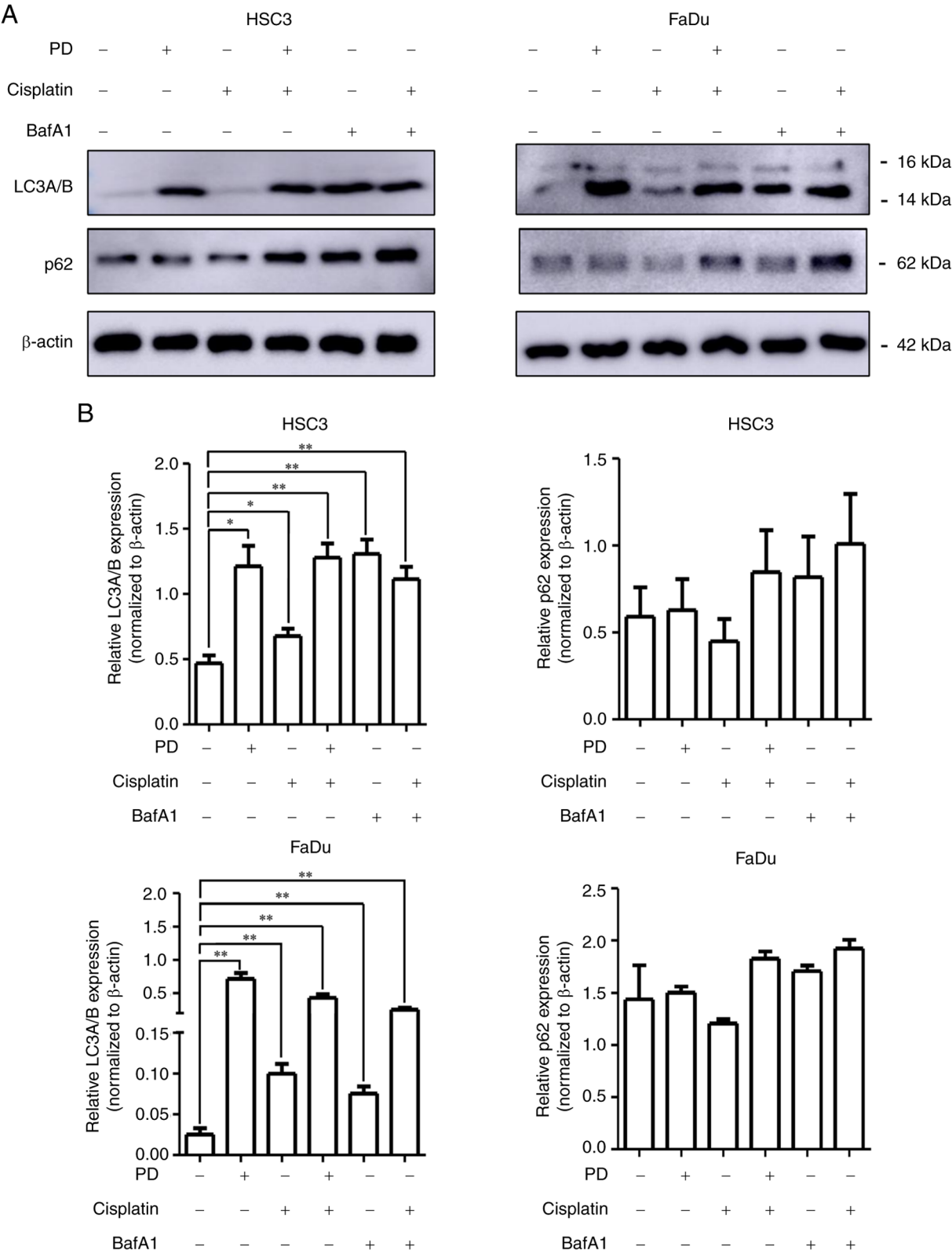


Figure 7. PD and cisplatin combination treatment induces autophagy arrest by inhibiting autophagosome degradation. HSC3 and FaDu cells were treated with PD (10 μ M), cisplatin (10 μ M) or combination treatment for 48 h, with or without BafA1. (A) Western blotting was performed to evaluate the expression levels of LC3A/B and p62. β -actin was used as a loading control. (B) Densitometric analysis of LC3A/B and p62 protein levels normalized to β -actin, performed using ImageJ software. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. Data are presented as the mean $\pm$ SD (n=3). *P<0.05, **P<0.01. BafA1, Bafilomycin A1; PD, Platycodin D.

