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Original Article

Flavopereirine exerts anti-cancer activities in various human thyroid cancer cells

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Abstract: Thyroid cancer (TC) stands out as the most prevalent endocrine malignancy globally, with a steadily increasing incidence. Its clinical manifestations include enlarged thyroid nodules, dysphagia, enophthalmos, and various other symptoms. While standard treatments such as thyroidectomy and radioiodine therapy effectively manage most cases of differentiated thyroid cancers (DTC), some recurrent cases of DTC or those involving poorly differentiated thyroid cancers (PDTC) require specialized interventions. However, existing drugs primarily address symptom management without offering a curative solution. Therefore, the development of a new therapeutic agent for these challenging cases is of utmost importance. Flavopereirine, derived from *Geissospermum vellosii*, has demonstrated promise as a potential anti-cancer agent across various human cancers. However, its specific anti-cancer effects on human thyroid cancer (TC) have remained unclear. Therefore, this study aims to investigate the anti-cancer activity of flavopereirine in human TC. The research findings revealed that flavopereirine effectively hinders the growth of human TC cells, induces cell cycle arrest, promotes apoptosis, and modulates autophagy. Moreover, the study delved into the underlying mechanisms by which flavopereirine influenced signaling pathways. To validate these anti-cancer effects, an *in vivo* zebrafish model was utilized, confirming the efficacy of flavopereirine against human TC cells. In summary, this study establishes that flavopereirine exhibits notable anti-human TC activities, positioning it as a promising therapeutic candidate for the treatment of human thyroid cancer.

Keywords: Flavopereirine, thyroid cancer, anti-cancer activity, apoptosis, autophagy

Introduction

Among endocrine tumors, thyroid cancer is the most common cancer, and the incidence rate is increasing [1]. Thyroid cancer can be derived as follicular epithelial cells and neuroendocrine cells (parafollicular or C cells) according to histopathology. Cancer with follicular epithelial cells include papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC) and anaplastic thyroid carcinoma (ATC). PTC and FTC are collectively called differentiated cancers, and ATC is classified as undifferentiated cancers. In addition, TC generated from neuroendocrine cells is medullary thyroid carcinoma (MTC). The incidence rate in women is about twice than men [2]. The Risk factors for TC include age, family history, and radiation exposure in childhood [2]. Among TC, PTC accounts for the vast majority, accounting for approximately 70-75%, and FTC accounts for the second highest rate, about 10% [2]. Moreover, Papillary cancer occurs more frequently in iodine-rich areas. In addition, undifferentiated cancer accounts for the least, about 2-5% [2].

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Currently, patients with PTC or FTC can be treated through total thyroidectomy and local lymph node dissection, followed by additional atomic iodine therapy, and the prognosis is very good. The more difficult aspects of clinical treatment are metastasized or recurrence PTC or FTC, as well as ATC. These patients show very poor effect of either chemotherapy or radiotherapy. Especially patients with ATC is extremely aggressive and lethal. It is one of the most malignant tumors in humans. Although its incidence rate is not high, unfortunately, the current therapeutic effect of ATC is still very poor, and most studies show that the average survival time is only 3 to 9 months [3]. Therefore, it is urgent to develop an effective therapeutic drug against ATC or patients with metastasized or recurrent PTA and FTC.

Flavopereirine, a β-carboline alkaloid which is isolated from the bark of *Geissospermum vellosii* [4]. Flavopereirine has antiplasmodial, antileishmanial activity and can selectively inhibit DNA synthesis of cancer cells [4-6]. Moreover, it also has been suggested for benign prostatic hyperplasia treatment [7]. Recent studies demonstrate that flavopereirine exerts anti-human cancer activity in glioblastoma, hepatoma, breast, colorectal and oral cancer cells through inhibition of cell proliferation, and induction of cell cycle arrest, cell apoptosis and autophagy [8-13]. The mechanisms including DNA synthesis inhibition and modulation of JAK-STAT, p53, Akt, p38, and Erk pathways have been reported to involve in flavopereirine mediated anti-cancer effects [8-10, 12, 13]. However, the detail mechanisms of flavopereirine regarding anti-cancer effect are still unclear, especially on human thyroid cancer cells. Therefore, in the present study, we aim to address whether flavopereirine can suppress human thyroid cancer cells and the underling mechanisms.

In this study, we demonstrate that flavopereirine administration could reduce cell proliferation in both ATC and ATC cells. It also showed more safety in skin normal fibroblast cell line. Moreover, a G0/G1 cell cycle arrest was determined in the cells with flavopereirine treatment. In addition, cells with flavopereirine treatment elevated a cellular apoptosis through both extrinsic and intrinsic caspase-dependent apoptosis pathways in both PTC and ATC cells. The activation of signaling pathways including Erk and p38, and the inhibition of Akt pathway were determined in the cells under flavopereirine treatment. Finally, the anti-thyroid cancer activity of flavopereirine was verified *in vivo* with a zebrafish model. Our results demonstrated that flavopereirine is a safe compound and exerts a potential anti-TC activity *in vitro* and *in vivo*.

Materials and methods

Cell lines and cell culture

Human multidrug-resistant PTC cell line (IHH-4) and ATC cell lines (KMH-2 and 8505c) were purchased from Japan Collection of Research Bioresources Cell Bank (JCRB), Japan. Human PDTC cell line (SW579) was purchased from Bioresource Collection and Research Center (BCRC), Taiwan. The FTC cell line (WRO) was provided by Prof. Jen-Der Lin [1]. IHH-4 and KMH-2 cells were incubated with Dulbecco's minimal essential medium (DMEM) + Roswell Park Memorial Institute (RPMI) (1:1) medium (Gibco, Gaithersburg, MD, USA); 8505c cells were cultured with MEM (Gibco); and SW579 and WRO cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), at 37°C in a 5% CO_2 incubator. The passage ranges for the cell lines used in this study were p05-p20 post-purchase.

Cell viability assay

The cells (5×10^3 cells/well) were cultured with the previous medium in a 96-well cell culture dish; after overnight incubation, the cells were maintained with the control medium (containing 0.01% DMSO) or flavopereirine (ChromaDex, Irvine, California, USA). After incubation for 24, 48 and 72 h, the cell survival was detected with a CCK-8 assay kit (Enzo Life Sciences, Farmingdale, NY, USA). Three independent assays were performed.

Colony formation assay

The cells were cultured with the previous medium in 6-well plates (10^3 cells/well), and the plates were incubated at 37°C incubator. And the cells were cultured with DMSO or flavopereirine. The colony formation of the cells and

the colony morphology were detected under 10% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) staining after 12 days incubation. The colony size and the colony number were examined.

Cell cycle examination

Cells were incubated with the previous medium containing either flavopereirine or DMSO for 6 and 18 h, and the cells were harvested and fixed in 70% ethanol overnight. After double washing with PBS, cells were labeled with propidium iodide (PI; Sigma-Aldrich) and incubated at room temperature in the dark for 30 min. DNA content was analyzed using FACScan (Becton Dickinson, San Diego, CA, USA) with Modfit. LT 3.3 software. Three independent assays were performed.

Cell apoptosis determination

Cells were incubated with the previous medium containing either flavopereirine or DMSO for various durations. Annexin-V (Sigma-Aldrich) and PI double staining was performed to detect apoptotic cells as our previous reports [14-17]. The cells were washed with PBS twice and centrifuged at 1500× *g* for 10 min. The cell pellets were resuspended with staining solution (2 μl Annexin-V-FITC and 2 μl PI in 100 μl binding buffer) and incubated at room temperature in darkness for 15 min. Annexin-V or PI fluorescent intensities were analyzed by FACScan (Becton Dickinson), and 10,000 cells were assessed in each sample. Three independent assays were performed.

Western blotting

To verify the mechanisms involving in flavopereirine mediated cellular apoptosis, cells were incubated with DMSO or flavopereirine, and the total cell lysate was collected and the proteins were separated with a sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were then transferred on a polyvinylidene fluoride membrane, and the expressions of the proteins were examined after blocking with a primary antibody and with a secondary antibody. The experimental process could refer to our previous paper [17].

The primary antibodies used in this study including caspase-3, -8, -9 (#9662, #9746, #9502, Cell Signaling, Danvers, MA, USA), Bid (GTX60429, GeneTex, Hsinchu City, Taiwan), Bcl-xL (#2762, Cell Signaling), LC3 (PM036, Medical and Biological Laboratories, Nagoya, Japan), p62 (AP2183b, Abgent, San Diego, CA, USA), BNIP3 (GeneTex), Akt (sc-8312, Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphor-Akt (sc-7985R, Santa Cruz Biotechnology), mTOR (#5562, Cell Signaling), phosphor-mTOR (#2475, Cell signaling), Erk (#4696, Cell Signaling), phosphor-Erk (#9106, Cell Signaling), p38 (#9212, Cell Signaling), phosphor-p38 (#9211, Cell Signaling), JNK (#9252, Cell Signaling), phosphor-JNK (#9251, Cell Signaling) and poly (ADP-ribose) polymerase (PARP; #9542, Cell Signaling) were assessed with Western blotting. The expression of GAPDH (GTX100118, GeneTex) was determined as a loading control in the Western blotting. To verify whether caspase activation was participated in flavopereirine-mediated cellular apoptosis, the pan-caspase inhibitor, Z-VAD-fmk (BioVision, Mountain View, CA, USA), was used and the cellular apoptosis was assessed using FACScan after double staining with FITC-labeled Annexin-V and PI.

Cellular autophagosome determination

The cells were cultured on a cover slip in a 10 cm culture dish, after overnight incubation in a 37°C incubator. The cells were incubated with medium containing DMSO or flavopereirine for 24 h, and the cellular autophagosomes were examined with immunostaining with LC3 antibody (Medical and Biological Laboratories) and fluorescein isothiocyanate-conjugated secondary antibodies (GeneTex) at 4°C overnight. The nucleus was labeled with 4',6-diamidino-2-phenylindole (Sigma-Aldrich). Autophagosome determination was performed as previously reported with laser confocal scanning microscopy (LSM800, ZEISS, Oberkochen, Germany) [1, 18].

Zebrafish xenograft assay

Zebrafish (Danio rerio) was purchased from Academia Sinica (TZCAS, Taipei) for this study. Adult and larval zebrafish were cared for and maintained according to the animal care regulations and standard protocols at the animal center (Kaohsiung Medical University Hospital, Kaohsiung, Taiwan). Kaohsiung Medical

University Hospital's Institutional Animal Care and Use Committee approved the use of zebrafish (IACUC Approval No. 111090) in accordance with the principles of 3Rs (Reduction, Replacement, and Refinement). DiI dye was used to label human thyroid cancer cells (IHH-4 and KMH-2) for injection into zebrafish to track with fluorescence microscopy. Minor modifications were made to the procedure based on a previous study [19].

Statistical analysis

Data analysis was carried out through GraphPad Prism 8 software package. Data was expressed as mean ± standard deviation. Differences between the control group and experimental groups were determined by one-way analysis of variance (ANOVA) with Fisher's least significant difference test. In the *in vivo* study, the Mann-Whitney *U*-test was used. $P < 0.05$, $P < 0.01$ or $P < 0.001$ are considered to indicate statistical significance.

