Deltonin induced both apoptosis and autophagy in head and neck squamous carcinoma FaDu cell

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Received June 25, 2014 / Accepted November 19, 2014

For decades, despite the advancement of medical science, the prognosis of head and neck squamous cell carcinoma (HNSCC), has not improved. Deltonin is one of the major active components of Dioscorea Zingiberensis Wright that has been used for anthrax, rheumatic heart disease, rheumatoid arthritis etc. By employing HNSCC FaDu cell and normal human epidermal keratinocyte, we investigate deltonin efficacy and associated mechanism in both cell culture and nude mice xenografts. Deltonin treatment selectively prevents proliferation of FaDu cells by cell-cycle arrest and induction of apoptosis, via activating checkpoint kinase Chk1and Chk2 as well as caspases 8, 9 and 3. Meanwhile, we found that treatment with deltonin induced autophagy, which played a protective role against deltonin-induced apoptosis. Further studies revealed that deltonin activated autophagy by Akt-mTOR signaling. Additionally, xenograft model showed that administration of deltonin significantly inhibited tumor growth and prolonged survival of tumor bearing mice. Our studies suggested that deltonin might be a potential chemotherapeutic agent against HNSCC, which might contribute to clinical application and pharmacological study of deltonin in future anti-cancer research.

Key words: deltonin, anti-tumor, head and neck squamous cell carcinoma, apoptosis, autophagy

Almost all (95%) head and neck cancers are squamous cell carcinomas (HNSCC)[1]. HNSCC is the sixth most common type of cancer and cancer-related death that accounts for 6% of all cancer cases [2-3]. Current standard therapy options for HNSCC patients contains surgery, radiation, and chemotherapy, which undergoing multiple significant progresses in recent years. However, in the last three decades, the mortality rate of HNSCC is still high (5-year survival rate is less than 50%). In addition, although some patients are clinically cured, the survivors usually suffer voice disorder and dysphagia caused by surgery and radiotherapy—the most commonly used treatments for HNSCC [4]. To improve prognoses and the patients’ quality life, it is urgent to develop more reliable and safer treatments for patients with HNSCC.

Many evidence-based medicines are originally derived from extracts of traditional medical plants; it is therefore reasonable to suppose that investigations in this area may offer fruitful and relatively inexpensive sources of possible treatments [5]. Deltonin (diosgenin-3-O-β-d-glucopyranosyl (1→4)-[α-L-rhamnopyranosyl (1→2)]-β-d-glucopyranoside) is a major spirostanol glycoside constituent in Dioscorea Zingiberensis C.H. Wright (DZW), an important and widely used traditional Chinese medicine. As a widely distributed plant in China, DZW in nature have a variety of interesting biological activities and its rhizome has been used as a traditional Chinese medicine for anthrax, rheumatic heart disease, rheumatoid arthritis etc [6]. Steroidal saponins are identified as the major active components of DZW, including zingiberensis new saponin, diosgenin, trillin, gracillin [7-8]. Among these steroidal saponins, deltonin is one of the two main constituents [9]. In its chemical structure, diosgenin-3-O-β-D-glucopyranosyl(1→4)-[α-L-rhamnopyranosyl (1→2)]-β-D-glucopyranoside, is a large hydrophobic structure contributes to transportation across the cell membrane, while the sugar moiety determines hydrophilicity [10]. Recently, many reports showed that deltonin elicited an anti-tumor effect in several tumor cell lines, including SKOV3, B16, PC-3, LL2, CT26, A549 and MDA-
MB-231[11], but its anti-tumor spectrum is still limited and the mechanism remains unclear.

Autophagy is a highly regulated homeostatic process involved in turnover of long-lived proteins and whole organelles by lysosomal activity that eliminates excess or damaged organelle and some pathogens[12]. The formation of double-membrane autophagosome initiated by PI3 kinase type III-Atg6/Beclin 1 cascade is the main characteristic of autophagy[13]. Formation and expansion of the autophagosome are mediated by two ubiquitin-like conjugation systems: the Atg8/LC3 –phosphatidylethanolamine conjugate system and the Atg12-Atg5 conjugate system[14]. Autophagy signaling can be activated by multiple signaling pathways in response to numerous forms of cellular stress including hypoxia, starvation, chemical insults or radiation[15]. Of them, the Akt-mTOR signaling pathway is considered as a classic negative regulator of autophagy by initiation of the vesicular double-membrane formation[16]. The molecular crosstalk between apoptotic and autophagic signaling pathway is complicated. Both pathways share several same or relational genes that are critical for their respective function[17]. It has been reported that Atg5, which is indispensable in autophagosome formation, switches autophagy to apoptosis response to death stimuli[18, 19]. In addition, Bcl-2 and Bcl-xL, 2 typical apoptosis negative regulators, can retard autophagy progression by binding to the BH3 domain of Beclin 1[20].

