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8-O-acetylharpagide, an active compound isolated from *A. taiwanensis* selectively induced G2/M phase arrest and radiosensitivity in hypopharyngeal cancer cells

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ABSTRACT

Hypopharyngeal cancer (HPC) is a rare but most malignant subtype of head and neck squamous cell carcinoma (HNSCC). Conventional chemo-radiotherapy is the primary approach for the treatment of HPC, while the efficacy is limited and complications. Previously, we have isolated an active compound (AT-1) identified as 8-O-acetylharpagide from *Ajuga taiwanensis*. Because Ajuga extracts are both edible and known for their anti-cancer properties, it is worthwhile to investigate whether AT-1 can inhibit cancers of otolaryngological origin. In this study, we compared the cell killing effects of AT-1 on FaDu HPC cells and periodontal ligament (PDL) cells. The results showed that AT-1 was more toxic on FaDu cells (IC_{50} = 0.88 mM) than on PDL cells (IC_{50} = 1.65 mM), yielding a selectivity index (SI) ≈ 1.85. AT-1 caused significant G2/M phase arrest in FaDu cells with a dose-dependent manner. Concomitantly, high concentration of AT-1 could induce necrosis-like fraction and late apoptosis in FaDu cells. Notably, AT-1 did not influence the cell cycle redistribution and cell death in PDL cells under the same condition of treatment. Furthermore, AT-1 enhanced the radiosensitivity of FaDu cells rather than PDL cells, suggesting that AT-1 induced G2/M phase arrest remains sensitive to ionizing radiation known as a principle of radiobiology. Taken together, current data indicate that AT-1 raises selectively efficacy on HPC cells by increasing G2/M phase arrest, apoptosis, as well as radiosensitivity.

1. Introduction

Hypopharyngeal cancer (HPC) is a rare and aggressive malignancy arising from the hypopharynx, the lower portion of the pharynx that connects the oropharynx to the esophagus. HPC accounts for 2–14 % of whole head and neck squamous cell carcinoma (HNSCC), while it is typically diagnosed at advanced stages with approximately 70 % of cases suffered from lymphatic invasion and poor prognosis after chemoradiotherapy [1–3]. Squamous cell carcinoma (SCC) constitutes over 95 % of HPC based on the histological subtype, and mainly locates the pyriform sinuses [4]. The management of HPC involves a multidisciplinary approach, including surgery, radiation therapy, and systemic chemotherapy. However, treatment of HPC remains a challenge because of highly dissemination and complex anatomy of the hypopharynx that

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reduces therapeutic efficacy and increase complications.

Ajuga genus referred to as bugleweed in the Lamiaceae family, comprising over 60 species distributed across Europe, Asia, Africa, and North America. Several species of Ajuga plants are recognized for their bioactive compounds and potential therapeutic applications. Phytochemical investigations of Ajuga plants have revealed the presence of bioactive compounds such as neo-clerodane diterpenoids, iridoid glycosides, phenolics, flavonoids, phytoecdysteroids, anthocyanidin-glucosides, ergosterol-5, 8-endoperoxide, withanolides, phenylethanoid glycosides, quinols, sphingolipids and triterpenoids that highlight their potentials for against tumors, oxidative stress, parasites, fungi, microorganisms, inflammation, arthritis and diarrhea [5–19]. These medical properties are reported from various research groups from different countries focusing on individual local Ajuga species. *A. taiwanensis* is a perennial herbaceous plant belonging to the Lamiaceae family, endemic to Taiwan and commonly found throughout the island from sea level to 2000 m [20]. *A. taiwanensis* may also possess pharmacological properties as mentioned above, whereas it remains relatively understudied compared to other species in the Ajuga genus. It has been reported that the extract of *A. taiwanensis* exhibits anti-senescence activity in senescent human dermal fibroblasts (HDFs), and an active compound abundantly existed in n-butanol sub-fraction of the extract shows similar effects at low concentration [21]. This active compound was named AT-1 and identified as 8-O-acetylharpagide, which is involved in antifungal effect of Ajuga plants [22]. Whether this compound exhibits other effects, such as anticancer is interesting to be studied.

The 8-O-acetylharpagide isolated from *A. decumbens* exhibits cancer chemopreventive activity on carcinogen induced murine skin tumors and hepatic tumors [23]. The pharmacokinetics has been analyzed in rats and mice by oral administration, and showed that 8-O-acetylharpagide can be quickly absorbed and metabolized via oral route [24,25]. However, it is still largely unknown if 8-O-acetylharpagide displays the effect on otolaryngological related cancers.

In this study, we demonstrated that AT-1 active compound exhibited significant cytotoxicity in HPC FaDu cells over 0.5 mM. On the other hand, normal periodontal ligament (PDL) cells were less sensitive to AT-1 using the same dose range. We further found that AT-1 induced significant G2/M phase arrest in FaDu cells with a dose-dependent manner from 0.5 mM to 1 mM, but did not influence the cell cycle of PDL cells even using the highest concentration of AT-1. AT-1 also induced stronger apoptosis and radiosensitivity in FaDu cells than in PDL cells, suggesting that AT-1, known as 8-O-acetylharpagide is an effective agent on anti-HPC cells with low toxicity to normal cells. The preclinical and clinical advantage of this active compound is discussed.