Results

Flavopereirine suppresses cell proliferation in various human thyroid cancer cells

To evaluate the antitumor capability of flavopereirine in human thyroid cancers, four types of human thyroid cancer cells including PTC, FTC, PDTC, and ATC cell lines were used, and the cell viability assay was performed with CCK-8 assay. These cells were incubated with control medium or with flavopereirine containing medium, and the data showed that growth inhibition of these cells with flavopereirine treatment with a dosage- and time-dependent manner (**Figure 1** and Supplementary Figure 1). The IC_{50} values of flavopereirine in these cells were showed in **Table 1**. The growth inhibition effect of flavopereirine showed IHH-4 > KMH-2 > 8505c > WRO > SW579 cells (**Table 1**), suggesting that PTC and ATC cells were more sensitive for flavopereirine treatment. To further investigate the growth inhibition activity of flavopereirine in PTC and ATC cells, IHH-4, 8505c and KMH-2 cells were incubated with flavopereirine, and the colony formation assay was performed. The data illustrated that both PTC and ATC cells show a growth inhibition with a dosage-dependent manner after flavopereirine treatment (**Figure 2**). These results demonstrated that flavopereirine exerts an anti-cancer property in human thyroid cancers especially on multiple drugs resistance PTC and ATC cells.

Flavopereirine modulates cell cycle in human thyroid cancer cells

Because flavopereirine inhibits tumor growth in PTC and ATC cells (**Figures 1** and **2**), therefore, whether flavopereirine could regulate cell cycle in these cells was further investigated. IHH-4, 8505c and KMH-2 cells were incubated with flavopereirine, and the cell cycle was examined with flowcytometry. **Figure 3** showed that flavopereirine treatment induced G0/G1 phase arrest in IHH-4 cells, and caused S phase arrest in both 8505c and KMH-2 cells. Here, we demonstrated that incubation with flavopereirine could assist cell cycle arrest in PTC and ATC cells.

Flavopereirine actuates cellular apoptosis in human PTC and ATC cells

To address whether flavopereirine exerted anti-human thyroid cancer activity was through cellular apoptosis induction, IHH-4, 8505c and KMH-2 cells were incubated with flavopereirine and the PI/Annexin-V double staining and flowcytometry analysis was conducted to evaluate apoptotic cells. The result showed that flavopereirine could induce cellular apoptosis with a dose- and time-dependent manner in these cells (**Figure 4**). Moreover, flavopereirine mediated apoptosis in these cells was IHH-4 > KMH-2 > 8505c, suggesting the sensitive ranking of flavopereirine treatment in these cells. This data was consistence with the growth inhibition result (**Table 1**).

Because flavopereirine actuated apoptosis in PTC and ATC cells (**Figure 4**), a Western blotting was conducted to evaluate the underling mechanisms involving in flavopereirine mediated apoptosis. IHH-4, 8505KMH-2 cells were incubated with flavopereirine, and the expressions of caspase-8, caspase-9, caspase-3 and PARP were determined. The result demonstrated that flavopereirine could cause caspase-8, -9, and -3 activation as well as induce PARP activation (**Figure 5**). Further investigation demonstrated that blocking caspase activation with Z-VAD-FMK could reduce flavopereirine mediated activation of aspase-8, -9, -3 and further activation of PARP (**Figure 5**).

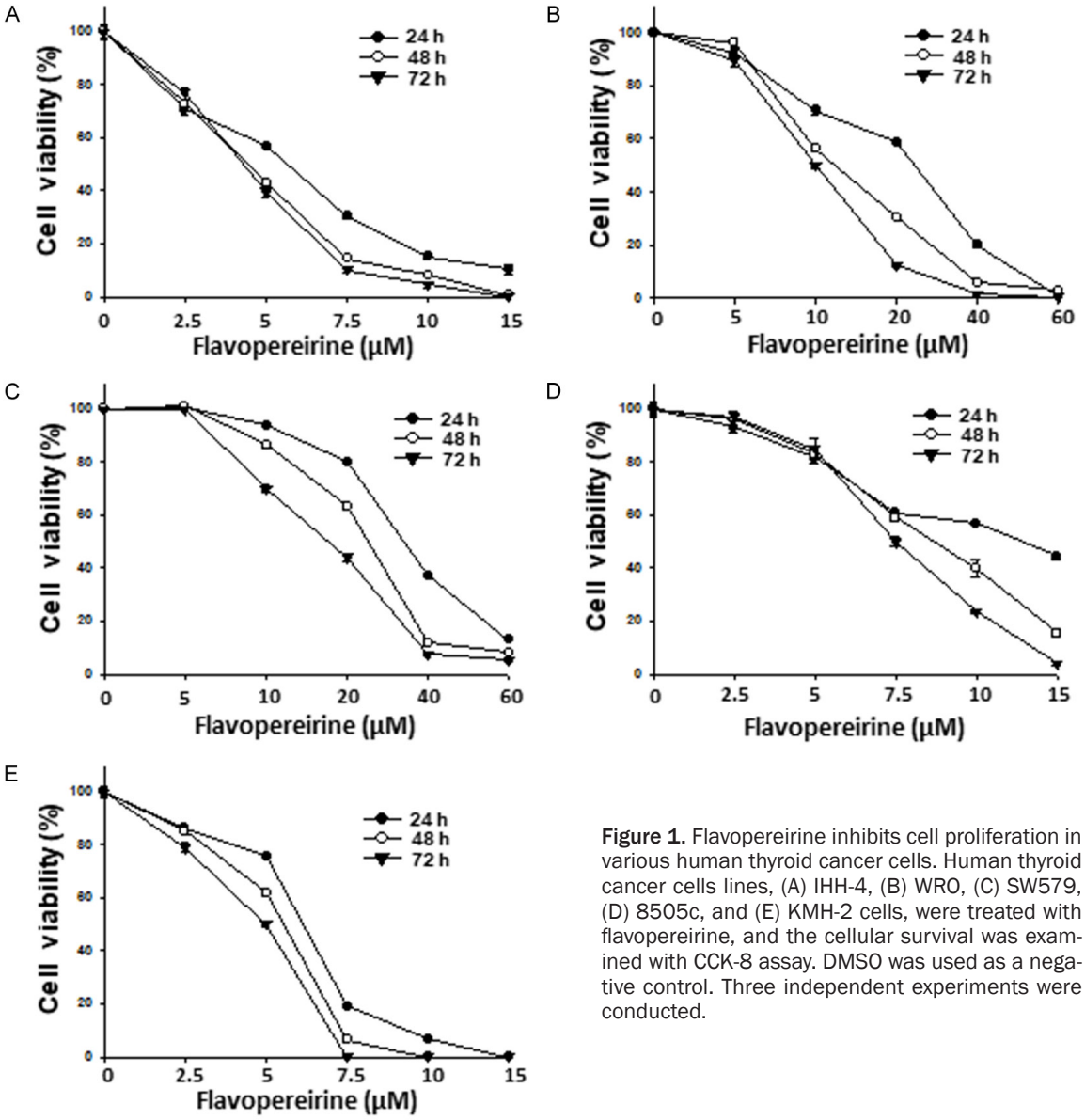


Figure 1. Flavopereirine inhibits cell proliferation in various human thyroid cancer cells. Human thyroid cancer cells lines, (A) IHH-4, (B) WRO, (C) SW579, (D) 8505c, and (E) KMH-2 cells, were treated with flavopereirine, and the cellular survival was examined with CCK-8 assay. DMSO was used as a negative control. Three independent experiments were conducted.

Table 1. IC_{50} values of flavopereirine in human thyroid cancer cells

	Time (h)	Cell line				
		IHH-4	WRO	SW579	8505c	KMH-2
Flavopereirine (μM)	24	5.6	24.4	26.2	12.8	6.1
	48	4.4	12.4	25.2	8.7	5.5
	72	4.3	10.0	17.7	7.5	5.0

This result suggested that flavopereirine could activate a caspase-dependent apoptosis in PTC and ATC cells (**Figure 4**). Moreover, flavopereirine induced cellular apoptosis was through both extrinsic and intrinsic caspase-dependent pathway.

To verify whether flavopereirine mediated anti-TC effect was through apoptosis induction, cells were incubated with flavopereirine; Z-VAD-FMK was used to block flavopereirine mediated caspase activation, and the cellular phenomena and apoptotic cells were determined

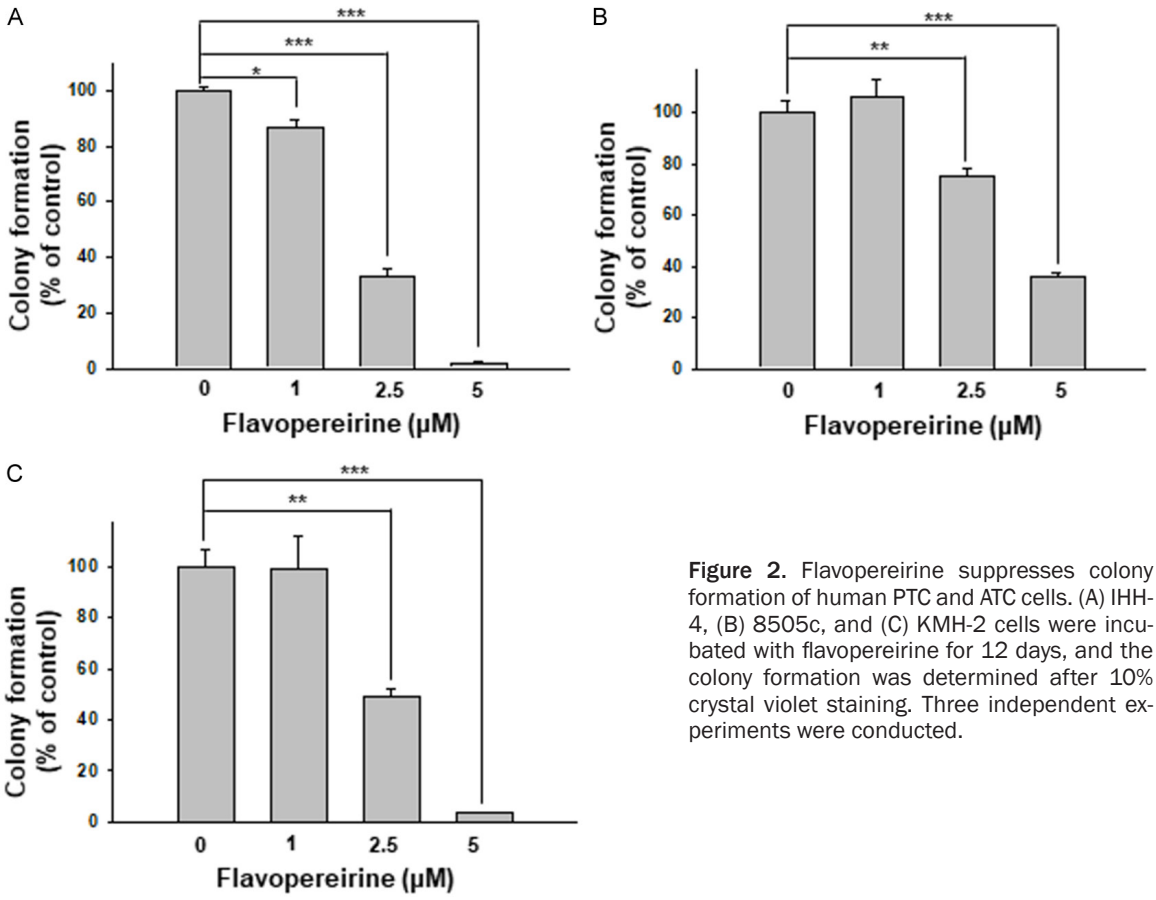


Figure 2. Flavopereirine suppresses colony formation of human PTC and ATC cells. (A) IHH-4, (B) 8505c, and (C) KMH-2 cells were incubated with flavopereirine for 12 days, and the colony formation was determined after 10% crystal violet staining. Three independent experiments were conducted.

with microscopy and flowcytometry. The data showed that blocking of caspase activation with Z-VAD-FMK could partially reverse flavopereirine mediated cell number lose, cell morphological change as well as apoptosis in IHH-4, 8505c, and KMH-2 cells (**Figure 6**). Altogether, we demonstrated that flavopereirine treatment exerts anti-human TC activity through induction of intrinsic and extrinsic caspase-dependent apoptosis.