In the present study, we aimed to use isolated deltonin from DZW and investigated its roles in antagonizing HNSCC. We evaluated its effects against hypopharyngeal carcinoma cell line FaDu as indicated by proliferation, cell-cycle arrest in G2/M phase, apoptosis, and autophagy induced by apopto-

FaDu tumor-bearing mice. Our studies could provide the groundwork for future studies on the implication of autophagy in deltonin-mediated anticancer activities.

Materials and methods

Chemical and reagents. The deltonin was provided from the Institute for Nanobiomedical Technology and Mem-

brane Biology of Sichuan University (Chengdu, Sichuan, China); its purity, as determined by HPLC, was above 98%; its architecture is shown in Figure 1. GAPDH antibody, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium (MTT), dimethylsulfoxide (DMSO), FITC-dextran and tamoxifen (TAM) were purchased form Sigma Aldrich St. Louis, MO, USA. Primary antibodies against were purchased from the following sources: checkpoint kinase 1/2 (Chk1/2) were from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-Chk1 (Ser296 and Ser345), phospho-Chk2 (Thr68), caspase-3, caspase-9, Bcl-2, Akt, phosphor-Akt, caspase-8, Erk1/2 and p-Erk1/2 (Cell Signaling Technology, Beverly, MA, USA), Beclin 1, β-actin (Santa Cruz, CA, USA), Atg5, Atg7 (Abcam, Cambridge, MA, USA), mTOR, phospho-mTOR (Milli-
pore Corporation, Bedford, MA, USA), secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture. Human hypopharyngeal carcinoma cell line FaDu, was obtained from the American Type Culture Collection (ATCC). Cells were cultured in RPMI 1640 medium (Life Technologies, Bedford, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Auckland, New Zealand), 1 mmol/L sodium pyruvate, 50 µg/mL streptomycin, and 50 U/mL penicillin at 37°C in an atmosphere of 5% CO2. Normal human epidermal keratinocytes were purchased from ScienCell (USA) and cultured Clonetics™ KGM™ Keratinocyte Growth Medium (Lonza, USA).

Cell viability assay (MTT dye assay). The cytotoxicity of deltonin on hypopharyngeal carcinoma cells was determined by MTT assay. FaDu cells were seeded at a density of 5x10^4 per well in 96-well plates (Costar Corning, Rochester, NY, USA). After 24 h incubation, cells were treated with deltonin in various concentrations (0.5, 1, 2, or 5 µM); vehicle DMSO (< 0.05%) was used as a control. Five wells were used for each concentration. At 24, 48 and 72 h, the absorbance at 570 nm was measured with SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA), using wells without cells as blanks. All experiments were performed in triplicate. The concentration- and time-dependent curves of the deltonin-treated hypopharyngeal cancer cell lines were generated as the percent cell growth inhibition, using the following formula: the cell inhibitory rate = ([control cell A570] – [treated cell A570])/ [control cell A570] × 100%. The 50% inhibiting concentration (IC_{50}) was calculated by SPSS for windows software version 16.0 (IBM, USA).

Cell cycle and apoptosis assays. Flow cytometry was used to analyze cell cycle arrest and apoptosis induction effect of
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deltonin as previously described[21, 22]. Briefly, 2×10⁵ FaDu cells were cultured in 2 mL of medium/well in 6-well plates (Costar Corning, Rochester, NY, USA). According to the results of previous MTT screening, after overnight incubation, the cells were treated with 1, 2 or 5 μM (both for apoptosis analysis and cell-cycle analysis) of deltonin; vehicle DMSO (< 0.05%) was used as control. After 24 h, for cell-cycle analysis, cells were harvested by trypsinization, fixed with 70% ethanol at 4°C overnight, stained with propidium iodide and analyzed by FAC Sort cytometer (ESP Elite, Beckman Coulter, Fullerton, CA.). The apoptotic index was assessed using Annexin V-FITC Apoptosis kit, following the protocol provided by the manufacturer.