2. Materials and methods

2.1. Cell lines

Human HPC FaDu cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA). Human periodontal ligament (PDL) fibroblasts were purchased from ScienCell Research Laboratories (Cat#2630, Carlsbad, CA, USA) and cultured in α-modified Eagle's medium (α-MEM, 12571063; Gibco, Thermo Fisher Scientific, MA, USA). Both cell lines were supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, and 50 U/mL penicillin, and were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 and passaged reaching 80 % confluence. Cell viability was assessed by counting before and after drug treatment. For viability assays, 5×10^5 cells were seeded in 10-cm dishes and allowed to adhere overnight.

2.2. Preparation of active compound

The active compound (AT-1) was purified and identified from the n-butanol sub-fraction partitioned from ethanol-extracted *A. taiwanensis* as reported before [21]. AT-1 was dissolved in dimethyl sulfoxide (DMSO) to prepare stock and working solutions.

2.3. Rounded cell Counting

Rounded cells were identified and quantified from microscopic images using ImageJ software (NIH, USA). The "Analyze Particles" function was applied after setting an appropriate threshold to distinguish cells from the background. Size and circularity parameters were adjusted to exclude debris and non-cellular objects. Cell counts were recorded and presented as the number of rounded cells.

2.4. Cell proliferation

Cell proliferation was measured using a hemocytometer. Data were expressed as absolute cell numbers

2.5. Cytotoxicity assay

Cytotoxicity was assessed using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In brief, cells (7500 per well) were seeded in a 96-well plate and treated with AT-1 at concentrations of 0.01–1 mM for 24 h. After treatment, MTT solution (1 mg/mL) was added to each well and incubated for at least 4 h. The reaction was terminated by adding DMSO, and the optical density at 570 nm (OD_{570}) was measured using a multi-well plate reader. Each condition was tested in at least six replicates, and each experiment's mean value was calculated. Cell viability was expressed as a percentage relative to the control group (treated with 1 % DMSO, set as 100 %). Selectivity Index (SI) was calculated as SI=IC_{50}(normal, PDL)/IC_{50}(tumor, FaDu); SI > 1 indicates tumor selectivity.

2.6. Measurement of intracellular ROS

The intracellular levels of reactive oxygen species (ROS) were assessed using 2′,7′-dichlorofluorescin diacetate (DCFDA), a membrane-permeable fluorescent dye that reacts with hydroxyl radicals, peroxyl radicals, and other ROS. Cells were plated in a 96-well black plate with a transparent bottom and incubated with the drug for 24 h. After treatment, the culture medium was discarded, and 20 μM DCFDA was added to each well. Cells were then incubated at 37 °C for 30 min, followed by the removal of excess dye before fluorescence measurement. Fluorescence intensity was measured using a multimode microplate reader (TECAN 200/200Pro, TECAN Group Ltd., Männedorf, Switzerland) with excitation/emission wavelengths of 495/535 nm. ROS levels were quantified by measuring fluorescence intensity (Ex/Em = 495/535 nm), normalized to background fluorescence, and adjusted for cell viability.

2.7. Cell cycle analysis

Cells treated with AT-1 were fixed in ice-cold 75 % ethanol (1×10^6 cells/3 mL) at 4 °C for at least overnight. Fixed cells were then treated with RNase A and incubated at 37 °C for 30 min. The cell pellets were resuspended in propidium iodide (PI, 20 μg/mL) and analyzed by flow cytometry. DNA content was measured using a CytoFLEX flow cytometer (Beckman Coulter, Inc., CA, USA), and DNA histogram were processed with the CytExpert software (Beckman Coulter, Inc., CA, USA).

2.8. PI/Annexin V-FITC dual staining

After 24 h of AT-1 treatment, cells were harvested and centrifuged.

The cell pellet (1×10^5 cells) was resuspended in 100 μL of 1 × binding buffer and stained with 2 μL Annexin V-FITC and 2 μL PI from the Annexin V-FITC Apoptosis Detection Kit (APOAF, Sigma-Aldrich, USA) for 15 min at room temperature in the dark. Apoptosis was analyzed by flow cytometry (CytoFLEX, Beckman Coulter, USA) and quantified using the CytExpert software (Beckman Coulter, Inc., CA, USA).

