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Basic Study

Deltonin enhances gastric carcinoma cell apoptosis and chemosensitivity to cisplatin *via* inhibiting PI3K/AKT/mTOR and MAPK signaling axis

Yang L *et al*. Deltonin and GC

Abstract

BACKGROUND

As an active ingredient derived from *Dioscorea zingiberensis* C.H. Wright, deltonin has been reported to show anti-cancer effects in a variety of malignancies.

AIM

To investigate its role and mechanism of action in suppressing gastric carcinoma (GC) cell apoptosis and chemosensitivity to cisplatin.

METHODS

GC AGS, HGC-27, and MKN-45 cells were treated with deltonin and then subjected to flow cytometry and 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide assays for cell apoptosis and viability determination. Western blotting was conducted to examine alterations in apoptosis-related proteins (Bax, Bid, Bad, Fas), DNA repair-associated proteins (Rad51 and MDM2), and phosphatidylinositol 3-kinase/protein kinase B/mammalian target of the rapamycin (PI3K/AKT/mTOR) and p38-mitogen-activated protein kinase (MAPK) axis proteins. Additionally, deltonin's influence on GC cell chemosensitivity to cisplatin was evaluated both *in vitro* and *in vivo*.

RESULTS

Deltonin treatment weakened viability, enhanced apoptosis, and dampened DNA repair in GC cell lines in a dose-dependent pattern. Furthermore, deltonin mitigated PI3K, AKT, mTOR, and p38-MAPK phosphorylation. HS-173, an inhibitor of PI3K, attenuated GC cell viability and abolished deltonin inhibition of GC cell viability and PI3K/AKT/mTOR and p38-MAPK pathway activation. Deltonin also promoted GC cells' chemosensitivity to cisplatin *via* repressing GC cell proliferation and growth and accelerating apoptosis.

CONCLUSION

Deltonin can boost GC cells' chemosensitivity to cisplatin *via* inactivating p38-MAPK and PI3K/AKT/mTOR signals.

Key Words: Deltonin; Gastric carcinoma; Cisplatin; Apoptosis; Chemotherapy; Axis

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Core Tip: Chemoradiotherapy is currently the mainstay of clinical treatment for advanced gastric carcinoma (GC). However, chemoradiotherapy is difficult to achieve the desired results due to the challenges of early diagnosis of GC and the characteristics of advanced distant metastasis and drug resistance. This study attempts to improve the efficacy of the clinical treatment of GC from a pharmacological mechanism perspective.

INTRODUCTION

Gastric carcinoma (GC) is a digestive tract cancer prevalent worldwide, ranking second in cancer-related deaths[1]. Currently, it is still associated with a high incidence and mortality rate in developing countries[2]. There are several risk factors for GC, including diet patterns, smoking and drinking, family genetic history, and *Helicobacter pylori* infection[3-5]. At present, chemoradiotherapy is the main clinical treatment for advanced GC. However, owing to the challenges in the early diagnosis of GC and the features of distant metastasis and drug resistance in the advanced stage, it is difficult for radiotherapy to achieve the expected results[6]. This experiment attempted to enhance the efficacy of GC clinical treatment from the perspective of drug mechanism.

Deltonin, an active ingredient in traditional Chinese medicine, is derived from *Dioscorea zingiberensis* C.H. Wright, and shows anti-cancer effects on many malignancies like colon cancer and breast cancer[7]. For instance, deltonin activates autophagy through the protein kinase B/mammalian target of the rapamycin (AKT/mTOR) axis

and prevents FaDu, a head and neck squamous cell carcinoma cell line, from proliferating through cell cycle arrest and apoptosis induction, thus boosting cell apoptosis[8]. Moreover, through reactive oxygen species (ROS)-mediated mitochondrial disorders and extracellular signal-regulated kinase/AKT axis, deltonin restrains human breast carcinoma cell proliferation and promotes cell apoptosis[9]. Although previous studies have demonstrated that deltonin functions in most cancers, there are few studies on its role in GC cells and relevant mechanisms.

The phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signal is activated in multiple tumors and regulates various processes such as tumor cell growth, apoptosis, migration, invasiveness, autophagy, and survival[10]. Currently, this signaling pathway is deemed to be a crucial therapeutic target for tumors. Some studies have verified that apigenin inhibits the PI3K/AKT/mTOR axis to suppress liver cancer cell proliferation, thus eliciting autophagy in liver cancer cells and facilitating cell apoptosis[11]. Diallyl disulfide inhibits the PI3K/AKT/mTOR signal to elicit G2/M phase arrest of human osteosarcoma cells, as well as their apoptosis and autophagic death[12]. p38 mitogen-activated protein kinases (p38-MAPK), as a type of serine/threonine MAPK, participate in the signaling cascades of cytokines and stress cell responses and influences the occurrence, metastasis, and drug resistance of tumor cells[13,14]. For instance, diosgenin suppresses ovarian cancer cell activity by modulating the PI3K/AKT/p38-MAPK axis-associated protein profiles[15]. Another example is inotilone, which inhibits lung carcinoma cell migration and invasiveness through ROS-mediated PI3K/AKT/p38-MAPK axis[16]. Thus, both p38-MAPK and PI3K/AKT/mTOR signals play essential regulatory roles in multiple malignancies. Nevertheless, whether deltonin influences drug resistance and disease progression in GC *via* the two signals still needs further investigation.

This study aims at investigating the underlying anti-tumor function of deltonin in GC cells. Our experiments revealed that deltonin boosted cell apoptosis and improved the chemosensitivity of GC cells to cisplatin. Furthermore, deltonin inhibited PI3K/AKT/mTOR and p38-MAPK signal activation. Thus, our work provides new

therapeutic avenues to explore for patients with GC undergoing end-stage chemotherapy.