Western blot analysis. Cells (2.5×10⁵) were seeded in 6-well plates (Costar Corning, Rochester, NY, USA). After 24 h, the cells were incubated with various concentrations of deltonin (0.1 μM, 2 μM or 5 μM) for 48 h, or with deltonin of 5μM for different times (24 h, 48 h or 72 h). Proteins were extracted on ice in radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) with protease inhibitor (Roche Diagnostics, Basel, Switzerland). Equal amounts of protein (40 μg) in the cell extracts were fractionated by 12% SDS-PAGE denaturing gels using Mini-Protean Bio-Rad II System (Bio-Rad, Hercules, CA, USA), and then transferred onto PVDF membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked in 20 mM Tris-buffered saline-0.1% Tween (TBST) buffer containing 5% non-fat milk at room temperature for 1 h, and then probed with specific primary antibodies overnight at 4°C. After washing with TBST for 45 min, membranes were incubated with an appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature. Finally, immunoblot signals were visualized using enhanced chemiluminescence (Millipore, Bedford, MA, USA). Densitometry readings for three independent blots were taken with Quantity One software (Bio-Rad, Hemel Hempstead, Herts, UK) for statistical analysis.

In vivo nude mice FaDu xenograft assay. The tumor model we used in this study has been described previously [23]. Briefly, 5 × 10⁵ FaDu cells per 0.2 ml were s.c. injected into 4- to 6-wk-old nude mice (Beijing HFK Bioscience Co., LTD, Beijing, China). All experiments conformed to the animal care and use guidelines of the Institute's Animal Care and Use Committee of Sichuan University. The mice were randomly divided into three groups of 10 mice. One week after FaDu implantation, the treatment groups received their first doses of deltonin dissolved in a vehicle (0.5% carboxy methylcellulose/0.1% Tween-80) (Sigma–Aldrich, USA) in saline solution. Deltonin dosage and administration schedules were based on our preliminary toxicologic and pharmacokinetic studies[24]. Briefly, deltonin was given via intraperitoneal administration to tumor-bearing mice at 40 and 80 mg/kg every day for 3 weeks (between day 8 and 28). In parallel, the control group received the vehicle. General clinical observations of the mice, including determination of body weight and tumor growth, were made every three days. Tumor size was measured by the modified ellipsoid formula: \((\pi/6) \times AB^2\), where A is the longest and B is the shortest perpendicular axis of an assumed ellipsoid corresponding to tumor mass [25]. The mice were humanely euthanized when they became moribund, as defined by: weight loss >10-15%, lethargy or ruffled fur. The sacrifice date was recorded to calculate the survival time. For detection of toxicity effects of deltonin on mice, deltonin (80mg/kg) was intraperitoneal administered to Balb/c mice once per day for 8 weeks.

Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling detection. The Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed to study DNA fragmentation in ex vivo-incubated FaDu cells or tumor tissue. Analysis of apoptotic cells in ex vivo-incubated FaDu cells was performed using an apoptotic cell detection kit following the manufacturer's directions (Promega, Madison, Wisc., USA). To analyze apoptotic processes in tumor tissues, TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche Diagnostics) on paraffin-embedded tissue harvested from tumors per manufacturer's instructions. DAPI counterstain was used to identify cells with intact nuclei. TUNEL-positive cells had pyknotic nucleus with dark green fluorescent staining, pointed apoptosis. All fluorescence imaging was performed and digitally captured at ×100 magnification on a fluorescent microscope (Olympus, Tokyo, Japan).

Data analysis and reproducibility. Statistical analysis was performed with the SPSS software system (SPSS for Windows, version 13.0; SPSS Inc, Chicago, IL). The data of cell cycle distribution, cell apoptosis, cell viability and TUNEL-positive cells were analyzed by one-way ANOVA followed by post hoc tests. Non-parametric Mann–Whitney U-test was used to compare the data of quantifying bands on western blot. Survival curves were statistically analyzed using Kaplan–Meier test. Data were expressed as means ± S.E. A P value < 0.05 or < 0.01 was considered significant.