2.9. Western blot analysis

Proteins (25–30 μg) were separated by 12 % SDS-PAGE and transferred to NC membranes. The membranes were blocked with blocking buffer (4 % skimmed milk powder dissolved in TBST) for 1 h, then incubated at 4 °C overnight with the following primary antibodies: anti-GAPDH (1:5000, Genetex Inc., Irvine, CA, USA), anti-BCL2 (1:1000, Genetex Inc., Irvine, CA, USA), Akt (pan) (1:1000, Cell Signaling Technology, Danvers, MA, USA), Phospho-Akt (Ser473) (1:1000, Cell Signaling Technology, Danvers, MA, USA), p21 Cip1 (1:1000, Genetex Inc., Irvine, CA, USA), Cyclin B (1:2000, BD, Franklin Lakes, NJ, USA), anti-CHK1 (1:4000, Genetex Inc., Irvine, CA, USA), and anti-CDK1 (1:1000, BD, Franklin Lakes, NJ, USA). After washing with TBST, membranes were incubated with an appropriate HRP-conjugated secondary antibody: anti-mouse IgG, Peroxidase-conjugated or anti-rabbit IgG, Peroxidase-conjugated (1:10,000, Millipore Co., MA, USA) at room temperature for 2 h. Protein expression levels were detected using enhanced chemiluminescence (Genetex Inc., Irvine, CA, USA) and visualized with the ImageQuant LAS-4000 chemiluminescent imaging system (GE Healthcare, Buckinghamshire, UK). Protein expression levels were quantified using ImageJ software. Target protein intensities were normalized to GAPDH levels.

2.10. Assessment of radiosensitivity

Cells were seeded at a density of 7500 cells per well in a 96-well culture plate and pretreated with 1 mM AT-1 for 24 h. After exposure to 0, 2, 4, 6, or 8 Gy using an X-ray irradiator (X-RAD 225, Precision X-Ray, North Branford, CT, USA) at a dose rate of 1.34 Gy/min, the medium was replaced with fresh culture medium. Cells were then incubated for an additional 48 h before MTT assay (1 mg/mL) to each well and incubating for at least 4 h. The reaction was terminated by adding DMSO, and the optical density at 570 nm (OD570) was measured using a multi-well plate reader. Each condition was tested in at least ten replicates, and the mean value was calculated for each experiment. Cell viability was expressed as a percentage relative to the control group (0 Gy, set as 100 %).

2.11. Statistical analysis

Data were compared using one-way analysis or two-way analysis of variance (ANOVA), or unpaired *t*-test. Data were presented as mean ± standard error of the mean (SEM) from independent experiments. Statistical analyses were performed using GraphPad Prism 10.1.2 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. AT-1 exhibited stronger cytotoxic effects on HPC cells than PDL cells –

We have previously demonstrated that AT-1 can suppress senescent human dermal fibroblasts (HDFs) as low as 5 μM [21]. This effect was further demonstrated in an aging mouse gavaged with low dose AT-1 that significantly reduced SA-β-gal staining in several aging tissues (Supplementary Fig. 1). Nevertheless, little is known about the dose responses of AT-1 on cell viability, including normal cells and cancer cells. Here we compared the dose responses of AT-1 on human FaDu cancer cells and normal PDL cells using MTT assay. The results showed that FaDu cells were more sensitive to AT-1 than PDL cells after 0.5 mM treatment, while cell viability was significantly enhanced in PDL cells using low dose AT-1 (0.01mM–0.1 mM) for 24 h (Fig. 1A). The IC_{50} of AT-1 for FaDu cells and PDL cells were calculated as 0.88 mM and 1.65 mM, respectively. AT-1 exhibited higher cytotoxicity in FaDu than in PDL, with a selectivity index SI ≈ 1.85, preferential activity toward cancer cells.

Cell rounded changes were visualized under the phase contrast microscope, and it revealed that AT-1 caused dramatic cellular round-up and floating in than in PDL cells (Fig. 1B). Cell rounding is a well-recognized feature of mitotic entry which accompanies G2/M accumulation [26]. Rounded cells were further quantified and confirmed the significant effects of AT-1 on FaDu cells using ImageJ software (Fig. 1C). Additionally, the cell proliferation rate of FaDu cells was significantly suppressed by AT-1 (Fig. 1D). We next examined the level of reactive oxygen species (ROS) in cells treated with AT-1. However, the results showed that AT-1 did not increase ROS in both FaDu cells and PDL cells at the dose that could significant induce cytotoxicity (0.5mM–1mM) (Fig. 1E). Therefore, AT-1 induced cytotoxicity at high dose is not related to escalation of oxidative stress.

3.2. AT-1 selectively induces G2/M phase arrest in HPC cells rather in PDL cells

Next, we examined if AT-1 would affect the cell cycle distribution in FaDu cells and PDL cells. The DNA histogram showed that a dramatic G2/M phase arrest and increase of sub-G1 population were detected in FaDu cells treated with 1 mM AT-1 (Fig. 2A). On the contrary, the cell cycle distribution of PDL cells was little changed using the same concentration of AT-1 (Fig. 2B). We also showed that AT-1 exhibited a dose-dependent effect on G2/M phase arrest of FaDu cells (Supplementary Fig. 2). The percentages of cell cycle phases were quantified and compared in these two cells with or without the treatment of AT-1, and it also showed that sub-G1 phase representing apoptosis was mostly increased in AT-1 treated FaDu cells (Fig. 2C). In addition, the Western blot analysis showed that the expressions of several G2/M phase associated molecules, including CHK1, cyclin B, CDK1 and p21 were altered and correlated to G2/M phase arrest in FaDu cells but not in PDL cells treated with AT-1 (Fig. 2D). These data suggest that AT-1 can selectively induce G2/M phase arrest in cancerous cells but not in normal cells.