Flavopereirine interferes cellular autophagy in human thyroid cancer cells

We also further investigation whether flavopereirine could modulate cellular autophagy in human PTC and ATC cells. IHH-4, 8505c, and KMH-2 cells were incubation with flavopereirine, and the expression of LC3-II (a biomarker of autophagosome) and p62 (a marker of autophagic flux) was examined with Western blotting. The data showed that LC3-II was elevated in all the cells under flavopereirine treatment with a dosage- and time-dependent manner (**Figure 7**). Importantly, the expression of p62 was decreased in the cells with autophagy induction after flavopereirine treatment (**Figure 7**). These data suggesting that flavopereirine might elevate autophagy through autophagosome formation as well as autophagic flux driving.

To confirm whether autophagosome formation was elevated in the cells with flavopereirine treatment, cells were incubation with flavopereirine, and the cells were immunostaining with LC3 antibody and the autophagosome formation was determined with confocal microscopy. The rapamycin was used to induce cellular autophagy, and the autophagy inhibitor, 3-MA, was used to suppress autophagy. **Figure 8A** demonstrated an autophagosome increasing in IHH-4, 8505c and KMH-2 cells with rapamycin as well as flavopereirine treatment. Moreover, the autophagosome formation was inhibited in the cells with 3-MA coincubation (**Figure 8A**). The data demonstrated that treatment with flavopereirine could induce auto-

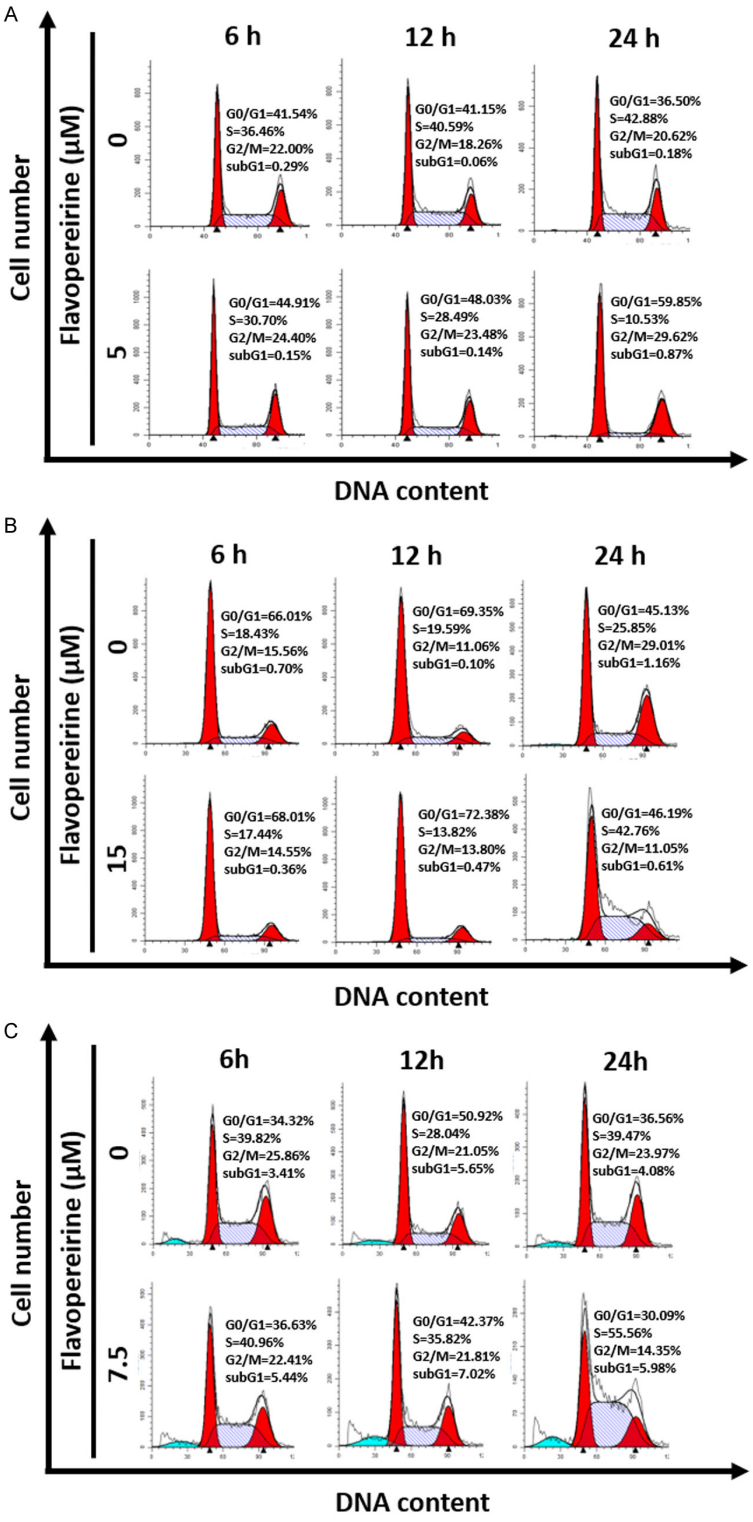
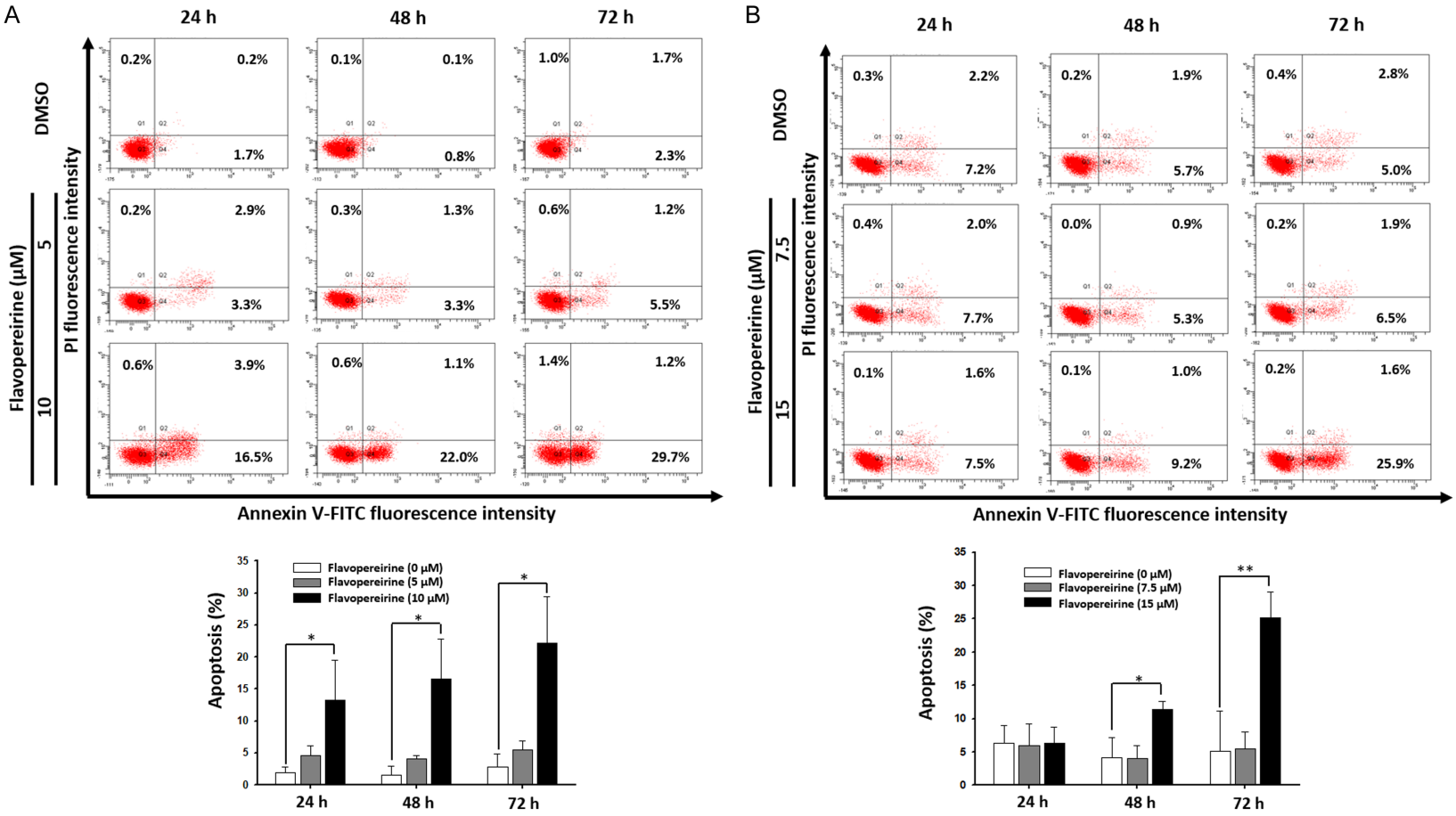


Figure 3. Flavopereirine modulates cell cycle in human PTC and ATC cells. (A) IHH-4, (B) 8505c, and (C) KMH-2 cells were administrated with flavopereirine, and the cell cycle regulation was investigated with flowcytometry analysis.

phagosome formation in PTC and ATC cells. However, autophagosome increasing might cause with autophagosome accumulation under autophagic flux blocking. Therefore, we further to evaluate whether autophagic flux was happen in the cells with flavopereirine treatment. IHH-4 cells were incubated with flavopereirine, and the expression of LC3-II and p62 were examined with Western blotting (**Figure 8B**). Moreover, rapamycin was used as an autophagy inducer, 3-MA was used as an autophagy inhibitor, and bafilomycin was used as an autophagic flux blocker. The data showed that bafilomycin could block autophagic flux and cause LC3-II as well as p62 accumulation; however, cells with flavopereirine treatment could increase LC3-II level, but decrease the expression of p62 (**Figure 8B**). Moreover, cells with coincubation with flavopereirine and bafilomycin, the expressions of both LC3-II and p62 were accumulated (**Figure 8B**). Altogether, these results demonstrated that treatment with flavopereirine in human PTC and ATC cells could elevate cellular autophagy.

