Deltonin, a Steroidal Saponin, Inhibits Colon Cancer Cell Growth *in Vitro* and Tumor Growth *in Vivo* via Induction of Apoptosis and Antiangiogenesis

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Key Words

Steroidal saponin • Deltonin • Anti-tumor • Colorectal Cancer • Apoptosis • Antiangiogenesis

Abstract

Deltonin, a steroidal saponin, isolated from *Dioscorea zingiberensis* Wright (DZW), has shown high-cytotoxic activity in cancer cells. However, its mechanisms and *in vivo* anti-cancer effects remain unknown. In the present study, we evaluated the effects and explored the anti-tumor mechanisms of deltonin on a panel of colon cancer cell lines and in a mouse model of murine colon cancer C26. Deltonin had more cytotoxic effect on C26 cells than 5-fluorouracil had, promoting dramatic G2-M phase arrest and apoptosis in C26 cells in a concentration-dependent manner; oral administration of deltonin significantly inhibited the tumor growth and prolonged survival of the tumor bearing mice. The deltonin treatment caused a noticeable apoptosis in tumor tissue, which associated with increased levels of Bax, activated caspase-3, caspase-9, and cleaved poly (ADPribose) polymerase, decreased pro-caspase-8, pro-caspase-9, Bcl-2 expression levels and extracellular signal-regulated kinase-1/2 activity; and dose-dependently

inhibit angiogenesis. In conclusion, the findings in this study demonstrated that deltonin is an effective natural agent for cancer therapy, which may be mediated, in part, by induction of apoptosis, as well as involve mitogen-activated protein kinase pathways, and inhibition of angiogenesis.

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Introduction

Colorectal cancer (CRC) is now the fourth most common cancer in men and the third most common cancer in women worldwide, and in developing countries such as China, the incidence would be a growing one because of the environmental factors and increased proportion of older age classes [1, 2]. Surgical resection is still the only effective treatment for localized disease; however, most colorectal cancer patients have regional or distant metastasis at the time of their initial presentation. Besides surgical resection, treatment strategies for high-risk patients are still mainly based on adjuvant or neoadjuvant use of 5-fluorouracil (5-fu) and folinate as chemotherapeutic agents alone or in combination with radiotherapy.

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Unfortunately, use of the above standard therapeutic protocols results only in a moderate decline in mortality and the risk to sustain a recurrence disease remains high [3, 4]. The medicinal use of plant extracts seems to be a more natural, less expensive approach and in general involves minimal unwanted side effects [5]. Hence, evaluation of naturally occurring phytochemicals agents that can reduce the risk and retard or inhibit the development of colon cancer could lead to new strategies for colon cancer chemoprevention and therapy.

Dioscorea zingiberensis Wright (DZW) is a widely distributed medicinal plant in China. Its rhizome has been known as a traditional Chinese medicine for a long time and used as a folk treatment for cough, anthrax, rheumatic heart disease, rheum, arthritis, tumefaction and sprain [6]. Steroidal saponins are the major active components of DZW, including diosgenin, trillin, gracillin, diosgenin diglucoside protogracillin, deltonin, *zingiberensis* newsaponin and so on [7-9]. Deltonin is one of the two main constituents of these steroidal saponins [10]. Its chemical structure is diosgenin-3-O- β -D-glucopyranosyl (1 $\rightarrow$ 4)-[α -L-rhamnopyranosyl (1 $\rightarrow$ 2)]- β -D-glucopyranoside, in which diosgenin is a large hydrophobic structure contributes to the molecular transported across the cell membrane of an organism, while the sugar moiety mainly determines the hydrophilicity of molecular [11]. According to the previous report, the parent compound of deltonin, diosgenin, has been shown to render cytotoxic effects against many cancer cell lines [12]. As a derivate of diosgenin, deltonin also has a strong antitumor capability [13]. However, its antitumor effects have not been clarified clearly. In attempting to further elucidate the antitumor activity and mechanisms by which deltonin works, we isolated deltonin from DZW and studied the role of it in inhibiting tumor cell growth both *in vitro* and *in vivo*. We evaluated the effects of deltonin on cell proliferation, cell cycle arrest and apoptosis in a panel of colon cancer cell lines. Specially, we assessed the antitumor efficacy of deltonin on apoptosis, extracellular signal regulated kinase (ERK1/2) activity, and antiangiogenesis in C26 tumor bearing mice.