3.3. Effects of AT-1 on induction of apoptosis in HPC cells

We have shown that high concentration of AT-1 exhibited stronger effects on induction of cytotoxicity on FaDu cells than on PDL cells. Additionally, the ratio of sub-G1 phase of FaDu cells was also dramatically increased by AT-1 treatment. We further used PI/Annexin V dual staining kit to detect the level of necrosis-like and apoptosis fraction after the treatment of AT-1. H_2O_2 (100 μM) was used as a positive control. The flow cytometry histogram of PI/Annexin V dual staining revealed that AT-1 induced a high level of necrosis-like and apoptosis fraction in FaDu cells, whereas the effect was relatively low in PDL cells (Fig. 3A). Additionally, each phase of stained cells was quantified and classified, revealing that AT-1 significantly induced necrosis-like and apoptosis fraction in FaDu cells (Fig. 3B). On the other hand, these effects were barely detected in PDL cells (Fig. 3C). The anti-apoptotic molecules including AKT, phosphorylated AKT and Bcl-2 were also down-regulated in FaDu cells treated with AT-1 (Fig. 3D). Therefore, current data indicate that AT-1 not only induced G2/M phase arrest but also apoptosis in HPC cells.

3.4. AT-1 increases the radiosensitivity of HPC cells but not that of PDL cells

As G2/M phase is the most radiosensitive phase of the cell cycle, we are interested in investigating if AT-1 can also enhance the