Flavopereirine modulates ERK, p38 and AKT/mTOR signaling pathways in human thyroid cancer cells

In our previous study, flavopereirine regulates AKT, p38 and ERK signaling pathways in human breast cancer cells has been demonstrated. Here, we evaluated the expressions and the activations of ERK, JNK, p38, AKT, and mTOR in IHH-4, 8505c and KMH-2 cells under flavopereirine treatment. The result showed that phospho-ERK and phospho-p38 were increased but the expression of phospho-AKT and phospho-mTOR were decreased in all the cells under flavopereirine treatment (**Figure 9A**). However, the


A
24 h
48 h
72 h
DMSO
5
10
Flavopereirine (μM)
PI fluorescence intensity
0.2%
0.2%
1.7%
0.1%
0.1%
0.8%
1.0%
1.7%
2.3%
0.2%
2.9%
3.3%
0.3%
1.3%
3.3%
0.6%
1.2%
5.5%
0.6%
3.9%
16.5%
0.6%
1.1%
22.0%
1.4%
1.2%
29.7%
Annexin V-FITC fluorescence intensity
B
24 h
48 h
72 h
DMSO
7.5
15
Flavopereirine (μM)
PI fluorescence intensity
0.3%
2.2%
7.2%
0.2%
1.9%
5.7%
0.4%
2.8%
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1.9%
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9.2%
0.2%
1.6%
25.9%
Annexin V-FITC fluorescence intensity
Apoptosis (%)
Flavopereirine (0 μM)
Flavopereirine (5 μM)
Flavopereirine (10 μM)
24 h
48 h
72 h
Apoptosis (%)
Flavopereirine (0 μM)
Flavopereirine (7.5 μM)
Flavopereirine (15 μM)
24 h
48 h
72 h

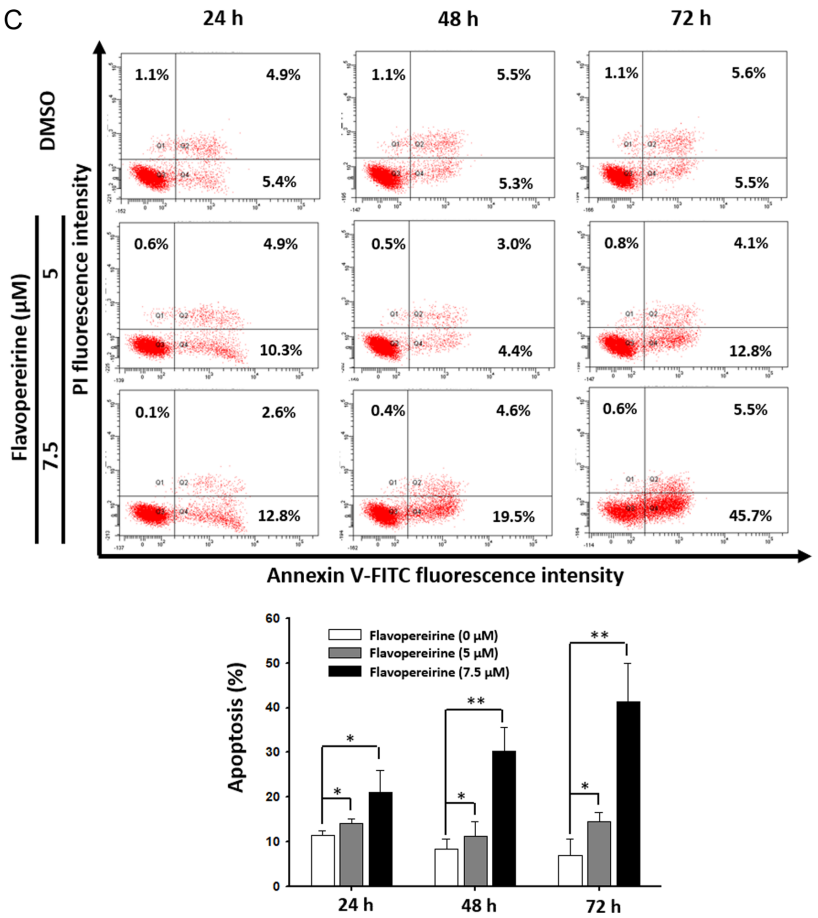


Figure 4. Flavopereirine induces cellular apoptosis in human PTC and ATC cells. (A) IHH-4, (B) 8505c, and (C) KMH-2 cells were incubated with flavopereirine, and the apoptotic cells were examined with flowcytometry analysis after PI/Annexin-V staining. Representative result of three independent experiments.

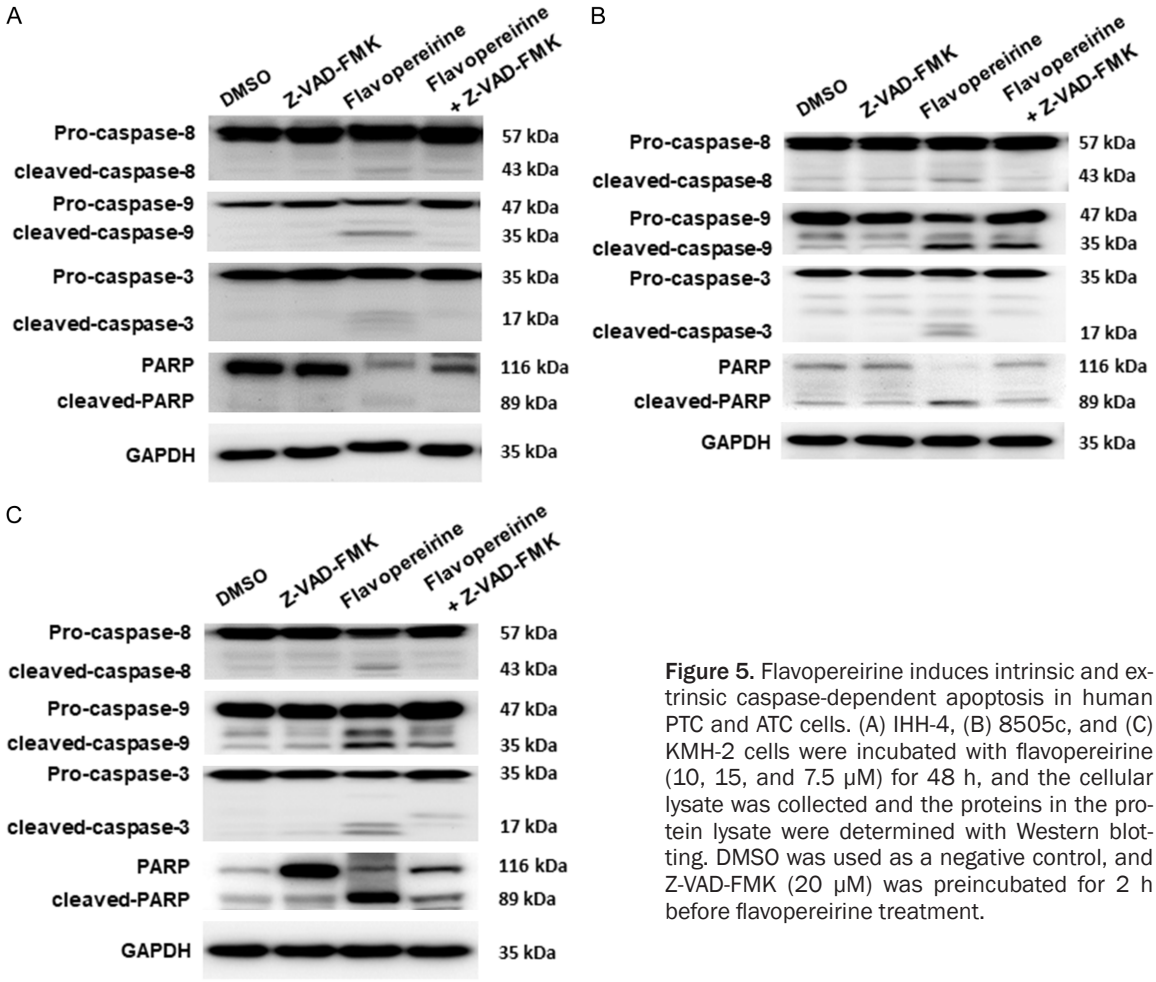
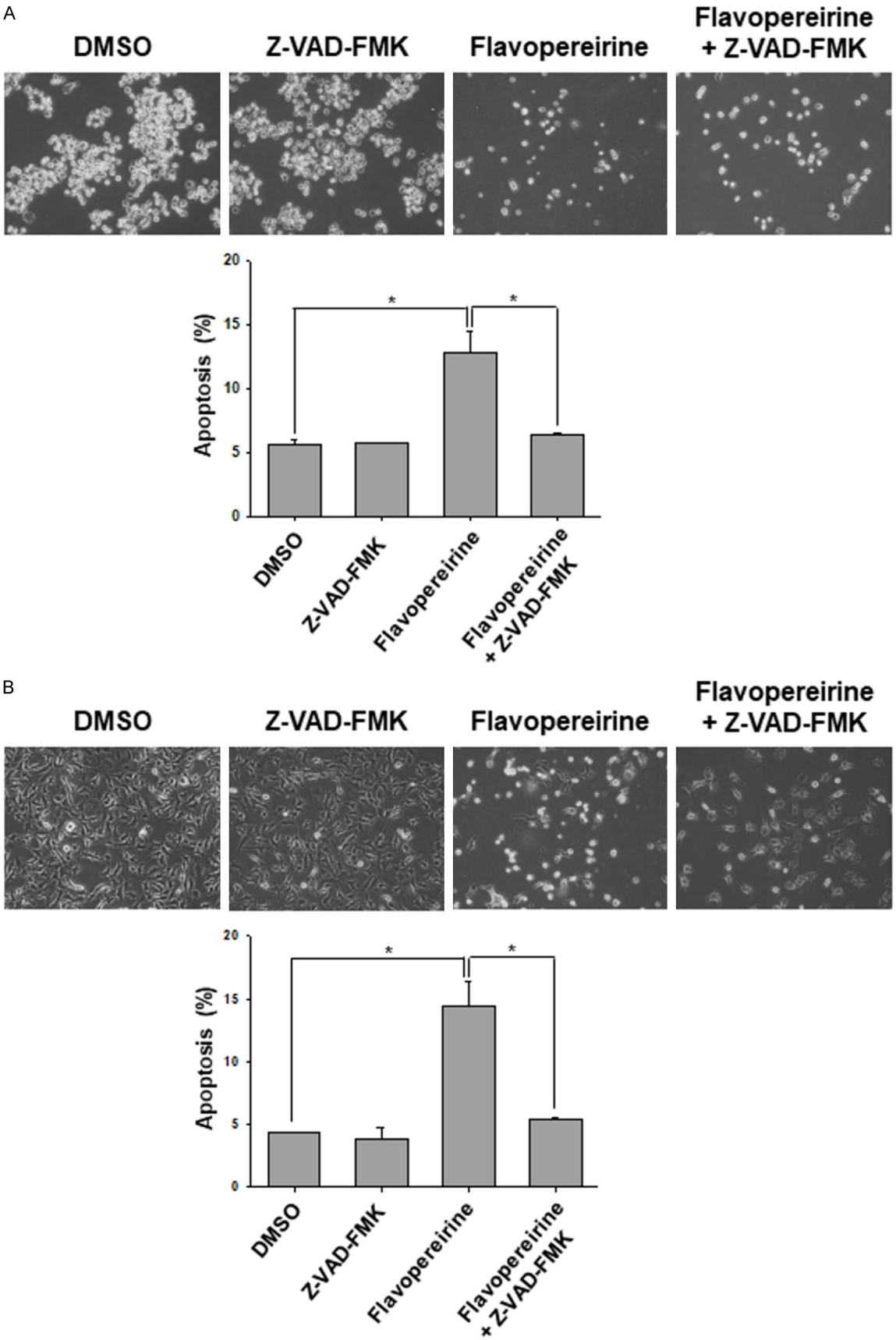


Figure 5. Flavopereirine induces intrinsic and extrinsic caspase-dependent apoptosis in human PTC and ATC cells. (A) IHH-4, (B) 8505c, and (C) KMH-2 cells were incubated with flavopereirine (10, 15, and 7.5 μM) for 48 h, and the cellular lysate was collected and the proteins in the protein lysate were determined with Western blotting. DMSO was used as a negative control, and Z-VAD-FMK (20 μM) was preincubated for 2 h before flavopereirine treatment.

activation of JNK pathway was not affected in the cells with flavopereirine incubation (**Figure 9A**). Moreover, the total protein of ERK was elevated, but decreased of AKT and mTOR proteins in the cells with flavopereirine treatment, suggested that incubation of flavopereirine might modulate protein synthesis of ERK, AKT and mTOR (**Figure 9A**). Further investigation using PD98059 and SB203589 to suppress flavopereirine mediated ERK and p38 activation in KMH-2 cells, showed reducing ERK activation in KMH-2 cell under flavopereirine treatment could reduce flavopereirine mediated cellular apoptosis (**Figure 9B**). However, inhibition p38 activation in KMH-2 cells with flavopereirine treatment didn't reverse cellular apoptosis (**Figure 9C**). In addition, elevation of AKT activity with a constitutive active form construction in KMH-2 cell [14] with flavopereirine treatment could significantly reduce flavopereirine mediated cellular apoptosis (**Figure 9D**). These results demonstrated that incubation with flavopereirine in human PTC and ATC cells might regulate the expressions and/or activations of ERK, p38, and AKT/mTOR signaling pathways. Moreover, the activation of ERK pathway and the inhibition of AKT pathway might be the upstream mechanisms in flavopereirine mediated cellular apoptosis.