Results

Cytotoxic effects of deltonin in cultured FaDu cell cells. To evaluate the effect of deltonin on cell viability of human hypopharyngeal cancer cell line FaDu, we initially treated FaDu cells with various doses of deltonin (0.5 to 5 μM) for different time (24 h to 72 h), respectively. Cell proliferation was measured by MTT assay. Figure 2A demonstrates that deltonin inhibited cellular proliferation in a dose and time dependent manner. With deltonin treatment inhibition rate was 15±3%, 29±9%, 45±4% and 63±5%, 12±2%, 30±9%, 46±3% and 60±4%, 10±3%, 31±9%, 59±2% and 65±3% at 0.5, 1, 2, 5μM for 24, 48 and 72 h respectively. Importantly, similar deltonin concentrations inhibited slightly the growth of normal human epidermal keratinocytes after 72 h, with a inhibition rate 27±4% (Figure 2B). This data suggested deltonin selectivity toward HNSCC cells only. Deltonin showed an IC₅₀ (median growth inhibitory concentration value) of 3.43 μM after 24 h of
treatment, implying that deltonin elicits marked cytotoxic effects on FaDu cells.

Effects of deltonin on cell cycle distribution and apoptosis of FaDu cell. Flow cytometric analysis showed that deltonin caused G2/M arrest in FaDu cell but did not affect on the cell cycle of NHEK cells (Figure 3A and 3B). FaDu cells treated with different concentrations (0.5 μM, 1 μM, 2 μM and 5 μM) of deltonin for 24 h, 48 h, or 72 h. Inhibition rate of deltonin was detected by MTT assay. Deltonin apparently inhibited proliferation of hypopharyngeal carcinoma cells in a time- and dose-dependent manner 24 h, the corresponding quantity was 60.3%. A dramatic hypodiploid population of FaDu cells appeared after treatment with deltonin for 24 h using flow cytometric analysis. The results indicated that deltonin is effective on FaDu cells, which warrants further study of the mechanism of deltonin at cellular and molecular levels.

Effects of deltonin on caspase-3 signal pathway activation. By western blot analysis, we determined the change of these crucial apoptosis-related proteins in FaDu cells exposure to deltonin treatment. FaDu cells were treated with deltonin at different concentrations (0, 1, 2 or 5 μM) for 24 h, and with 5 μM deltonin for 48 h or 72 h to obtain the total proteins for western blot analysis. As shown in Figure 4A and 4B caspase-9 and -3 were activated by deltonin treatment, the levels of procaspase-8, procaspase-9 and -3 decreased, and levels of the cleaved caspase-3, -9 accumulated in a dose-dependent way of deltonin.

Deltonin on expression of antiapoptotic factor Bcl-2 and proapoptotic protein Bax. We then sought delineate further the mechanisms that underlie the combined effects of deltonin on FaDu cell apoptosis. We detected the effect of deltonin on expression of a major anti-apoptotic protein Bcl-2 Figure 4A and 4B. Compared with control group, deltonin treatment reduced the level of Bcl-2 expression significantly (P<0.05) (Figure 4A and 4B). Furthermore, increased levels of proapoptotic protein Bax was also associated with increased deltonin doses (Figure 4A and 4B).

Deltonin down-regulates p-ERK1/2 expression. The extracellular signal-regulated kinase-1/2 (ERK1/2) pathway is related to tumor growth and survival, and inhibition of ERK1/2 pathway has been shown to induce apoptosis in FaDu tumor cells [26-28]. As shown in Figure C and 4D, Compared with control group, deltonin reduced the expression of p-ERK1/2 protein in dose dependency.

Deltonin induced LC3 turnover in FaDu cells. Due to its localization and aggregation on autophagosomes, the lipidated form of LC3 transforming from LC3-I to LC3-II has been considered to be an autophagosomal marker [29-30]. To investigate whether deltonin treatment induced processing of LC3-I (18 kDa) to LC3-II (16 kDa), western blot analysis was performed. In Figure 5A, LC3 positive spots were accumulated in deltonin-treated FaDu cells, and the accumulation was more prominent while the dose of deltonin increased. As shown in Figure 5B, LC3-II was accumulated in deltonin-treated FaDu cells, and the accumulation was more prominent while the dose of deltonin increased. We also found that deltonin upregulated expression of Beclin 1 in a dose-dependent manner.