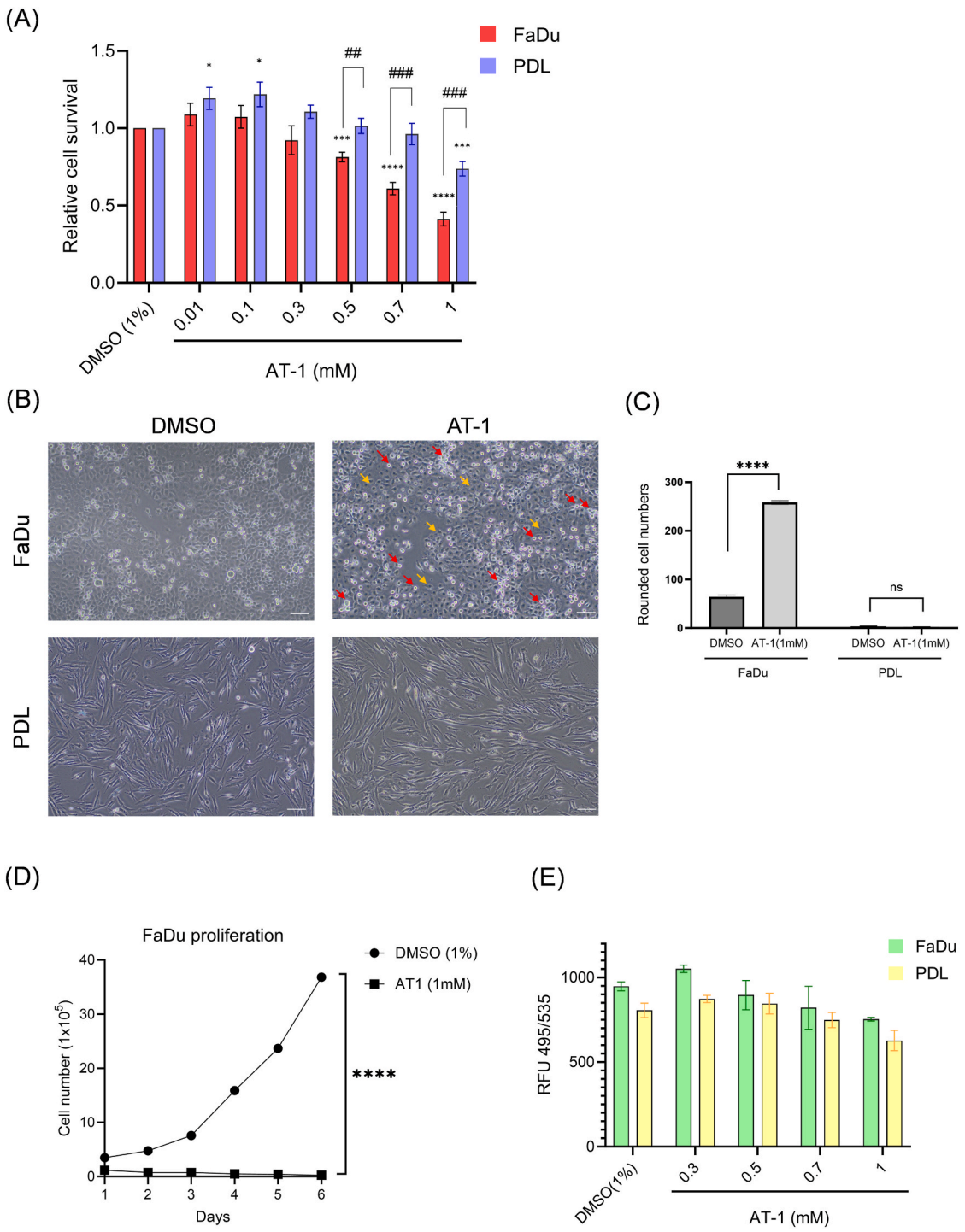

Fig. 1. Effects of AT-1 on FaDu cells and PDL cells. (A) Cell viability was assessed using the MTT assay after FaDu cells and PDL cells were treated with different concentrations of AT-1 for 24 h. (B) Images of FaDu and PDL cells treated with AT-1 (1 mM) or DMSO for 24 h. Red arrows indicate rounded, mitosis-like cells and yellow arrows indicate unchanged cells for demonstration. Scale bar = 100 μm. (C) Quantification of rounded cells from cell images. (D) Comparison of cell proliferative rates of FaDu cells treated with AT-1 or DMSO. (E) Quantification of intracellular ROS levels using the DCFDA fluorescence assay. Data are normalized to cell viability (mean ± SEM, n = 3). * and #: $p < 0.05$, ** and ###: $p < 0.005$, ##: $p < 0.01$, ***: $p < 0.001$, and ****: $p < 0.0001$ compared to the control group.

radiosensitivity of HNSCC cells that never reported before. Because conventional colony formation assay can not be applied to normal PDL cells, we used MTT assay to compare the survival curves of FaDu cells and PDL cells treated with 1 mM AT-1 and incremental radiation dose. The results showed that AT-1 indeed enhanced the radiosensitivity of FaDu cells but not that of PDL cells (Fig. 4). Hence, AT-1 induced G2/M phase arrest in HNSCC cells can contribute to the radiosensitivity, suggesting that AT-1 would be a radiosensitizer in cancer treatment.

4. Discussion

Previously, we have isolated the active compound AT-1 from *A. taiwanensis* and demonstrated its capacity on suppression of cell senescence using low concentration (5 μM). In the same study, we have also demonstrated that oral administration of AT-1 can suppress senescent phenotypes in aging mice [21]. In this study, we showed that AT-1 inhibited the viability of FaDu cells but not normal PDL cells over