Flavopereirine inhibits the growth of thyroid cancer cells in vivo

To further verify the anti-cancer activity of flavopereirine in human PTC and ATC cells, a in vivo zebrafish xenotransplantation model was conducted. The IHH-4 and KMH-2 cells were labeled with DiI, and the *in vivo* cancer growth was monitored to illustrate the response of flavopereirine treatment (**Figure 10**). Zebrafish larvae were randomly divided into several groups that received flavopereirine at indicated


A
DMSO
Z-VAD-FMK
Flavopereirine
Flavopereirine + Z-VAD-FMK
Apoptosis (%)
0
5
10
15
20
*
*
DMSO
Z-VAD-FMK
Flavopereirine
Flavopereirine + Z-VAD-FMK
B
DMSO
Z-VAD-FMK
Flavopereirine
Flavopereirine + Z-VAD-FMK
Apoptosis (%)
0
5
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DMSO
Z-VAD-FMK
Flavopereirine
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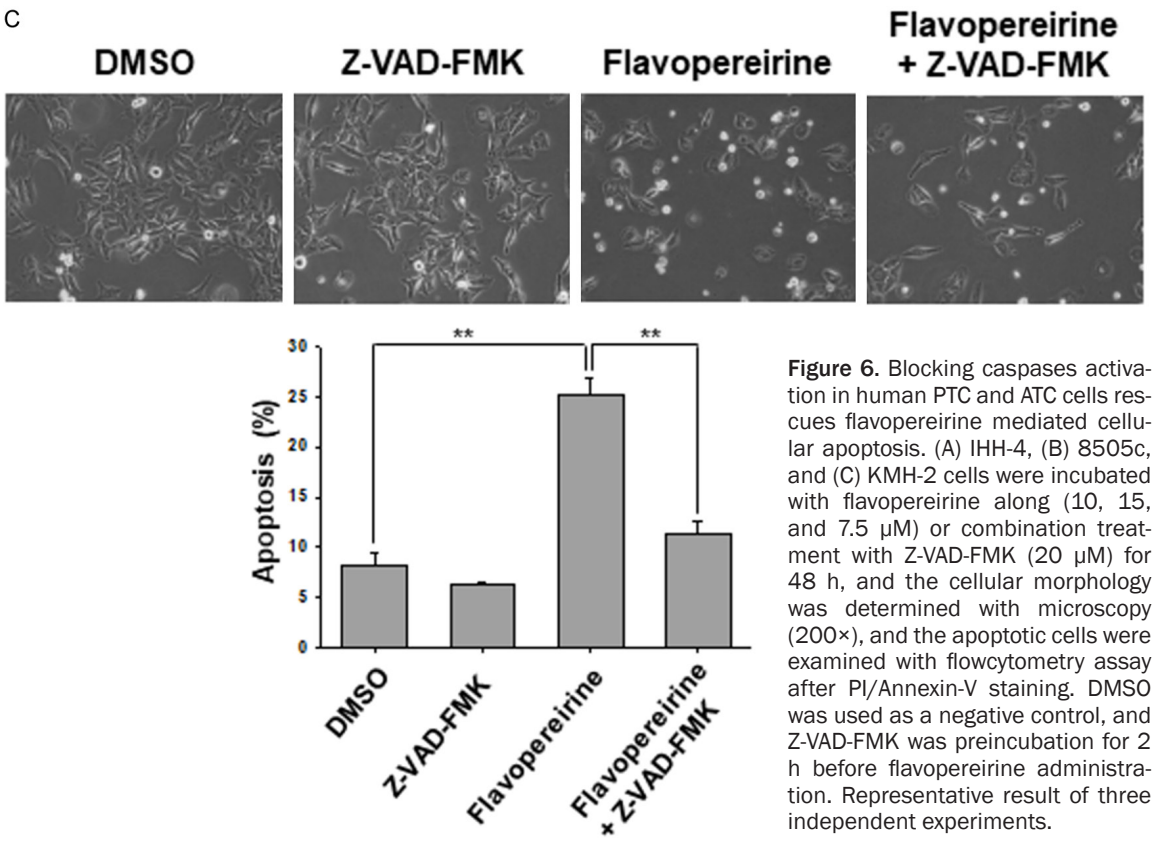

Figure 6. Blocking caspases activation in human PTC and ATC cells rescues flavopereirine mediated cellular apoptosis. (A) IHH-4, (B) 8505c, and (C) KMH-2 cells were incubated with flavopereirine along (10, 15, and 7.5 μM) or combination treatment with Z-VAD-FMK (20 μM) for 48 h, and the cellular morphology was determined with microscopy (200×), and the apoptotic cells were examined with flowcytometry assay after PI/Annexin-V staining. DMSO was used as a negative control, and Z-VAD-FMK was preincubation for 2 h before flavopereirine administration. Representative result of three independent experiments.

concentrations, and a control group (untreated xenografted zebrafish larvae) was present. There was no developmental toxicity observed with flavopereirine throughout the experiment (**Figure 10A**). Compared with the control, flavopereirine inhibited tumor growth *in vivo* significantly (**Figure 10B** and **10C**), indicating that flavopereirine could be an effective and safe treatment for thyroid cancer cells *in vivo*.

Discussion

Flavopereirine is a natural alkaloid isolated from the plant *Geissospermum vellosii* [4]. Moreover, *Geissospermum vellosii* has long been used by oncologic patients and integrative medicine practitioners in South America [20]. Steele et al. is the first to isolate a β-carboline alkaloid flavopereirine from the bark of *Geissospermum sericeum* and reported its anti-plasmodial activity [4]. Moreover, flavopereirine also exerts anti-leishmania in the previous report [6]. In addition, flavopereirine can suppress the expressions of androgen receptor (AR), and androgen-associated proteins such as steroid 5 alpha-reductase 1 (SRD5A1) and prostate specific antigen (PSA), suggesting that flavopereirine can be used to reduce testosterone-induced BPH development [7]. Furthermore, we and others reported that flavopereirine has an anti-tumor property in human glioblastoma, colon, hepatoma, breast, and oral cancer cells [8-13]. In the present study, we demonstrated that flavopereirine exerts an anti-human thyroid cancer activity in various types of human TC cells. This study is particularly significant as it demonstrates that flavopereirine exhibits optimal therapeutic effects against PTC cells with multidrug resistance and the most malignant ATC cells *in vitro* and *in vivo*, suggesting that flavopereirine could be a good therapeutic candidate for the patients with TC that are clinically challenging to treat.

Although flavopereirine inhibits AR, SRD5A1, and PSA to suppress testosterone-induced BPH has been demonstrated [7], the activation of AR in thyroid cancer cells could decrease cellular proliferation and induce senescence

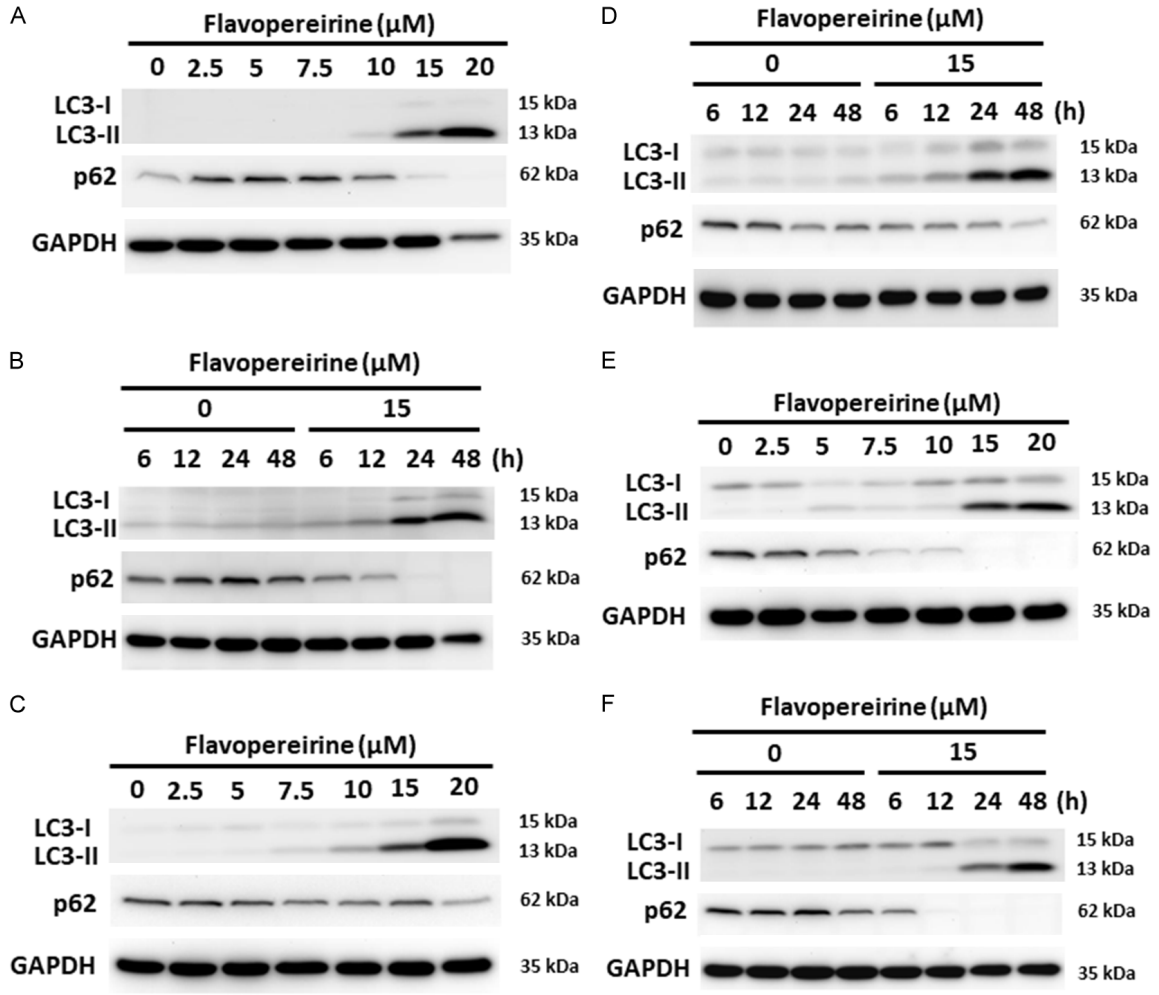

Figure 7. Flavopereirine induces cellular autophagy in human PTC and ATC cells. (A and B) IHH-4, (C and D) 8505c, and (E and F) KMH-2 cells were treated with flavopereirine (A, C and E) for 24 h or the indicated condition. The total cell lysate was collected and the proteins in the lysate were assessed with Western blotting. DMSO was used as the negative control.