Materials and Methods

Reagents

The deltonin was obtained from the Institute for Nanobiomedical Technology and Membrane Biology of Sichuan University (Chengdu, Sichuan, China). Briefly, deltonin was isolated from the rhizomes of DZW, using silica gel, macroporous adsorption resin, Sephadex LH-20, RP-C18 column

chromatography and vacuum system (Büchi, Switzerland). Its structure was identified on the basis of its spectral data (1D and 2D NMR, MS and IR experiments) and its purity determined by HPLC was above 98%. 5-fluorouracil (5-Fu), 3-[4,5-Dimethylthiazol-2-yl]-2,5 -diphenyltetrazolium(MTT), dimethylsulfoxide (DMSO), GAPDH antibody and FITC-dextran were purchased from Sigma Chemical Co. OCT compound was obtained from Sakura Finetechnical Co. (Tokyo, Japan). Primary antibodies against p44/42 MAPK (Erk1/2) (137F5), phospho-p44/42MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E), Akt, phospho-Akt (Ser473), caspase-3, caspase-8, and caspase-9, poly (ADPribose) polymerase (PARP) were purchased from Cell Signaling Technology (Beverly, MA); Bcl-2 (C-2), Bax (P19), and secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ECL detection system was from Amersham (Arlington Heights, IL). All other chemicals were of the highest quality commercially available.

Cell Lines and culture

Human colon cancer cell lines SW480, SW620, LOVO and mouse colon cancer cell line C26, were obtained from the American Type Culture Collection. Cells were cultured in RPMI 1640 or Dulbecco's modified Eagle medium (Life Technologies, Bedford, MA) supplemented with heat-inactivated fetal bovine serum (FBS, Gibco, Auckland, New Zealand), 100units/ml penicillin, and 100 units/ml streptomycin in a humid chamber at 37°C under 5% CO₂. Treated cells were cultured in fresh medium.

MTT assay

The cytotoxicity of deltonin on colon cancer cells was determined by the MTT assay [14]. Cells were seeded at a density of 3×10^3 per well in 96-well plates (Costar Corning, Rochester, NY). After incubation overnight, cells were treated with deltonin in various concentrations (0.5, 1, 2, 4, 8 μ M), and carrier DMSO (< 0.05%) was used as a control, 5 wells were included in each concentration. Every 24 h the absorbance at 570 nm was measured with SpectraMax M5 (Molecular Devices), using wells without cells as blanks. All experiments were performed in triplicate. The concentration-and time-dependent curves of the deltonin-treated colon cancer cell lines was generated as the % cell growth inhibition, using the following formula: % inhibition = (A570 of control - A570 of treated cells) / A570 of control cells $\times$ 100%. The 50% inhibiting concentration (IC50) was calculated by SPSS software version13.0.

Cell cycle and apoptosis assays

Flow cytometry was used to analyze the cell cycle arrest and apoptosis induction effect of deltonin as previously described [15, 16]. Briefly, 2×10^5 C26 cells were cultured in 2mL of medium/well in 6-well plates (Costar Corning, Rochester, NY). After incubation overnight, cells were treated with 1, 2 and 4 μ mol/L (or 0.2, 0.4 and 0.8 μ mol/L for cell-cycle analysis) of deltonin and carrier DMSO (< 0.05%) was used as a 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 AnnexinV-FITC Apoptosis kit

(Keygentec Nanjing, China) following the manufacturer's instructions. For each analysis, 30,000 events were acquired on a forward and side scatter gate. Annexin V⁺ PI⁺ cells represented the early apoptotic populations; Annexin V⁺ PI⁺ cells represented either late apoptotic or secondary necrotic populations. Data acquisition and analysis were performed in FACS using list mode software (Becton Dickinson).