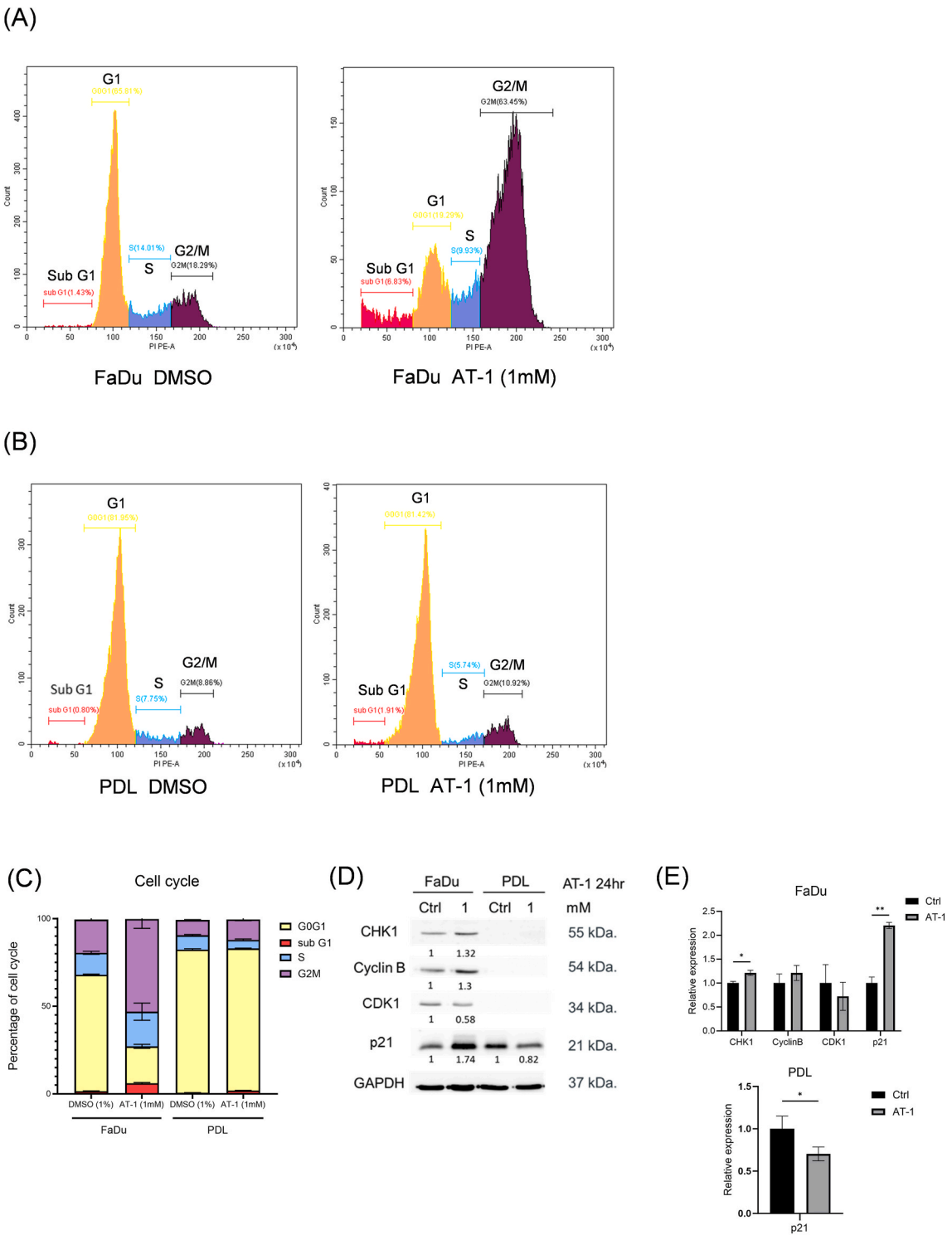

Fig. 2. AT-1 induced G2/M phase arrest in FaDu cells but not PDL cells. (A) and (B) Flow cytometric analysis of cell cycle distribution in FaDu cells and PDL cells treated with or without AT-1 for 24 h, respectively. (C) Quantification of cell cycle phases (Sub G1, G0/G1, S, G2/M) in FaDu and PDL cells. Data were presented as mean ± SEM from three independent experiments. (D) Western blot analysis of cell cycles related proteins in FaDu cells and PDL cells treated with AT-1. Cyclin B1, CDK1, p21, and CHK1 protein expression levels were normalized to GAPDH. (E) Summary bar graphs for Western blot quantification (mean ± SEM, n = 3). * and #: $p < 0.05$, ** and ###: $p < 0.005$ compared to the control group.

0.5 mM via induction of G2/M phase arrest. Although low dose of AT-1 suppresses cell senescence, whether high dose of AT-1 would influence senescence of FaDu cells is interesting to investigate in the future. The chemical form of AT-1 is known as 8-O-acetylharpagide, which has been reported to inhibit the proliferation of A431 skin cancer cells with IC_{50} at 0.31 mM [27]. It suggests that AT-1 is not a highly toxic active compound but is still effective on reducing the viability of different types of cancer cells at millimolar range. Importantly, the cytotoxicity of AT-1 on normal PDL cells is extremely low, suggesting that the complications would be minimized using this active compound for cancer treatment.

As reported previously, low concentration of AT-1 could suppress ROS in senescent HDFs [21]. This effect was still detected in both FaDu