Deltonin inhibited the Akt-mTOR signaling pathway in FaDu cells. Akt-mTOR signaling is considered atypical negative regulator of autophagy [31]; therefore, we investigated whether phosphorylation of both Akt and mTOR was involved in deltonin-induced autophagy in FaDu cells. As shown in Figure 5C and 5D, deltonin treatment resulted in a noticeable inhibition of both Akt (S473) and mTOR (S2448).
Figure 3. Deltonin promotes G2-M phase arrest and induces apoptosis in FaDu cells. (A) Deltonin induces G2-M phase arrest in FaDu cells. Quantification of cell cycle distribution analyzed by one-way ANOVA followed by post hoc tests. (B) Effects of Deltonin on cell cycle distribution of NHEK cells. (C) Effect of Deltonin on the expression of checkpoint kinase Chk1/Chk2. (D) Assessment of apoptosis in deltonin-treated FaDu cells. After 24h of deltonin treatment, induction of apoptosis was determined by flow cytometric analysis of Annexin V-FITC and propidium iodide staining. Cells in the lower right quadrant indicate Annexin V+/PI+ early apoptotic cells; cells in the upper right quadrant indicate Annexin V+/PI+ late apoptotic cells. (E) Quatification of apoptosis in deltonin-treated FaDu cells analyzed by one-way ANOVA followed by post hoc tests. Each bar is representative of mean±S.E. of three samples for each treatment. * P<0.05, ** P<0.01 vs. control group. (F) A representative result of (C), the relative levels of expression for these proteins were evaluated by densitometry and assessed using Mann–Whitney U-test for non-parametric data.
phosphorylation in FaDu cells. Meanwhile, deltonin-mediated dephosphorylation of Akt and mTOR was more pronounced with the dose of deltonin increased.

**Inhibition of autophagy enhanced deltonin-induced apoptosis in FaDu cells.** To explore the biological effect of autophagy in deltonin-mediated apoptotic cell death, a well-known autophagy inhibitor chloroquine was utilized to disrupt and prevent completion of autophagy. In this round, cells were treated with deltonin, chloroquine alone or in combination. Our study showed that chloroquine enhanced deltonin-in-

![Figure 4](image)

**Figure 4.** Deltonin induced cell apoptosis in FaDu cells through western blot analysis. Quantitative analysis was performed by densitometric scanning. Bars represent the mean ± S.E. of 3 different experiments. *P < 0.05; **P < 0.01 vs. control group. (A) Deltonin activated caspase-3 signal pathway, reduced expression of antiapoptotic factor Bcl-2 and up-regulated expression of proapoptotic protein Bax. GAPDH was used as a loading control. (C) Deltonin resulted in a significant activation (phosphorylation) of ERK1/2. GAPDH was used as a loading control. (E) Expressions of caspase-9 and caspase-3 increased gradually in a time-dependent manner after treatment with deltonin. GAPDH was used as a loading control. (B), (D), (F) A representative result of (A), (C) and (E) respectively. The relative levels of expression for these proteins were evaluated by densitometry. Non-parametric Mann–Whitney U-test was used to compare the data in A, C and E.
Deltonin-induced suppression of FaDu cells growth significantly (P<0.01) (Figure 6A). In line with this observation, deltonin-induced apoptotic cell death was augmented in the presence of chloroquine, which was demonstrated by TUNEL assays to detect DNA fragmentation (Figure 6B and 6C).

Deltonin’s in vivo anti-tumor effects in a FaDu xenograft model. Finally, we tested the in vivo anti-tumor effect of deltonin in a FaDu xenograft model as described in material and methods. The FaDu xenograft mice group that received indicated i.p. injection of deltonin treatment showed a great