In vivo evaluation

The tumor model we used in this study has been described previously [17, 18]. Briefly, 5×10^5 C26 cells were s.c. injected into 4- to 6-wk-old male BALB/c mice (Sichuan University Animal Center, Chengdu, China). All experiments conformed to the animal care and use guidelines of the Institute's Animal Care and Use Committee. The mice were randomly divided into four groups of 10 mice. One week after C26 implantation, the treatment groups received their first doses of deltonin dissolved in a vehicle solution of DMSO (< 0.1%) and diluted in saline solution. Deltonin dosage and administration schedules were based on our preliminary toxicologic and pharmacokinetic studies. Briefly, deltonin was given via oral administration to tumor-bearing mice at 10, 20 and 40 mg/kg every day for 3 weeks (between day 8 and 28). In parallel, the control group received the vehicle (DMSO, < 0.1%) in saline solution. The same quantity of saline solution containing DMSO was used in these groups. General clinical observations of the mice, including determination of body weight and tumor growth, were made twice weekly. To determine tumor size, we measured two perpendicular diameters of the tumor in centimeters by calipers. Tumor volume was estimated using the formula $0.52 \times a \times b^2$, where "a" is the long diameter and "b" is the short diameter. The mice were sacrificed when they became moribund, and the sacrifice date was recorded to calculate the survival time. To detect the apoptosis protein expression and microvessel density, tumor tissues were removed, fixed in 10% formalin or not and snap-frozen in liquid nitrogen immediately.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling detection

Analysis of apoptotic cells in tumor tissue was performed by Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining using an apoptotic cell detection kit following the manufacturer's directions (Promega, Madison, Wisc., USA). TUNEL-positive cells had pyknotic nucleus with dark green fluorescent staining, pointed apoptosis. Images of the sections were taken by a fluorescence microscope (Olympus, Tokyo, Japan).

Western blot analysis

Western blot analysis was performed as described previously [19]. Briefly, tumor tissues were lysed in a buffer containing 50 mM Tris-HCL (pH 7.4), 1% (v/v) NP-40, 0.25% (v/v) Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenyl-methylsulfonyl fluoride (PMSF), 1 mg/ml leupeptin, 1 mg/ml pepstatin and 1 mg/ml aprotinin, 1 mM NaF, 1 mM Na₃VO₄ (Applygen Technologies Inc, Beijing, China), following the manufacturer's instructions. The protein concentration was

Fig. 1. The structure of deltonin, diosgenin-3-O- β -D-glucopyranosyl (1 $\rightarrow$ 4)-[α -L-rhamnopyranosyl (1 $\rightarrow$ 2)]- β -D-glucopyranoside.

determined by a modified Bradford assay kit (Applygen Technologies Inc, Beijing, China). The samples containing equal amounts of protein were denatured and subjected to electrophoresis in a 10% or 12% sodium dodecyl sulfate-acrylamide gel followed by electroblot transfer to PVDF membrane (Bio-Rad, Hercules, CA). For immunodetection, the membranes were blocked with 5% (w/v) nonfat milk and 0.1% Tween 20 (v/v) in TBS and probed with the following antibodies: rabbit anti-phosphorylated PARP (1:500), rabbit anti-Bax antibody (1:1000), rabbit anti-caspase3 antibody (1:1000), rabbit anti-caspase8 antibody (1:1000), rabbit anti-MAPK antibody (1:1000), rabbit anti-phosphorylated MAPK (pMAPK) antibody (1:1000), rabbit anti-AKT antibody (1:1000), and mouse anti-phosphorylated AKT antibody (1:1000), mouse anti-caspase9 antibody (1:1000), mouse anti-Bcl-2 antibody (1:1000), mouse anti-GAPDH antibody (1:1000). Goat anti-rabbit IgG antibody conjugated to horseradish peroxidase or anti-mouse IgG antibody conjugated to horseradish peroxidase were used (1:5000) as the secondary antibodies. Protein bands were visualized using the Enhanced Chemiluminescence Plus Western Blotting Detection System (Amersham Life Science).

Immunohistochemistry and alginate-encapsulate tumor cell assay.

To explore whether the antitumor effect involved the inhibition of angiogenesis, detection of vessel density in tumor tissue and angiogenesis was done as described previously [20]. Frozen sections were used to determine vessel density with an anti-CD31 antibody. In addition, an alginate-encapsulate tumor cell assay was done. Briefly, male BALB/c mice were injected s.c. with 0.1 ml of alginate beads containing 1×10^5 C26 tumor cells into both dorsal sides of the back. Two days latter, mice were dosed orally with deltonin at 10, 20 and 40 mg/kg every day. After 12 days, mice were injected intravenously with 0.1 ml of a 100 mg/kg FITC-dextran solution. Alginate beads were rapidly removed and photographed 20 min after FITC-dextran injection. The uptake of FITC-dextran was measured as described [21].