Autophagy arrest-mediated apoptotic cell death induced by PD and cisplatin co-treatment. Given that ROS accumulation and autophagic flux inhibition are known to be closely

associated with cellular stress (44-46), the present study investigated whether the reduced cell viability observed following PD and cisplatin co-treatment was mediated by the induction

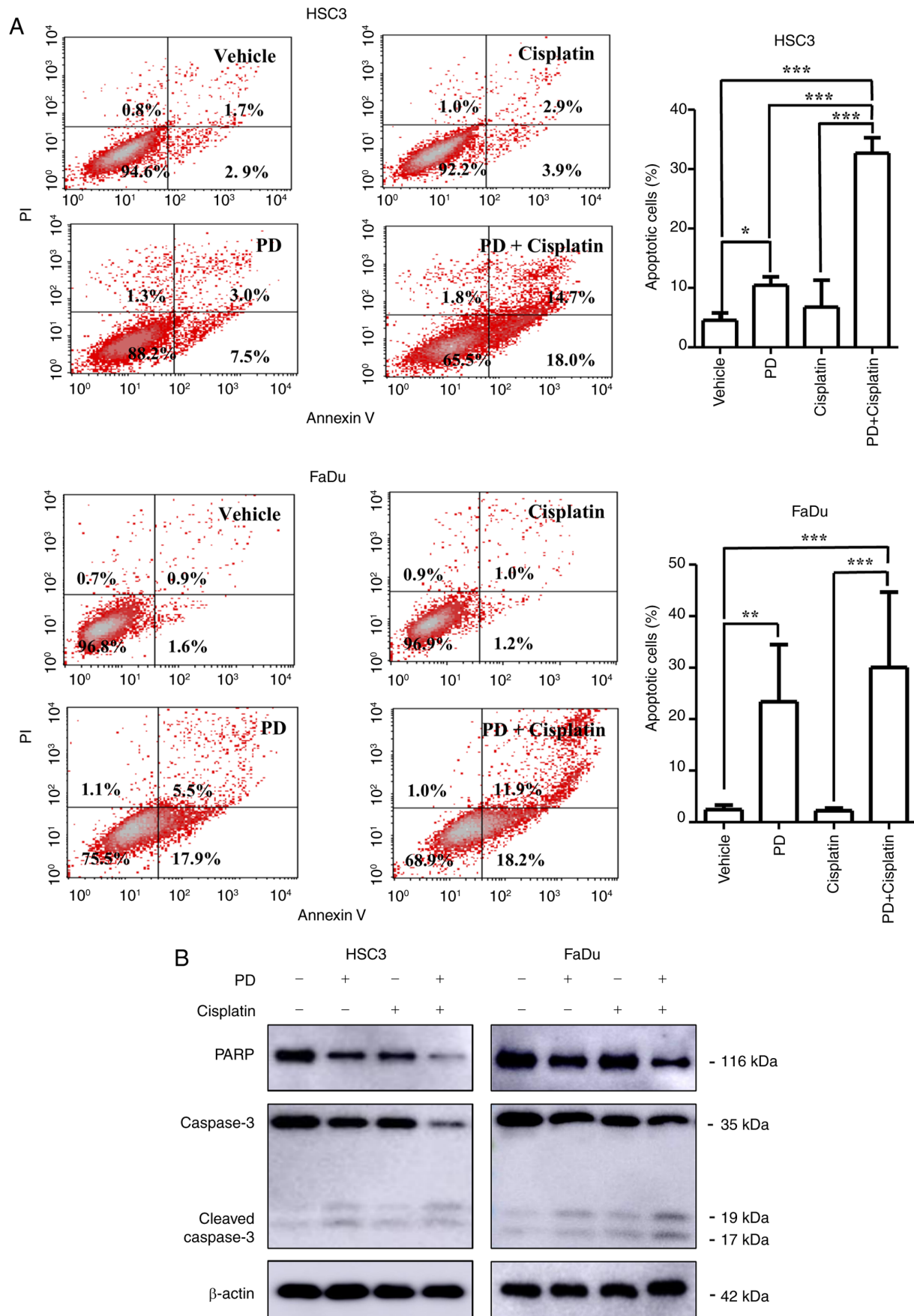


Figure 8. PD and cisplatin combination treatment induces apoptotic cell death in head and neck squamous cell carcinoma cells. (A) Apoptosis was analyzed by annexin V/PI staining followed by flow cytometric analysis in HSC3 and FaDu cells treated with PD (10 μ M), cisplatin (10 μ M) or their combination for 48 h. Combination treatment markedly increased the percentage of apoptotic cells. Data are presented as the mean $\pm$ SD (n=3). *P<0.05, **P<0.01, ***P<0.001. (B) Western blot analysis was performed to evaluate the levels of apoptosis-related proteins PARP and cleaved-caspase 3. β -actin was used as a loading control. PD, Platycodin D; PARP, poly(ADP-ribose) polymerase.