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**Figure 5.** Deltonin induced LC3 turnover and activated autophagy-related genes in gastric cancer cells. (A) FaDu cells were transfected with pEGFP-LC3 plasmid and treated with DMSO (<0.1%), 5 μM tamoxifen (positive control) or indicated concentrations of deltonin for 48 h. GFP-LC3 positive cells were defined as that cells have five or more GFP-LC3 punctate dots. The percentage of GFP-LC3 positive cells with GFP-LC3 dots and the average number of GFP-LC3 dots per cell were assessed from 100 random fields. (B) Immunoblot analysis of LC3 and Beclin 1 expression from lysates of FaDu cells treated with various concentrations of deltonin for 48 h. β-actin was used as a loading control. (C) Relative levels of expression for these proteins were evaluated by densitometry. Non-parametric Mann–Whitney U-test was used to compare the data in C and E. All data were representative of three independent experiments. Values are means ± S.E.* P< 0.05; ** P< 0.01 vs. control group.
inhibition in tumor growth, which is demonstrated by reduced tumor size (Figure 7A) and improved mice survival and (Figure 7B), indicating a significant anti-tumor ability by deltonin in vivo. In deltonin-treated groups, 40 and 80 mg/kg led to reductions in tumor growth of 27.8% (P < 0.05) and 44.64% (P < 0.01) respectively, compared with the vehicle control (Figure 7A). There is an increase in survival in the deltonin-treated groups. At the terminus of the study, compared with the control, the 80 mg/kg group and 40 mg/kg group resulted in a 50-day survival rate of 20% and 15%, respectively (Figure 7B) (P < 0.05). In addition, there are no significant differences in weight among the groups, indicating lack of toxicity-dependent weight loss in tumor-bearing mice treated with deltonin (Figure 7C). We also observed deltonin caused significant tumor cell apoptosis in Fadu tumor-bearing mice. TUNEL assay was performed to measure cell apoptosis in tumor tissue. Deltonin-treated tumor tissue showed significantly increased number of positive-cells than the control groups (Figure 7D).

To examine the toxicity of deltonin, mice were sacrificed 8 weeks after consecutively oral administration of 80 mg/kg deltonin. Then main organs including heart, liver, spleen, lung and kidney were taken for histological examination by H&E staining. As shown in Figure 8, we found no significant change of histopathology in deltonin treated group.

**Discussion**

In the present study, we found that deltonin exhibited cytotoxic effect against a hypopharyngeal squamous cell Fadu both...
Deltonin induced both apoptosis and autophagy in FaDu cell in vitro and in vivo. In vitro, deltonin had a dose-dependent effect on cytotoxicity of FaDu cells by reducing viability in relation to control (Figure 2). By using flow cytometry analysis, deltonin interfered in cell cycle arrest and showed an increased proportion of hypodiploid DNA. Western blot analysis showed that deltonin induced apoptosis via ERK1/2 and Caspase kinase pathways. What’s more, to our surprise, we observed an induction of protective autophagy in FaDu cells while inducing apoptosis by deltonin. In vivo, deltonin treatment displayed anti-tumor activities in murine model of hypopharyngeal carcinoma xenografts.

Cell cycle control has been proven to be an important strategy in cancer therapy, while abnormalities of cell cycle regulators have been associated with many carcinogenic processes. There was a prominent increase in the G2/M DNA after deltonin treatment (Figure 3A), suggesting that deltonin could prevent FaDu cells from entering mitosis, and inhibit carcinoma cell growth by this pathway in a concentration-dependent manner. This is line with a Chk1/2 activation in FaDu cells exposed to deltonin (Figure 3C and F). In general, activated by Ataxia telangiectasia mutated protein (ATM) and ataxia telangiectasia and Rad3-related protein (ATR), Chk1 and Chk2 play a pivotal role in controlling progression of cells through different phase of cell cycle, which deter cells to enter new phase without the successful completion of the earlier phase [32,33]. In the G2/M checkpoint, the activated CHK1 or CHK2 phosphorylates and inhibits the cell division cycle 25 (Cdc25) phosphatase activity [34,35]. Our result probably of-
ferred a reasonable explanation in an earlier report on colorectal cancer, deltonin was reported to exhibit a promoting dramatic G2-M phase arrest in CT26 cell [24].

Inducing apoptosis is an important strategy to overcome cancer [36,37,38]. Previous studies proved that activations of caspase-9 and caspase-3 lead to apoptosis in FaDu cells [39,40]. Here, deltonin treatment altered expression levels of caspase-3, caspase-8 and caspase-9 in concentration- and time-dependent manners and also increased the expression of Bax in concentration dependent manner (Figure 4A and 4B). These findings suggested that deltonin treatment mediated cross-talk between the intrinsic and extrinsic apoptotic pathways by altering expression levels of several regulators of these pathways, such as caspase-9, caspase-3, Bax. ERK1/2 pathway is known to dictate cell fate during DNA damage, mitogenic stimuli, and survival [41]. ERK1/2 phosphorylates caspase-9 at Thr125 then inhibits caspase-9 processing and caspase-3 activation [42]. Inhibition of ERK1/2 phosphorylation is proved to blockade CXCL12-induced FaDu migration and chemoinvasion, suggesting that activation of ERK1/2 is an important step in the signal that leads to increased migration and chemoinvasion of HNSCC [43]. A very recent research showed that ERK1/2 MAPK mediated FaDu cell death via a caspase-independent mechanism [44]. We found that the level of p-ERK1/2 protein was reduced in FaDu cells treated with deltonin (Figure 4C and 4D), consistent with the previous study of Kian Ang et al., that the decreased activity of p-ERK1/2 in hypopharyngeal cancer cells was accompanied by cell death in HNSCC [45]. The reduction of phosphorylated ERK1/2 activation was accompanied with the activation of caspase-9 and -3 (Figure 4E and 4F). These results suggested that deltonin may suppress hypopharyngeal squamous cell carcinoma through targets including the supporting cell-survival function of ERK and activating apoptotic signals. Further study should be done to elucidate the role of p-ERK1/2 in deltonin induced apoptosis.