Statistical analysis and reproducibility

The results are given as the means $\pm$ standard error (S.E.) and analyzed for statistical significance using analysis of

Fig. 2. The cytotoxic effects of deltonin on colon cancer cell lines. (A) Colon cancer cells were treated with various concentrations of deltonin for 24, 48, and 72 h, respectively. Inhibition rate of deltonin was detected by MTT assay. It appeared that deltonin inhibited the proliferation of colon cancer cells in a time- and dose-dependent manner; (B) Dose-effect curves for deltonin and the clinical antitumor drug 5-fu on the C26 cell line for 24, 48, and 72 h. Deltonin shows stronger cytotoxicity than 5-fu. Values are means $\pm$ S.E. from three experiments, $P < 0.05$ vs. control group. Table 1 shows the IC50 (means $\pm$ SD, n=3) of colon cancer cell lines after 48h deltonin treated.

variance (ANOVA), followed by Scheffe’s test formultiple comparisons. A P value < 0.05 or < 0.01 was considered significant.

Results

Deltonin treatment inhibits the growth of colon cancer cells

The chemical structure of deltonin is shown in Fig. 1. To investigate the cytotoxic effects of deltonin on colon cancer cells, we treated a panel of colon cancer cell lines that included SW480, SW620, LOVO and C26 with deltonin. As shown in Fig. 2A, 0.5-8 $\mu\text{mol/L}$ deltonin treatment resulted in significant cell proliferative inhibition in all treated cell lines. In addition, prolonged exposure of cancer cells to deltonin increased its inhibitory effect. It appeared that deltonin inhibited the proliferation of colon cancer cells in a time- and concentration-dependent manner. We compared the cytotoxic effects of deltonin with 5-fu in C26 cells. As indicated in Fig. 2B, deltonin showed more significant inhibitory effect than 5-fu. The maximum inhibition ratio attained with 2 $\mu\text{mol/L}$ deltonin treatment was 88.24%, compared with the 57.97% inhibition produced by 2 $\mu\text{mol/L}$ 5-fu. Deltonin treatment also had lower IC50 values (1.22 $\mu\text{mol/L}$) than 5-fu had (5.38 $\mu\text{mol/L}$). Furthermore, after 48h of deltonin treatment, the IC50 values of all cell lines are $< 2.5\mu\text{mol/L}$ (Table1).

Deltonin promotes G2-M phase arrest and induces apoptosis in C26 cells

To determine the mechanism involved in deltonin inhibition of tumor cell growth, we examined the effects of deltonin on cell cycle and apoptosis in C26 cells. Flow cytometric analysis indicated that after 24h of deltonin treatment the population of cells trapped in the G2-M

Tumor cell lines	Cell type	IC50(μM)
C26	mouse colon cancer cell line	1.22±0.22
SW-620	human colon cancer cell line	1.29±0.69
SW-480	human colon cancer cell line	1.30±0.05
LOVO	human colon cancer cell line	2.11±0.68

Table 1. IC50 of tumor cell lines after 48h deltonin treated.

Fig. 3. Deltonin promotes G2-M phase arrest and induces apoptosis in C26 cells. (A) Deltonin induces G2-M phase arrest in C26 cells. (B) Columns, means of three different experiments; bars, S.E., * $P < 0.05$, ** $P < 0.01$ vs. control group. (C) Assessment of apoptosis in deltonin treated C26 cells. After 24 h of deltonin treatment, the induction of apoptosis was determined by flow cytometric analysis of Annexin V-FITC and PI-staining. Cells in the lower right quadrant indicate Annexin-positive, early apoptotic cells, and cells in the upper right quadrant indicate Annexin-positive/PI positive, late apoptotic cells. (D) Columns, means of three different experiments; bars, S.E., * $P < 0.05$, ** $P < 0.01$ vs. control group.

phase increased in a concentration-dependent manner (Fig. 3 A and B).

Cell morphology evaluated under an inverted phase contrast microscope showed that deltonin treatment induced anoikis, a phenomenon in which cells shrink and detach from the culture surface, suggested deltonin probability induced apoptosis [22]. Flow cytometry analysis with an AnnexinV-FITC Apoptosis kit also indicated that deltonin treatment induced apoptosis in C26 cells in a concentration-dependent manner (Fig. 3C). Compared with the untreated C26 cells, 4μmol/L deltonin treatment resulted by up to 83.36% apoptosis incidence after a 24h treatment (Fig. 3D). Together, these results imply that deltonin inhibits the proliferation of colon cancer cells by cell cycle arrest or inducing apoptosis or both.