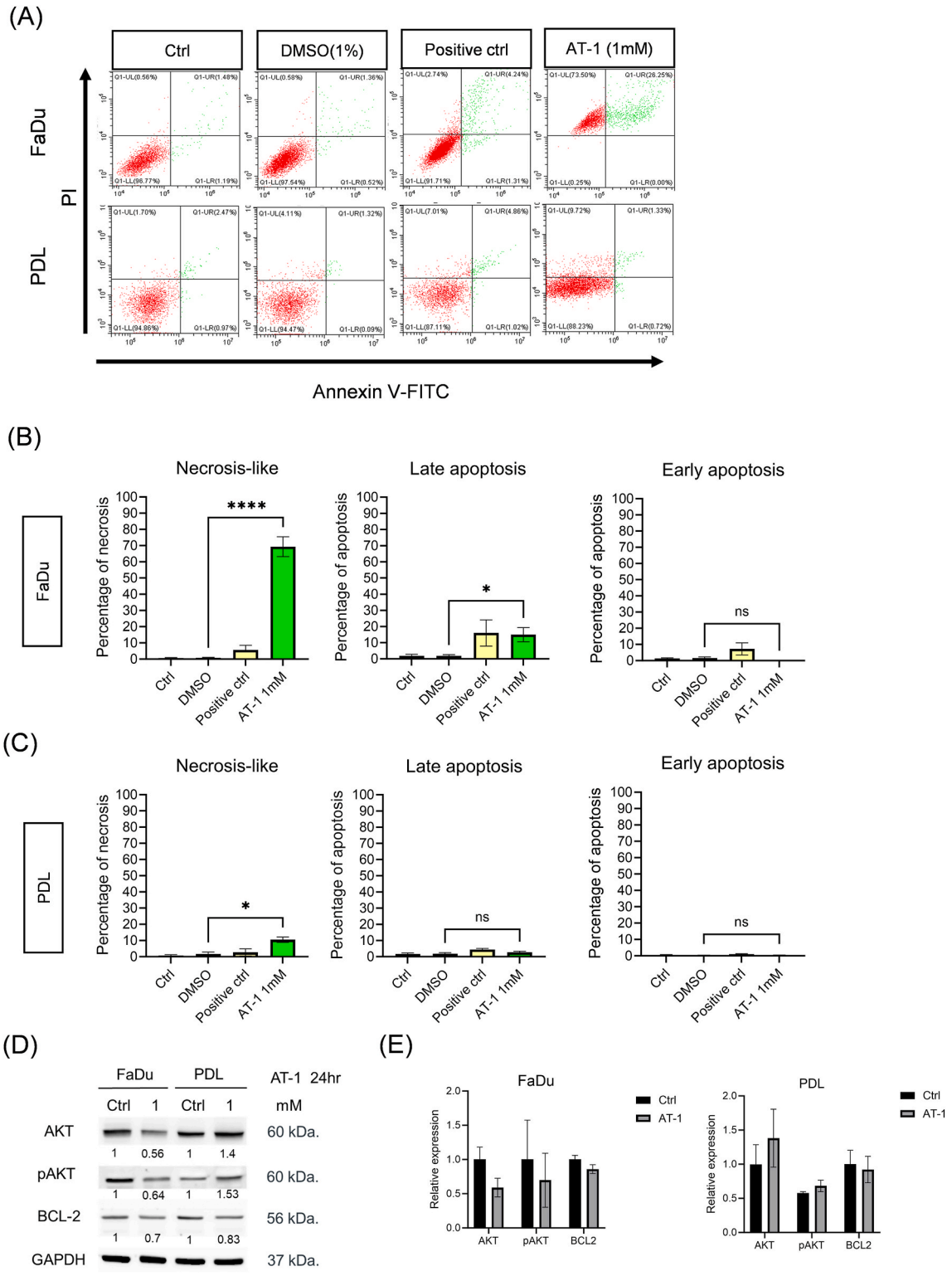

Fig. 3. Effects of AT-1 induced apoptosis in FaDu cells and PDL cells. (A) Flow cytometric profiles of annexin V/PI dual staining in FaDu cells and PDL cells with different treatments for 24 h. (B) and (C) Quantification of necrosis-like, late apoptosis, and early apoptosis in FaDu cells and PDL cells, respectively. (D) Western blot analysis of apoptotic related proteins in FaDu and PDL cells treated with AT-1. AKT, pAKT, and BCL-2 protein expression levels were normalized to GAPDH. (E) Summary bar graphs for Western blot quantification (mean ± SEM, n = 3) *: $p < 0.05$, ****: $p < 0.001$.

cells and PDLs treated with high concentration of AT-1, indicating that AT-1 mediated anti-tumor activity is not caused by increase of oxidative stress. Surprisingly, we found that AT-1 could dramatically induce G2/M phase arrest in FaDu cells rather PDL cells at the same concentration. The G2/M phase related biomarkers were barely detected in PDL cells compared to FaDu cells, suggesting that AT-1 would be more effective on inhibition of fast-growing cancer cells. A recent report found that 8-O-acetylharpagide isolated from *A. decumbens* Thunb could inhibit the growth of syngeneic breast tumors using murine 4T1 cells [28]. The same active compound has also been reported to exhibit anti-tumor