[21, 22]. Therefore, the androgen receptor pathway is not indicated as the mediator for the anti-thyroid cancer properties of flavopereirine. In the current study, we further demonstrating that flavopereirine modulated activation of Erk and p38, as well as suppression of Akt pathways are illustrated (**Figure 9**). Further investigation demonstrated that flavopereirine induced caspase-dependnet apoptosis in KMH-2 cells could be suppressed with manipulation of Erk and Akt pathways (**Figure 9B** and **9D**). These finding was similar with our previous study [10, 13], suggesting that flavopereirine may modulate these pathways to cause anti-TC activity. However, the biological effect of p38 signaling pathway modulation in the cells with flavopereirine treatment should be further investigation.

Furthermore, our earlier research illustrated that the augmentation of p53 activation during flavopereirine treatment in human colon cancer is responsible for the anti-colon cancer activity of flavopereirine [9]. However, inactivating mutations of p53 have been identified in merely 10% of thyroid carcinomas, predominantly in poorly differentiated and aggressive histotypes. In addition, the p53 gene has been identified in IHH-4 and KMH-2 cells with wild-type, and is missence mutation in 8505c cells. Therefore, whether flavopereirine can achieve its anti-thyroid cancer effects through the

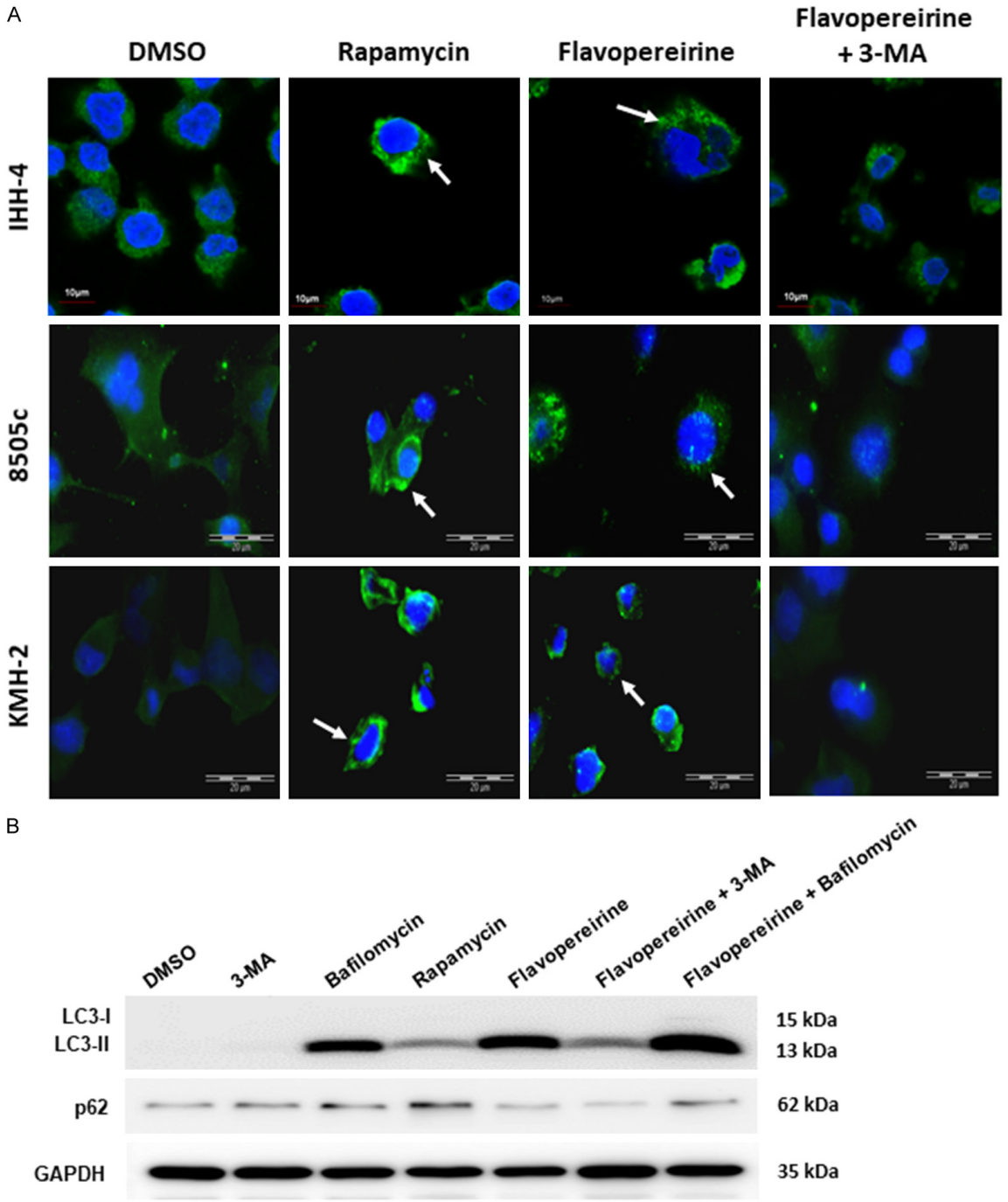

Figure 8. Flavopereirine induces autophagy as well as autophagosome formation in human PTC and ATC cells. IHH-4, 8505c and KMH-2 cells were incubated with flavopereirine (10, 15, and 7.5 μM) for 24 h, and (A) the autophagosomes in these cells were evaluated with immunofluorescence staining, and (B) the protein expressions were examined with Western blotting. DMSO was used as the negative control. 3-MA (5 μM) was the autophagy inhibitor, and the rapamycin (30 μM) was a autophagy inducer. Bafilomycin (50 nM) was used to be an autophagic flux blocker.

activation of p53 remains to be further investigated.

In addition, BRAF mutations are frequently identified in thyroid carcinoma, with a reported prevalence of 35-40% in anaplastic thyroid carcinoma [23]. The presence of BRAF mutations activates the mitogen-activated protein (MAP) kinase pathway, involving downstream proteins such as MEK and ERK. ERK activation ultimate-

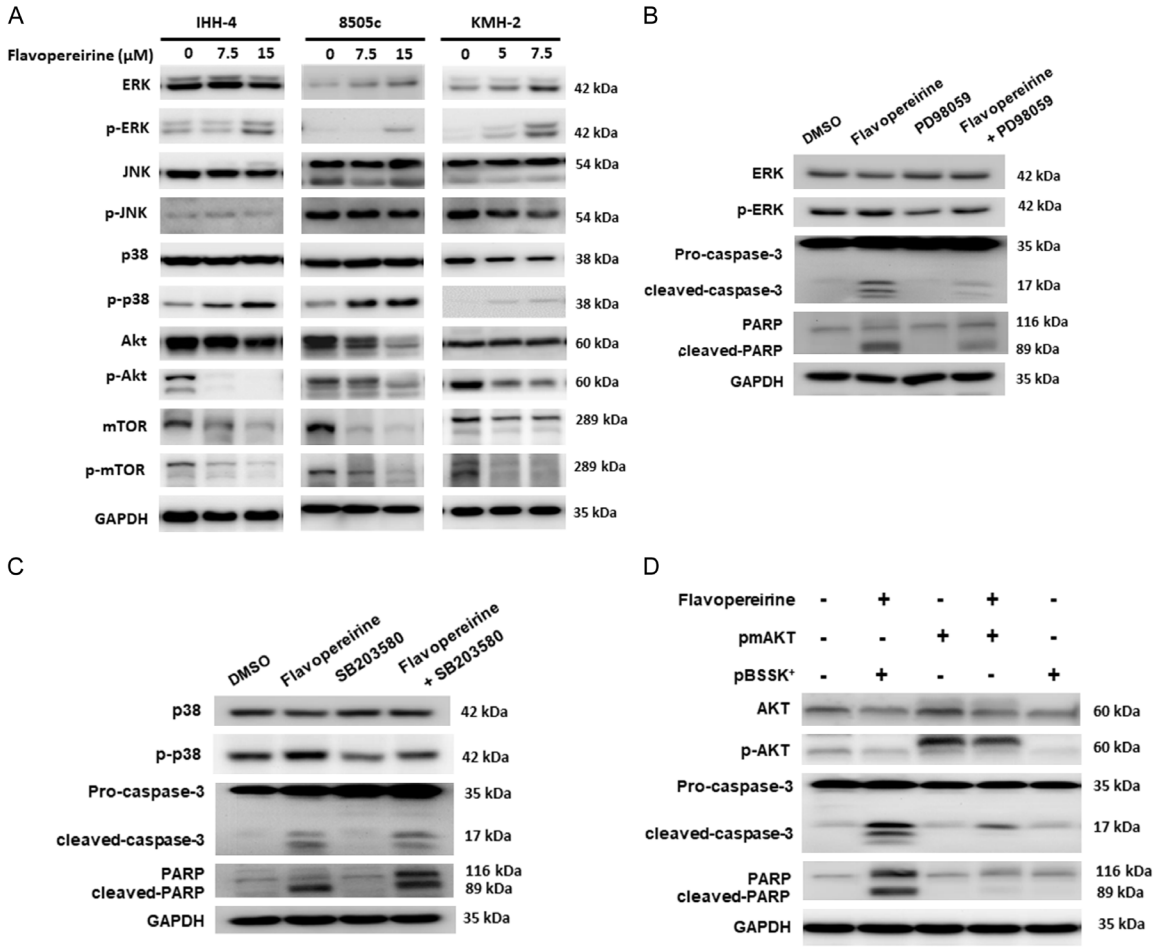

Figure 9. Flavopereirine modulating signaling pathways in human PTC and ATC cells. (A) IHH-4, 8505c and KHM-2 cells were administrated with flavopereirine for 48 h, and the total cell lysate was collected and the proteins in the lysate were examined with Western blotting. KMH-2 cells were co-incubated with (B) PD98059 and (C) SB203580 to block ERK and p38 activation, and the expressions and activations of ERK and/or p38, caspase-3, and PARP were investigated by Western blots after incubation with flavopereirine for 48 h. (D) KMH-2 cells were transfected with or without constitutively active AKT construct, and the expression and the activation of AKT, caspase-3, and PARP were investigated by Western blots after incubation with flavopereirine for 48 h. DMSO was used as a negative control. GAPDH was a loading control.

ly promotes cell proliferation and contributes to the oncogenesis of thyroid tumors [24]. Tyrosine kinase inhibitors (TKIs) targeting BRAF V600E, such as dabrafenib, and MEK1/2, such as trametinib, have been developed. The combined use of dabrafenib and trametinib has shown promising effects in various cancers with BRAF mutations, including melanoma, lung cancer, and ATC [25-27]. However, the impact of this combination on clinical practice on ATC and recurrence PTC needs thoroughly evaluated. Because flavopereirine treatment could suppress AKT/mTOR signaling pathway, and elevate ERK and p38 signaling pathways in both PTC and ATC cells (**Figure 9**). Therefore, whether flavopereirine combination with dabrafenib and trametinib can elevate therapeutic effect in recurrent PTC and ATC need further investigation.