Deltonin inhibits C26 derived-tumor growth and prolongs the survival of the tumor-bearing mice

Having shown that deltonin suppresses tumor cell growth, we investigated its antitumor effects in a mouse model of colon cancer. As shown in Fig. 4A, at the terminus of the experiment, the oral administration of deltonin at 20 and 40 mg/kg led to reductions in tumor growth of 37.7% and 56.7%, respectively, compared with that in control mice treated with vehicle solution of DMSO

(< 0.1%) diluted in saline solution. We measured the animal weight and found no significant difference among all the mice (Fig. 4B). We also observed appetite, fur, behavior etc. to evaluate the physical status and there were no changes in gross measures. In addition, H&E histological staining of the heart, liver, spleen, lung, and kidney indicated no significant differences between deltonin-treated and the control mice. Most intriguing, as shown in Fig. 4C, at the terminus of the study, compared with the control, the 40 mg/kg group and 20 mg/kg group resulted in a 50-day survival rate of 60% and 50%, respectively. Although the 10 mg/kg group showed a similar 50-day survival rate the control, the death date was obviously delayed. The control animals survived only 34.2 days on average, whereas deltonin-treated mice survived 52.5 days in 40 mg/kg group, 49.5 days in the 20mg/kg group and 39.2 days in 10mg/kg group. These data suggest that oral administration of deltonin had effects on inhibiting C26 derived-tumor growth and prolonging the survival of the tumor-bearing mice.

Deltonin induces cell apoptosis in tumor tissue

As deltonin induces cell apoptosis obviously *in vitro*, we observed if it also caused tumor cell apoptosis in animal. TUNEL assay was performed to detect tumor

Fig. 4. Deltonin inhibits C26 derived-tumor growth and prolongs the survival of the tumor-bearing mice. (A) Suppression of tumor growth in deltonin-treated groups, 20 and 40 mg/kg led to reductions in tumor growth of 37.7% and 56.7% respectively, compared with the vehicle control. Points, mean; bars, S.E., * $P < 0.05$, ** $P < 0.01$. (B) Lack of toxicity-dependent weight loss in tumor-bearing mice treated with deltonin. There are no significant differences in weight among the four groups. Values are means $\pm$ S.E. $P < 0.01$. (C) An increase in survival in the deltonin-treated groups, compared with the vehicle control ($P < 0.05$). At the terminus of the study, compared with the control, the 40 mg/kg group and 20 mg/kg group resulted in a 50-day survival rate of 60% and 50%, respectively.

Fig. 5. Deltonin induces cell apoptosis in tumor tissue. (A) Representative TUNEL data presented as the apoptotic cells of treated tumor tissue, which showed that deltonin treated induced a significant enhancement of apoptotic cells in contrast to control therapies (original magnification 400 $\times$). (B) Representative Western blot analysis for caspase -8,-9,-3 and Bcl-2, Bax, PARP levels in tumor samples. GAPDH was used as a loading control.

Fig. 6. Deltonin inhibits ERK1/2 activation. Representative western blot analysis for phosphorylated-Akt, phosphorylated-ERK, total AKT and total ERK levels in tumor samples. Phosphorylation of ERK1/2 and ERK levels were obviously inhibited by deltonin, whereas total ERK1/2 and Akt slightly decreased. GAPDH was used as a loading control.

cell apoptosis. As shown in Fig. 5A, deltonin-treated tumors showed significantly more apoptotic cells in the tumor tissues than the control groups. We also determined the change of the crucial apoptosis-related proteins in tumor tissue in response to deltonin treatment. As shown in Fig. 5B, with deltonin treatment, caspase-9 and -3 were

Fig. 7. Deltonin inhibit angiogenesis within tumors. (A-D) Frozen sections of tumor tissue were tested by immuno-histochemical analysis with anti-CD31 antibody (brown, original magnification 200×). (E) Vessel density was determined by counting per high power field in the sections stained with an antibody reactive to CD31 as described above. Vessel density in deltonin treated tumor tissues indicated a significant decrease compared with controls. Columns: means; bars: S.E. (* $P < 0.05$, ** $P < 0.01$); (F-I) Vascularization of alginate implants. FITC-dextran was quantified as described above. (J) FITC-dextran uptake of beads from deltonin treated mice indicated a significant decrease. Columns: means; bars: S.E., $n = 6$ for each group (** $P < 0.01$, compared with control group).

activated, the levels of procaspase-8, procaspase-9 and -3 decreased, and levels of the cleaved caspase-3, -9 accumulated in a dose-dependent way. Furthermore, increased levels of the proapoptotic protein Bax and a dramatic reduction of Bcl-2 levels were also associated with increased deltonin doses. According to these results, we propose that deltonin induces cell apoptosis in tumor tissues, which may explain why tumor volumes shrunk.