Of interest, we discovered autophagy enhancement by deltonin, in addition to apoptosis incitation (Figure 5). Monitoring conversion from LC3-I to LC3-II is widely used as a marker for autophagosome determination. Protein Atg6 (also called beclin 1) is considered to have a central role during the initiation of autophagosome formation. By interacting
with several cofactors (Atg14L, UVRAG, Bif-1, Rubicon, Ambra1, HMGB1, nPIST, VMP1, SLAM, IP3R, PINK and survivin), Beclin 1 regulate lipid kinase Vps-34 protein and promote formation of Beclin 1-Vps34-Vps15 core complexes, thereby inducing autophagy. As one of the classic pathway that regulates autophagy, mTOR activity inhibits autophagy. Akt-mTOR signaling pathway is the major negative signaling pathway against autophagy [31]. In addition, increasing levels of Akt activation correlates with an increase in the malignant potential of HNSCC [46]. Akt activation occurs by phosphorylation at T308 and S473 residues and has been validated as a critical step in the initiation and maintenance of metastatic tumors [47]. Chemical interaction of Akt is reported to cause apoptosis, and inhibit chemotaxis and migration FaDu cells in vitro [48]. When treated with deltonin, downregulated levels both dephosphorylated Akt at Ser473 and p-mTOR at s2448 were found in FaDu cells (Figure 5C and 5D), suggesting a profound effect of deltonin on Akt/mTOR signaling network in FaDu cells.

Although the relationship between apoptosis and autophagy is still unclear, there are crucial proteins that are relevant to both processes, implying some degree of crosstalk: Bcl2 and Bcl-XL are anti- apoptotic as well as being blockers of autophagy. Pattingre et al. reported that the anti-apoptotic protein, Bcl-2, interacts with the evolutionarily conserved autophagy protein, such as Beclin-1 [49]. Chloroquine is a lysosomotropic agent that has been suggested to inhibit autophagy by perturbing lysosomal function. Blocking autophagy by utilizing of chloroquine increase the susceptibility to FaDu cell to pro-apoptotic insults (Figure 6), suggesting that deltonin-induced autophagy plays a protective role in FaDu cells. Certain anticancer agents such as polyoxomolybdates-, platonin- or phenethyl isothiocyanate could induce autophagy to enhance chemotherapeutic efficacy [50,51,52], while others, i.e., arginine deiminase, suberoylanilide hydrazide (higher dose). Then histopathological analysis of several organs and serum biochemical parameters were analyzed. As shown in Figure 8, we found no significant difference of histopathology in deltonin group.

Our current study revealed a hitherto undescribed cellular response showing that deltonin induced autophagy antagonizing apoptotic cell death in FaDu cancer cells. In terms of the molecular machineries and signaling cascades, we offered evidence that involvement of Akt-mTOR signaling in deltonin-induced autophagic progression. This work will not only enrich our understanding of deltonin in cancer treatment, but also be helpful for the development of the whole family of saponins in disease treatment and structure modification.

**Supplementary information** is available in the online version of the paper.

Acknowledgments: We thank Dr. Xiao-Hua Wu from the Institute for Nanobiomedical Technology and Membrane Biology of Sichuan University, for kindly providing deltonin used in the present research. This work was supported by National Natural Science Foundation of China (No. 8100093).

**References**


Supplementary Information

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Supplementary Figure

Detonin causes G2/M arrest in FaDu cell after 24 hours exposure to varying doses at 1, 2, 5 μM respectively.