Furthermore, our earlier research illustrated that the augmentation of p53 activation during flavopereirine treatment in human colon cancer is responsible for the anti-colon cancer activity of flavopereirine [9]. However, inactivating mutations of p53 have been identified in merely 10% of thyroid carcinomas, predominantly in poorly differentiated and aggressive histotypes [28]. In addition, the p53 gene has been identified in IHH-4 and KMH-2 cells with wildtype,

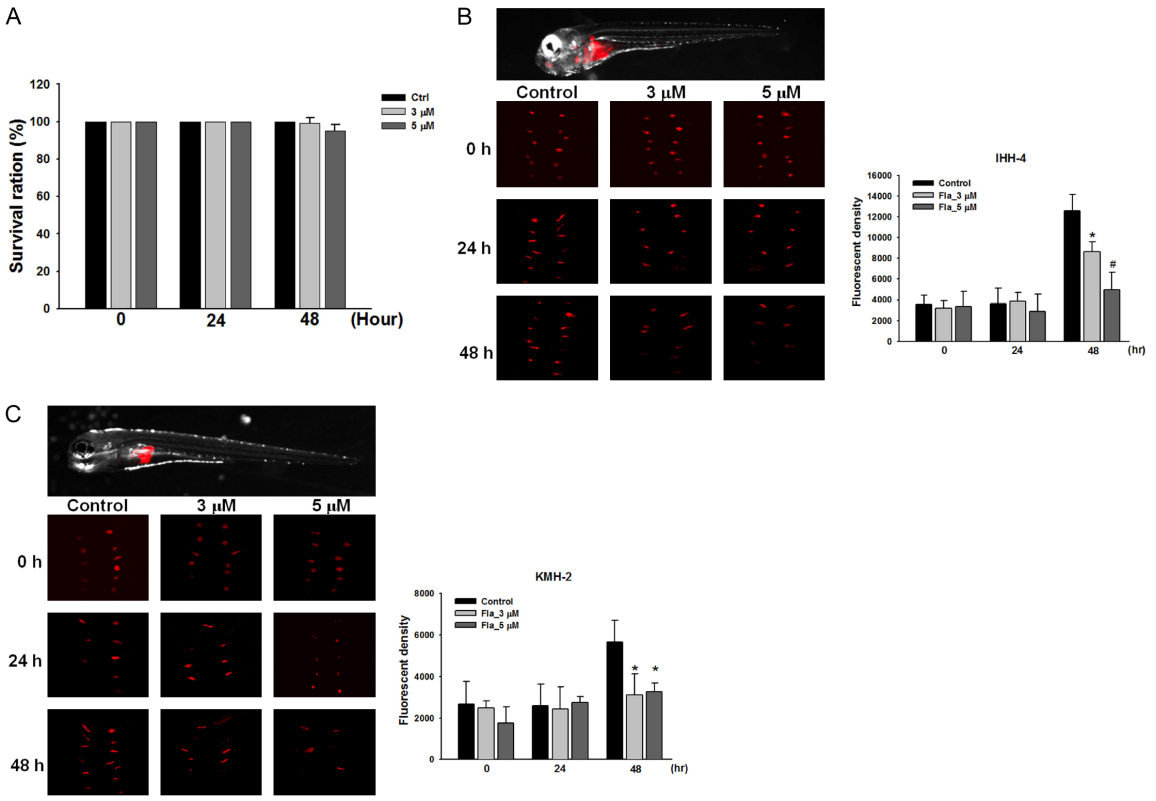

Figure 10. Flavopereirine inhibits tumor growth in a zebrafish xenograft model. (A) The toxicity effect of flavopereirine for zebrafish larvae at the indicated concentrations and time. *P < 0.05 compared with the control. (B) IHH-4 and (C) KMH-2 cells labeled with a red fluorescent dye (CM-DiI) were injected into zebrafish yolk sacs. The intensity of red fluorescence is proportional to the tumor size. Thyroid cancer xenograft zebrafish treated with flavopereirine and observed at 24 and 48 h post-treatment. Quantitative analysis of the tumor cell proliferation with or without flavopereirine treatment. *P < 0.05; **P < 0.01 compared with control cells. Data represent means ± SD.

and is missence mutation in 8505c cells [29]. Therefore, whether flavopereirine can achieve its anti-thyroid cancer effects through the activation of p53 remains to be further investigated.

Autophagy serves as a self-degradation mechanism responsible for eliminating surplus intracellular organelles and proteins, thereby contributing significantly to cellular homeostasis [30, 31]. Various compounds, including clinical chemotherapeutic agents and/or natural products, have the capacity to trigger autophagy [32]. Nonetheless, the induction of autophagy can manifest as either cytotoxic or cytoprotective [32], underscoring the intricate nature of autophagy induction in cancer cells.

The induction of autophagy by natural products is recognized as a dual-edged mechanism influencing the cellular destiny of human cancers [33, 34]. The intricate interplay between autophagy and apoptosis has garnered recent attention [33]. Various proteins and signaling pathways, such as p53, Bcl-2, DAPK, Akt/mTOR, and JNK pathways, serve as essential mediators facilitating the cross-talk between autophagy and apoptosis [33, 35].

In our earlier study, we provided evidence supporting the role of flavopereirine in inhibiting cellular autophagic flux through the AKT/p38 signaling pathway, thereby contributing to its anti-human breast cancer activity [13]. However, in our current research, we have demonstrated a contrasting effect, wherein flavopereirine is shown to enhance autophagy and autophagic flux in PTC and ATC cells (see **Figures 7** and **8**). Consequently, additional investigation is required to determine whether the modulation of autophagy by flavopereirine plays a role in its anti-properties against PTC and ATC.

In conclusion, we validated the anti-PTC and -ATC efficacy of flavopereirine using a zebrafish model. Our findings demonstrate that flavopereirine exhibits both safety and effectiveness as an in vivo anti-cancer agent. Consequently, further exploration of flavopereirine as a potential therapeutic agent for highly aggressive PTC and ATC cases is warranted.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

[1] Lin TH, Kuo CH, Zhang YS, Chen PT, Chen SH, Li YZ and Lee YR. Piperlongumine induces cellular apoptosis and autophagy via the ROS/Akt signaling pathway in human follicular thyroid cancer cells. Int J Mol Sci 2023; 24: 8048.

[2] Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T and Thun MJ. Cancer statistics, 2008. CA Cancer J Clin 2008; 58: 71-96.

[3] Veness MJ, Porter GS and Morgan GJ. Anaplastic thyroid carcinoma: dismal outcome despite current treatment approach. ANZ J Surg 2004; 74: 559-562.

[4] Steele JC, Veitch NC, Kite GC, Simmonds MS and Warhurst DC. Indole and beta-carboline alkaloids from Geissospermum sericeum. J Nat Prod 2002; 65: 85-88.

[5] Beljanski M and Beljanski MS. Selective inhibition of in vitro synthesis of cancer DNA by alkaloids of beta-carboline class. Exp Cell Biol 1982; 50: 79-87.

[6] da Silva E Silva JV, Cordovil Brigido HP, Oliveira de Albuquerque KC, Miranda Carvalho J, Ferreira Reis J, Vinhal Faria L, Coelho-Ferreira M, Silveira FT, da Silva Carneiro A, Percario S, do Rosario Marinho AM and Dolabela MF. Flavopereirine-an alkaloid derived from geissospermum vellosii-presents leishmanicidal activity in vitro. Molecules 2019; 24: 785.

[7] Liu J, Fang T, Li M, Song Y, Li J, Xue Z, Li J, Bu D, Liu W, Zeng Q, Zhang Y, Yun S, Huang R and Yan J. Pao pereira extract attenuates testosterone-induced benign prostatic hyperplasia in rats by inhibiting 5alpha-reductase. Sci Rep 2019; 9: 19703.

[8] Xu J, Wu Z and Huang J. Flavopereirine suppresses the progression of human oral cancer by inhibiting the JAK-STAT signaling pathway via targeting LASP1. Drug Des Devel Ther 2021; 15: 1705-1716.

[9] Li JM, Huang YC, Kuo YH, Cheng CC, Kuan FC, Chang SF, Lee YR, Chin CC and Shi CS. Flavopereirine suppresses the growth of colorectal cancer cells through P53 signaling dependence. Cancers (Basel) 2019; 11: 1034.

[10] Yeh HT, Tsai YS, Chen MS, Li YZ, Lin WC, Lee YR, Tseng YS and Sheu SM. Flavopereirine induces cell cycle arrest and apoptosis via the AKT/p38 MAPK/ERK1/2 signaling pathway in human breast cancer cells. Eur J Pharmacol 2019; 863: 172658.

[11] Chen SY, Chao CN, Huang HY and Fang CY. Flavopereirine inhibits hepatocellular carcinoma cell growth by inducing cell-cycle arrest, apoptosis, and autophagy-related protein expression. Anticancer Res 2020; 40: 6907-6914.

[12] Beljanski M, Crochet S and Beljanski MS. PB-100: a potent and selective inhibitor of human BCNU resistant glioblastoma cell multiplication. Anticancer Res 1993; 13: 2301-2308.

[13] Chen MS, Yeh HT, Li YZ, Lin WC, Lee YR, Tseng YS and Sheu SM. Flavopereirine inhibits autophagy via the AKT/p38 MAPK signaling pathway in MDA-MB-231 cells. Int J Mol Sci 2020; 21: 5362.

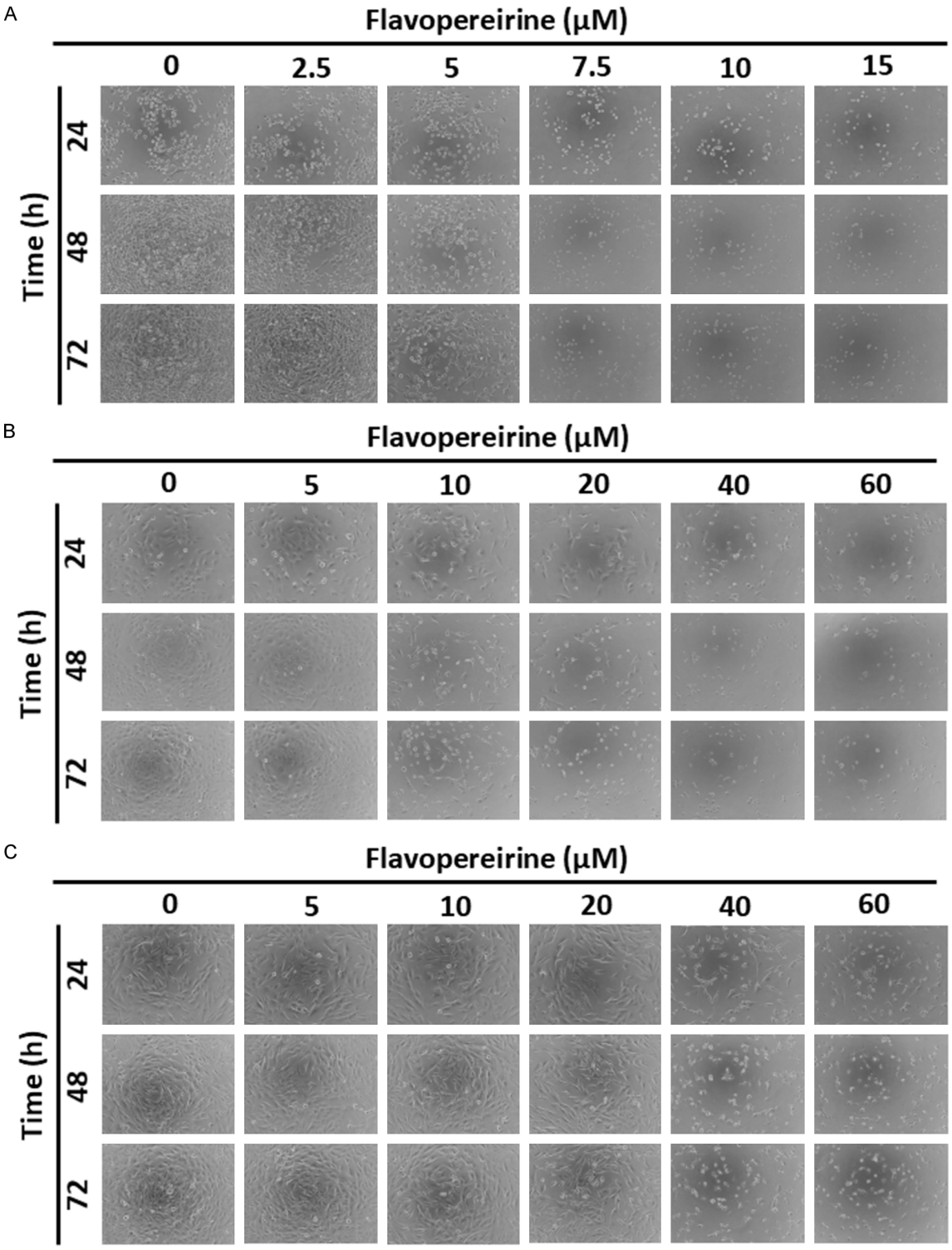
[14] Kung FP, Lim YP, Chao WY, Zhang YS, Yu HI, Tai TS, Lu CH, Chen SH, Li YZ, Zhao PW, Yen YP and Lee YR. Piperlongumine, a potent anticancer phytotherapeutic, induces cell cycle arrest and apoptosis in vitro and in vivo through the ROS/Akt pathway in human thyroid cancer cells. Cancers (Basel) 2021; 13: 4266.