Deltonin inhibits ERK1/2 and AKT activation

The extracellular signal-regulated kinase-1/2 (ERK1/2) pathway or Akt activation is known to be important in tumor growth and survival, and the inhibition of either has been shown to induce apoptosis in tumor cells [23-25]. So we analyzed if these two signal pathways were affected in deltonin-treated tumor bearing mice. As shown in Fig. 6, deltonin clearly reduced the phosphorylated levels of Akt and ERK1/2, whereas the levels of total Akt and ERK1/2 were not significantly altering. This result indicates that the anti-tumor effect of deltonin may be associated with inhibition of Akt and ERK1/2 activities.

Deltonin inhibits tumor angiogenesis

Immunohistochemical staining of the tumor tissue from deltonin treated mice with anti-CD31 antibody

showed significantly decreased microvessel density compared with control group (Fig. 7A-D). Vessel density was also determined by counting per high power field in the sections stained with an antibody reactive to CD31. As shown in Fig. 4E, vessel density reduced significantly in 10mg/kg, 20mg/kg and 40mg/kg deltonin treated groups (49.9 ± 3.9 /hpf, 30.8 ± 3.3 /hpf and 16.6 ± 1.9 /hpf, respectively) compared with control group (63.1 ± 4.52 /hpf). In addition, inhibition of angiogenesis could also be detected in the alginate-encapsulate tumor cell assay. As shown in Fig. 7F-I, vascularization of alginate beads was apparently reduced. Angiogenesis in alginate implants was quantified by measuring the uptake of FITC-dextran into the beads. Fig. 7J showed that FITC-dextran uptake was significantly decreased in deltonin treated mice when compared with controls. These results suggest that tumor angiogenesis was inhibited in deltonin treated mice, which resulted in the suppression of tumor growth.

Discussions

Dioscorea zingiberensis Wright is a widely distributed medicinal plant and its components have been used extensively for many years in China. Previously, we isolated total steroidal saponins from DZW and found it have the potential to reduce the risk of cardiovascular

diseases by antithrombotic action, and its traditional uses did not appear to have significant toxicity [9, 26]. We also found diosgenin could be a very useful compound to control hypercholesterolemia by both improving the lipid profile and modulating oxidative stress [27]. Deltonin is a major component of steroidal saponins from DZW and a derivative compound of diosgenin, which has been reported useful for cancer therapy, but its biology and mechanism of action in treating cancer are not well understood. In the present study, we investigated the effects of deltonin on the biology of tumor cells, and found that deltonin has significant anti-cancer activities against a panel of colon cancer cell lines and C26 is the most sensitive to deltonin treatment. Specifically, deltonin inhibited the viability of C26 cells at a low IC₅₀, even lower than 5-fu, a widely used chemotherapeutic medicine, suggesting that it has a powerful cytotoxic effect on tumor cells. As not all the compounds that exhibit antitumor activity *in vitro* effective *in vivo*, so we investigated the *in vivo* antitumor effect of deltonin, and confirmed that deltonin is a potent anticancer agent that can be oral administered.

Inhibiting proliferation is a critical mechanism of antitumor drug-induced cell death. Besides that, inducing apoptosis is another important strategy to overcome cancer [28-30]. In our study, we found that deltonin treatment inhibits the proliferation of colon cancer cells by inducing apoptosis or cell cycle arrest or both. 24 hours deltonin treatment increased the population of cells trapped in the G2-M phase in a concentration-dependent manner. This indicates that deltonin treatment can promote G2/M arrest and inhibits the proliferation of colon cancer cells. It was previously described that p21 induces cell cycle arrest by both p53-dependent and independent pathways [31]. As a proliferation inhibitor, p21 is poised to play an important role in preventing tumor development. We examined the expression levels of p21 in deltonin treated tumor tissues using western blotting; it's showed that the expression is decreasing in a concentration-dependent manner (data not show). This result indicating that deltonin treatment may block cell cycle in a time-dependent manner, which may not interrelate with p21, the other possible mechanisms is our further research.