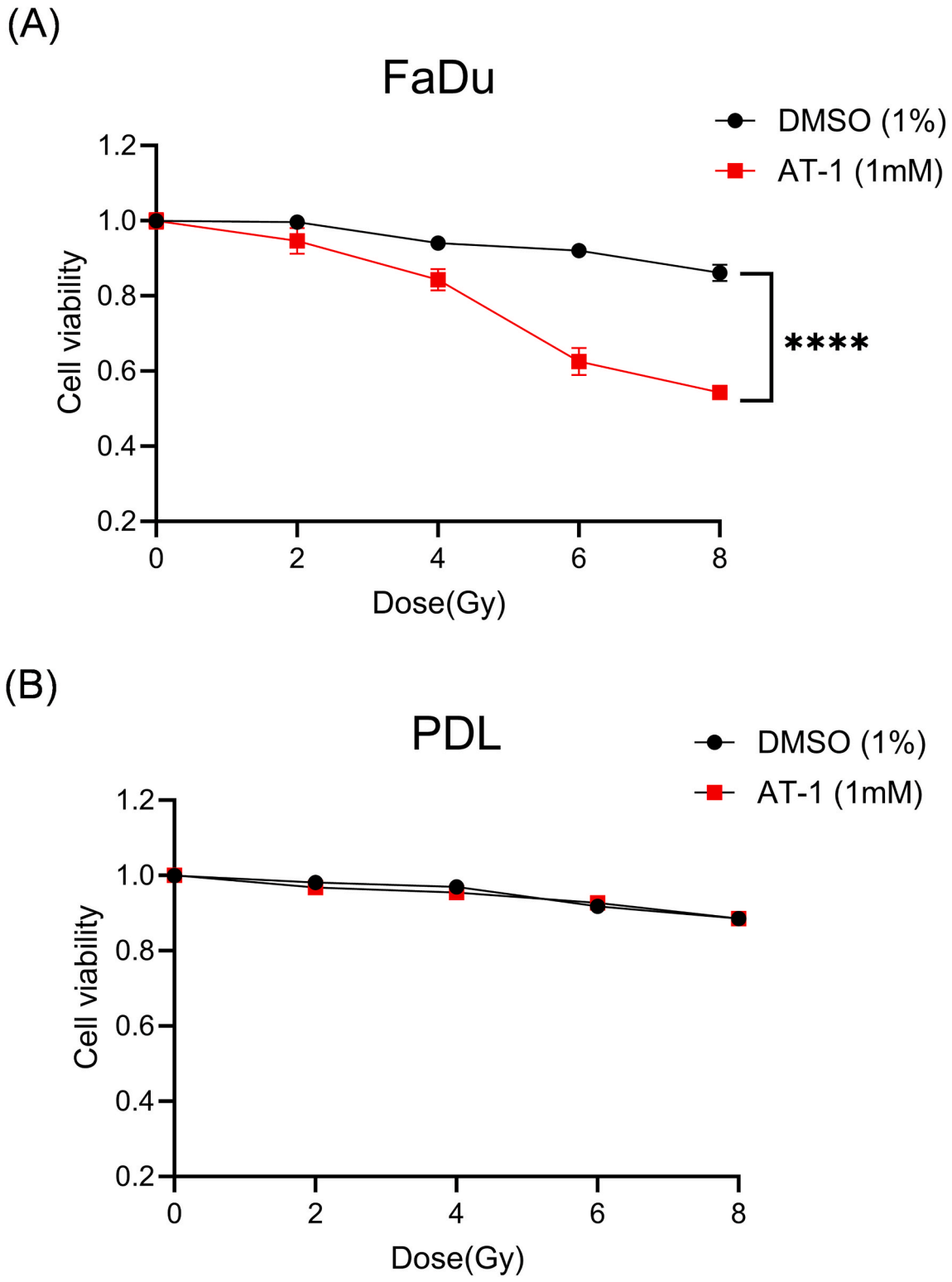

Fig. 4. Effects of AT-1 on radiosensitivity of FaDu cells and PDL cells. (A) and (B) MTT assay for analysis of the cell viability of FaDu cells and PDL cells irradiated with different doses of X-rays after AT-1 treatment, respectively. Cell viability was presented as fractions relative to the control group (0 Gy, set as 1). ****: $p < 0.0001$.

promoting activity in carcinogens-induced skin, liver and pulmonary tumors [23,29]. However, little is known whether these antitumor activities are all due to 8-O-acetylharpagide induced G2/M phase arrest. Our findings suggest that a further investigation of AT-1 on the cell cycle redistribution of different cancer types would be interesting and important for clinical application in the future.

In addition to the cell cycle effects, we also demonstrated that high concentration of AT-1 induced significant PI-positive in FaDu cells but not in PDL cells as determined using PI/Annexin V dual staining [30]. Despite G2/M phase arrest and apoptosis can be concomitantly induced by several anti-tumor compounds [31–34], little is known that 8-O-acetylharpagide can also cause the same effect. A domestic paper written in

simplified Chinese showed that 8-O-acetylharpagide induces G1 phase arrest and apoptosis in human colon cancer HCT116 cells [35]. To the best of our knowledge, 8-O-acetylharpagide as the AT-1 active compound can induce dramatic necrosis-like fraction in HPC cells has not been reported. PI positive primarily indicates loss of membrane integrity and is compatible with late apoptosis or secondary necrosis but not diagnostic of primary necrosis. Therefore, additional markers of necrosis is required for validation [36]. Notably, this cell killing event is not related to induction of cellular ROS that commonly found in other anticancer drugs [31–33]. Because AT-1 showed selective killing effect on HPC cells and normal cells, further investigation into the molecular mechanisms of necrosis and apoptosis uniquely triggered in cancer cells after treatment would be valuable.

G2/M phase is known as the most radiosensitive phase of the cell cycle. As expected, high concentration AT-1 only enhanced radiosensitivity in FaDu cells rather PDL cells. At present, most radiosensitizers are common chemotherapeutic agents, such as cisplatin, 5-fluorouracil, or taxanes [37]. Development of active compounds from Chinese herbs, such as curcumin, resveratrol, dihydroartemisinin and paclitaxel for sensitizing cancer cells to radiotherapy has been reported [38]. These active compounds are easily to be accepted by patients because they are mainly isolated from edible plants, and may be regarded health food or nutrients. As AT-1 showed high specificity to cancerous cells and low toxicity to normal cells, it would be an ideal radiosensitizer for radiotherapy. However, a promising preclinical study is required to demonstrate this perspective.

In summary, current data demonstrated that the active compound AT-1 would be an anti-cancer agent with potent radiosensitizing ability for HPC treatment, and non-toxic to normal cells. It is interesting to investigate whether other types of cancer cells exhibit similar response to AT-1 treatment in future studies.

CRediT authorship contribution statement

Wan-Yu Yang: Conceptualization, Data curation, Formal analysis, Validation, Writing – original draft, Writing – review & editing. **Yung-Cheng Wang:** Project administration, Supervision. **Yun-Lian Lin:** Conceptualization, Resources, Validation. **Kuei-Yuan Hou:** Project administration, Validation. **Wan-Chun Li:** Resources, Validation. **Yi-Jang Lee:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2025.102431.

Data availability

No data was used for the research described in the article.

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