[15] Hua SC, Chang TC, Chen HR, Lu CH, Liu YW, Chen SH, Yu HI, Chang YP and Lee YR. Reversine, a 2,6-disubstituted purine, as an anticancer agent in differentiated and undifferentiated thyroid cancer cells. Pharm Res 2012; 29: 1990-2005.

[16] Lu CH, Chen SH, Chang YS, Liu YW, Wu JY, Lim YP, Yu HI and Lee YR. Honokiol, a potential therapeutic agent, induces cell cycle arrest and program cell death in vitro and in vivo in human thyroid cancer cells. Pharmacol Res 2017; 115: 288-298.

[17] Tung CL, Chao WY, Li YZ, Shen CH, Zhao PW, Chen SH, Wu TY and Lee YR. Ivermectin induces cell cycle arrest and caspase-dependent

apoptosis in human urothelial carcinoma cells. Int J Med Sci 2022; 19: 1567-1575.
[18] Yu HI, Shen HC, Chen SH, Lim YP, Chuang HH, Tai TS, Kung FP, Lu CH, Hou CY and Lee YR. Autophagy modulation in human thyroid cancer cells following aloperine treatment. Int J Mol Sci 2019; 20: 5315.
[19] Chen HL, Lo YH, Lin CL, Lee TH, Leung W, Wang SW, Lin IP, Lin MY and Lee CH. Trichodermin inhibits the growth of oral cancer through apoptosis-induced mitochondrial dysfunction and HDAC-2-mediated signaling. Biomed Pharmacother 2022; 153: 113351.
[20] Yu J and Chen Q. The plant extract of Pao pereira potentiates carboplatin effects against ovarian cancer. Pharm Biol 2014; 52: 36-43.
[21] Jones ME, O'Connell TJ, Zhao H, Darzynkiewicz Z, Gupta A, Buchsbaum J, Shin E, Iacob C, Suslina N, Moscatello A, Schantz S, Tiwari R and Geliebter J. Androgen receptor activation decreases proliferation in thyroid cancer cells. J Cell Biochem 2021; [Epub ahead of print].
[22] Gupta A, Carnazza M, Jones M, Darzynkiewicz Z, Halicka D, O'Connell T, Zhao H, Dadafarin S, Shin E, Schwarcz MD, Moscatello A, Tiwari RK and Geliebter J. Androgen receptor activation induces senescence in thyroid cancer cells. Cancers (Basel) 2023; 15: 2198.
[23] Cabanillas ME, Ryder M and Jimenez C. Targeted therapy for advanced thyroid cancer: kinase inhibitors and beyond. Endocr Rev 2019; 40: 1573-1604.
[24] Begum S, Rosenbaum E, Henrique R, Cohen Y, Sidransky D and Westra WH. BRAF mutations in anaplastic thyroid carcinoma: implications for tumor origin, diagnosis and treatment. Mod Pathol 2004; 17: 1359-1363.
[25] Robert C, Grob JJ, Stroyakovskiy D, Karaszewska B, Hauschild A, Levchenko E, Chiarion Sileni V, Schachter J, Garbe C, Bondarenko I, Gogas H, Mandala M, Haanen JBAG, Lebbe C, Mackiewicz A, Rutkowski P, Nathan PD, Ribas A, Davies MA, Flaherty KT, Burgess P, Tan M, Gasal E, Voi M, Schadendorf D and Long GV. Five-year outcomes with dabrafenib plus trametinib in metastatic melanoma. N Engl J Med 2019; 381: 626-636.
[26] Planchard D, Besse B, Groen HJM, Souquet PJ, Quoix E, Baik CS, Barlesi F, Kim TM, Mazieres J, Novello S, Rigas JR, Upalawanna A, D'Amelio AM Jr, Zhang P, Mookerjee B and Johnson BE. Dabrafenib plus trametinib in patients with previously treated BRAF(V600E)-mutant metastatic non-small cell lung cancer: an open-label, multicentre phase 2 trial. Lancet Oncol 2016; 17: 984-993.
[27] Subbiah V, Kreitman RJ, Wainberg ZA, Cho JY, Schellens JHM, Soria JC, Wen PY, Zielinski C, Cabanillas ME, Urbanowitz G, Mookerjee B, Wang D, Rangwala F and Keam B. Dabrafenib and trametinib treatment in patients with locally advanced or metastatic BRAF V600-mutant anaplastic thyroid cancer. J Clin Oncol 2018; 36: 7-13.
[28] Malaguarnera R, Vella V, Vigneri R and Frasca F. p53 family proteins in thyroid cancer. Endocr Relat Cancer 2007; 14: 43-60.
[29] Landa I, Pozdeyev N, Korch C, Marlow LA, Smallridge RC, Copland JA, Henderson YC, Lai SY, Clayman GL, Onoda N, Tan AC, Garcia-Rendueles MER, Knauf JA, Haugen BR, Fagin JA and Schweppe RE. Comprehensive genetic characterization of human thyroid cancer cell lines: a validated panel for preclinical studies. Clin Cancer Res 2019; 25: 3141-3151.
[30] Glick D, Barth S and Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol 2010; 221: 3-12.
[31] Suzuki K and Ohsumi Y. Molecular machinery of autophagosome formation in yeast, Saccharomyces cerevisiae. FEBS Lett 2007; 581: 2156-2161.
[32] Gewirtz DA. The four faces of autophagy: implications for cancer therapy. Cancer Res 2014; 74: 647-651.
[33] Marino G, Niso-Santano M, Baehrecke EH and Kroemer G. Self-consumption: the interplay of autophagy and apoptosis. Nat Rev Mol Cell Biol 2014; 15: 81-94.
[34] Huang KJ, Kuo CH, Chen SH, Lin CY and Lee YR. Honokiol inhibits in vitro and in vivo growth of oral squamous cell carcinoma through induction of apoptosis, cell cycle arrest and autophagy. J Cell Mol Med 2018; 22: 1894-1908.
[35] Porta C, Paglino C and Mosca A. Targeting PI3K/Akt/mTOR signaling in cancer. Front Oncol 2014; 4: 64.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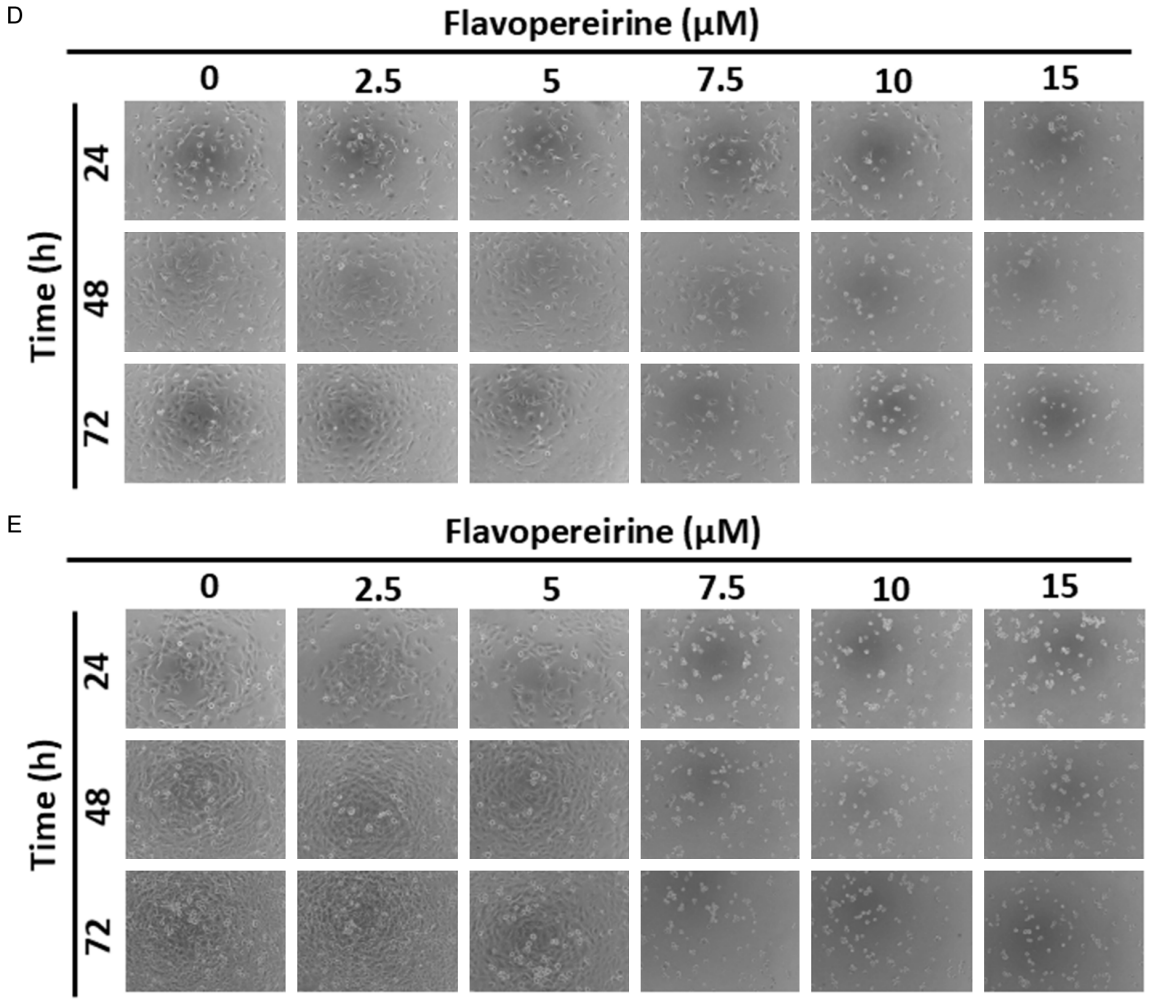

Supplementary Figure 1. Flavopereirine suppresses cell growth and modulates cellular morphology in human thyroid cancer cells. (A) IHH-4, (B) WRO, (C) SW579, (D) 8505c, and (E) KMH-2 cells were incubated with flavopereirine, and the cellular morphology was determined in the microscopy (100×).