It's generally accepted that activated caspase-8, an initiator caspase, can directly cleave the proenzyme forms of effector caspases such as caspase-3, -6 and -7, leading to activation of the latter. This pathway is often referred to as the extrinsic pathway of apoptosis signaling [32]. In this study, we found that deltonin treatment altered the expression levels of caspase-8 and -3, indicating that

deltonin may induce apoptosis through extrinsic pathway. Activation of caspase-8 also induces apoptosis through mitochondrial pathways, referred to as intrinsic pathways [33]. This apoptosis pathway is mainly regulated by the interplay between members of the Bcl-2 protein family, which is followed by the formation of the apoptosome complex and caspase activation. We also showed that deltonin treatment altered the expression of caspase-9, Bax, Bcl-2 and poly (ADP-ribose) polymerase, which are components of the intrinsic apoptotic pathway. Altogether, these data suggest that deltonin treatment may mediate cross-talk between the intrinsic and extrinsic apoptotic pathways by altering the expression levels of several regulators of these pathways, such as caspase-8, -9 and -3, Bcl-2, Bax, and poly (ADP-ribose) polymerase.

Signals from distinct signaling cascades of the MAPK family such as stress-activated protein kinase/c-jun NH2-terminal kinase, p38 MAPK, and ERK1/2 are known to dictate cell fate during DNA damage, mitogenic stimuli, and survival [34]. ERK1/2 also phosphorylates caspase-9 at Thr125 resulting in the inhibition of caspase-9 processing and caspase-3 activation [35]. In the present study, we found that the inhibitory effects of deltonin were associated with significantly decreased phosphorylated levels of ERK1/2 and Akt in a dose-dependent manner. The reduction of phosphorylated ERK1/2 activation accompanied with the activation of caspase-8, -9 and -3. These results suggest that deltonin may elicit its anti-tumor effect through multiple targets, which may include impeding the supporting cell-survival function of ERK and activating apoptotic signals.

Tumor angiogenesis is a complex and obligatory process for the growth and progression of solid tumors beyond the size limit (approximate 2mm diameter) imposed by simple diffusion for the nutrient supply. Limiting nutrient supply by the blockage of neovessels formation in tumor has been a useful strategy in cancer therapy [36, 37]. In 2004, Avastin became the first antiangiogenic drug approved by the US Food and Drug Administration (FDA) intended for combination use with standard chemotherapy for metastatic colon cancer [38]. Phytochemicals such as curcumin, genistein, and ginseng saponins were also found to exert inhibitory effect on the neovascularization, and these phytochemicals are effective at a relatively lower dose with relatively mild or undetectable side effect [39, 40]. In the present study, we found that deltonin could dose-dependently inhibit angiogenesis, which is consistent with a recent patent

report that deltonin could block angiogenesis by inhibiting bFGF-induced endothelial cell growth and migration [41]. Thus, in addition to induce tumor cell apoptosis, deltonin may also be developed as an angiosuppressive drug in controlling tumor growth and metastasis.

Furthermore, we showed that deltonin is a potent anticancer agent that can be oral administered. Recently, chemotherapy drugs are developing in the form of a pill that can be administered orally. Xeloda (capecitabine), a form of the chemotherapy drug 5-FU that can be administered orally, was invented because side effects of IV 5-FU may include pain and infection at the injection site. Results from recent clinical trials have demonstrated that the oral Xeloda is associated with fewer side effects than IV 5-FU while providing similar treatment outcomes and allowing patients to be treated at home [42-44]. The oral administration of deltonin dramatically decreased tumor sizes by 56.7% after a 3-week treatment period in a mice model. This attribute may confer advantages to deltonin that other i.v. or i.p. agents lack, including convenience, reduction of complications associated with long-term venous access, and cost savings. Nevertheless,

because the high incidence and mortality of colon cancer, additional studies using different colon cancer model systems should be used to fully establish the therapeutic potential of deltonin for colon cancer patients at all stages.

In conclusion, after isolating deltonin from DZW, we systematically show for the first time, to our knowledge, the anti colon cancer effects of deltonin. We have shown that deltonin can induce cancer cell apoptosis, inhibit ERK1/2 activation and exhibit angiosuppressive effect in a mice model of colon cancer. Of greatest interest to us was that deltonin displayed salient antitumor activity, and oral availability, indicating that deltonin may have great therapeutic potential in clinical settings.

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