of apoptosis. HSC3 and FaDu cells were treated with 10 μ M PD, cisplatin or their combination for 48 h and apoptosis was assessed by annexin V/PI double staining. In HSC3 cells, the proportion of apoptotic cells increased from 10.46 $\pm$ 1.42% with PD treatment and 6.79 $\pm$ 4.51% with cisplatin treatment to 32.69 $\pm$ 2.60% following combination treatment, which was statistically significant. Similarly, in FaDu cells, the proportion of apoptotic cells was elevated from 23.41 $\pm$ 11.08% with PD treatment and 2.23 $\pm$ 0.51% with cisplatin treatment to 30.04 $\pm$ 14.64% following combined treatment, which was also statistically significant (Fig. 8A). These results were consistent with the cell viability assay findings.

Apoptosis is regulated by the activation of caspase 3, which subsequently cleaves downstream substrates such as PARP. Therefore, the present study analyzed the levels of caspase 3 and PARP as representative apoptosis markers (47). Combined treatment with PD and cisplatin resulted in a decrease in the levels of total caspase 3 and PARP, accompanied by an increase in cleaved-caspase 3 levels, indicating enhanced apoptotic cell death (Fig. 8B).

Collectively, these results demonstrated that co-treatment with PD and cisplatin induced apoptotic cell death through ROS accumulation and autophagic flux inhibition in HNSCC cells.

Discussion

The present study aimed to assess whether the combination of PD and cisplatin enhanced antitumor efficacy and to evaluate the potential of PD as a sensitizer to cisplatin. The findings of the present study were as follows: i) Combination treatment with PD and cisplatin reduced cell viability and a synergistic effect between the two agents was confirmed; ii) the combination treatment also markedly suppressed colony formation of HNSCC cells; iii) combination treatment led to a significant increase in intracellular ROS levels; iv) furthermore, it impaired autophagic flux, as evidenced by the accumulation of LC3B and p62 proteins, indicating defective autophagosome degradation; and v) combination treatment with PD and cisplatin induced autophagy arrest-associated cell death in HNSCC cells. Collectively, the results suggested that the combination of PD and cisplatin induced cell death in HNSCC cells by increasing oxidative stress and inhibiting autophagic flux.

HNSCC is primarily treated with definitive radiotherapy or surgery followed by adjuvant chemotherapy to prevent recurrence (4). Cisplatin, a platinum-based chemotherapeutic agent, is widely utilized as a key component of concurrent chemoradiotherapy in patients with HNSCC (8). However, cisplatin treatment is associated with adverse side effects, including hair loss, nephrotoxicity and immunosuppression, which can compromise long-term therapeutic efficacy and markedly reduce the quality of life of patients (48). Furthermore, the high level of drug resistance reduces therapeutic efficacy and ultimately leads to tumor recurrence (49). As a result of these therapeutic limitations, the 5-year survival rate for patients with HNSCC remains <50% worldwide, based on global epidemiological data reported in recent decades (50). Therefore, there is a critical need to develop effective therapeutic strategies that can enhance antitumor effects while reducing cytotoxicity

toward normal cells. In this context, the present study also evaluated the cytotoxic effects of PD in normal human keratinocytes. Notably, PD treatment did not markedly reduce cell viability in normal cells at the concentrations used, as assessed using an MTT assay. This finding suggested that PD exerted limited cytotoxicity toward normal cells while enhancing antitumor effects in HNSCC cells, highlighting its potential as a selective cisplatin sensitizer.

Cisplatin is a widely used chemotherapeutic agent that induces cell death primarily by causing DNA damage and disrupting redox homeostasis, leading to increased intracellular ROS levels (31,51,52). Cancer cells respond to cytotoxic stress by activating protective mechanisms such as autophagy. Autophagy is a catabolic process that degrades and recycles damaged organelles and proteins to maintain cellular homeostasis (51-53). Therefore, autophagy may serve as a crucial target for regulating the therapeutic efficacy of anticancer treatments.

PD, a triterpenoid saponin derived from the root of PG, has been reported to demonstrate pharmacological effects across various types of cancer, including lung and colorectal cancers (18,54), particularly through autophagy-related anticancer mechanisms (55). However, the effects of PD have not been fully examined in HNSCC, particularly in combination with cisplatin. Based on this, we hypothesized that PD could sensitize HNSCC cells to cisplatin-induced cytotoxicity by inhibiting autophagy, and a series of *in vitro* experiments were conducted to verify this hypothesis. Combination treatment with PD and cisplatin markedly reduced cell viability compared with single treatments, demonstrating a synergistic effect. Microscopic observation showed a substantial increase in intracellular vesicle structures in cells treated with the combination of PD and cisplatin compared with the vehicle control and single treatments, which were assumed to be associated with autophagic activity. Accordingly, the expression of the autophagy-related markers LC3B and p62 was analyzed. The levels of both proteins were upregulated following combination treatment, with a particularly notable accumulation of p62.

Autophagy is initiated in response to cellular stress, leading to the formation of double-membraned autophagosomes that enclose damaged organelles and proteins. These autophagosomes subsequently fuse with lysosomes to form autolysosomes, where the enclosed contents are degraded and recycled (56). LC3B is a key component of the autophagosome membrane and serves a crucial role in its formation, while p62 functions as a selective cargo adaptor that is typically degraded during autophagic flux (56,57). Therefore, accumulation of p62 indicates disrupted autophagic degradation. The present study observed increased levels of both LC3B and p62 following combination treatment with PD and cisplatin, suggesting that PD inhibited autophagosome-lysosome fusion and disrupted autophagic flux, thereby impairing the clearance of damaged cellular components. This interpretation was further supported by the observation that the effects of PD on LC3B and p62 accumulation were comparable to those induced by Bafilomycin A1, a late-stage autophagy inhibitor that blocks autophagosome-lysosome fusion.

Cisplatin induces the generation of ROS, thereby promoting cytotoxicity through oxidative stress (31), while autophagy can serve as a protective mechanism by

eliminating ROS and supporting cell survival (58). However, in the present study, PD-mediated inhibition of autophagy may have compromised this protective mechanism, leading to the accumulation of cisplatin-induced ROS within the cells. To verify this hypothesis, the present study analyzed the mRNA expression levels of antioxidant genes such as HO-1, NQO1, SOD1 and SRXN1 (59-61). The expression of these representative antioxidant markers was markedly decreased following combination treatment, suggesting that cancer cells failed to effectively activate their antioxidant defense system in response to severe oxidative stress. This impaired response may have increased the susceptibility of the cells to ROS-mediated damage. Furthermore, measurement of intracellular ROS levels using DCF-DA staining followed by FACS analysis demonstrated a significant increase in ROS levels in cells treated with the combination of PD and cisplatin. These results indicated that PD-mediated inhibition of autophagic flux disrupted the clearance of cisplatin-induced ROS, thereby exacerbating oxidative stress and ultimately promoting cell death. To further support this hypothesis, the present study observed that activation of autophagy by rapamycin markedly reduced ROS accumulation induced by PD and cisplatin co-treatment. Although these findings are promising, they require further validation *in vivo*. Further studies should assess the pharmacokinetics, toxicity and therapeutic effects of PD in animal models of HNSCC, in addition to further clarifying the molecular mechanisms associated with autophagy inhibition. Nevertheless, the present study provided a compelling rationale for the development of PD-based combination therapies as a novel strategy to enhance the efficacy of current chemotherapeutic regimens in HNSCC.

In conclusion, the present study proposed a mechanism in which PD inhibited autophagic flux, thereby enhancing the cytotoxic effects of cisplatin-induced ROS stress. This led to ROS accumulation, downregulation of antioxidant gene expression, increased oxidative stress and ultimately accelerated cell death. These findings suggested that the combination of PD (10 μ M) and cisplatin (10 μ M), selected based on cell viability assays to allow evaluation of synergistic effects, may exhibit a potent synergistic effect in the treatment of HNSCC and provide a potential strategy for the development of innovative combination therapies.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MB and YGE conceived the study. MGJ and SGK developed the methodology. MB conducted the experiments. MKL and SIK performed the data analysis. MB drafted the original manuscript and YGE reviewed and edited the manuscript. YL and HJ contributed to data visualization and participated in data interpretation. YCL and JWL contributed to data validation and interpretation and critically reviewed the manuscript for important intellectual content. YGE supervised the project. MB and YGE confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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