MATERIALS AND METHODS

Cell culture

The culture medium of GC (AGS, HGC-27, and MKN-45) and human gastric epithelial (GES-1) cell strains, all from the Chinese Academy of Sciences, Shanghai, China, was RPMI1640 medium (Thermo Fisher Scientific, MA, United States) + 1% penicillin/streptomycin (Thermo Fisher Scientific) + 10% fetal bovine serum (FBS; Invitrogen, CA, United States), and the culture condition was 37 °C and 5% CO_2. Cells grew to logarithmic (log)-growth phase were trypsinized using 0.25% trypsin (Thermo Fisher HyClone, United States) and then harvested through centrifugation at 170 g for 5 min.

Cell treatment

The three GC cell lines were treated with cisplatin (Cat. no. 15663-27-1, Sigma-Aldrich, United States; 5 μg/mL)[17], deltonin (Cat.no. HYN2283, MedChemExpress; 0, 0.625, 1.25, 2.5, 5, 10, and 20 μM)[9,18], and/or HS-173 (a PI3K inhibitor; Cat. no. HY-15868, MedChemExpress; 1 μM)[19], or 740 Y-P (a PI3K activator; Cat. no. HY-P0175, MedChemExpress; 20 μM)[20]. Thereafter, the cells were harvested in preparation for the following experiments.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The three log-growth phase GC cell lines were inoculated into 96-well plates (4×10^3 cells/well, 100 μL) and incubated for 24 h under conditions of 100% humidity, 37 °C, and 5% CO_2 in air. They were then treated with cisplatin, deltonin, and/or the PI3K inhibitor HS-173; the control group was treated with phosphate buffered saline (PBS) of the same volume. Each group contained five replicates. Cells were immersed in 50 μL of 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT) (5 g/L) (Beyotime

Biotechnology, Shanghai, China) after a 24-h culture, and the supernatant was aspirated following a 4-h incubation at 37 °C. The cells were treated with DMSO at 150 µL per well, and then placed on a plate shaker. Ultimately, we used a microplate reader to examine each well's OD value at 450 nm at 24, 48, and 72 h.

Western blotting

After cell treatment mentioned in section 2.2 and cultivation in 6-well plates, the cells were subjected to two PBS washes and 30 min of lysis in 200 µL RIPA (Beyotime Biotechnology, Shanghai, China). Thereafter, the lysates were collected for a 15-min centrifugation at 14000 rpm to obtain total protein. Protein concentrations were measured using Bradford dye (Bio-Rad). Following 2 h of separation on a polyacrylamide gel by electrophoresis at a voltage maintained at 100 V, the protein samples were electroblotted onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, United States). They were then treated with 1 h of room temperature (RT) sealing with 5% nonfat-dried milk, followed by three 10-min Tris-buffered saline with 0.1% Tween® 20 detergent (TBST) rinses and overnight incubation at 4 °C with primary antibodies at 1:1000 dilution that were procured from Abcam (MA, United States): Anti-Bax (ab32503), anti-Bid (ab32060), anti-Bak (ab32371), anti-Fas (ab133619), anti-Rad51 (ab133534), anti-MDM2 (ab16895), anti-PI3K (ab32089), anti-mTOR (ab134903), anti-p-mTOR (ab137133), anti-p-PI3K (ab182651), anti-AKT (ab8805), anti-p-AKT (ab38449), anti-p38-MAPK (ab170099), anti-p-p38-MAPK (ab178867), and anti-β-actin (ab115777). Following TBST washes, the membranes were subjected to 1 h of RT incubation with horseradish peroxidase-labeled anti-rabbit secondary antibody (1:300 dilution). Thereafter, TBST was used to rinse the membranes again thrice (10-min rinses). Eventually, the membranes were imaged and the staining intensity was assessed using BeyoECL Plus (Beyotime Biotechnology, Shanghai, China) and ImageJ, respectively.

Flow cytometry

The human GC cell lines were harvested in the log growth phase and prepared as single-cell suspensions for inoculation in a 25 cm^2 culture flask. Following adherent culture overnight, the original medium was replaced with fresh medium containing 0.3% FBS for the experimental group and a comparable volume of PBS medium for the control group, followed by 24 h of incubation with 5% CO_2 and the temperature maintained at 37 °C and cell supernatant collection. Thereafter, the cells were subjected to cold PBS flushing for 3 times, trypsinization using EDTA-free trypsin, and harvesting. Then, the cells were treated as instructed by the Annexin V-PI Apoptosis Detection Kit (Yeasen Biotech Co., Ltd.) manuals. Subsequently, flow cytometry was performed within 1 h for analyzing cell apoptosis.

In-vivo experiments in nude mice

We acquired 12 female athymic BALB/c nude mice (6 wk old with a weight of 22-24 g) from Shandong University Experimental Animal Center (Jinan, China) and reared them under normal specific pathogen-free conditions (24 °C, 12-h/12-h light/dark regime, and free food and water). Then, AGS cells were administered hypodermically at 2×10^6 cells/0.1 mL PBS into mouse right back according to a previous study[21]. Seven days later, the animals were randomly distributed to the sham (treated with normal saline *via* intraperitoneal injection), cisplatin (once every three days at 3 mg/kg, for 3 times)[22,23], deltonin (once every three days at 50 mg/kg, for 3 times)[8], or the cisplatin (once every three days at 1.5 mg/kg, for 3 times) + deltonin (once every three days at 25 mg/kg, for 3 times) groups. During the following 28 d after drug treatment, a caliper was used for measuring the tumor volume (tumor volume = 0.5 × length × $width^2$) weekly. Four weeks later, the nude mice were sacrificed using 30 mg/kg phenobarbital sodium, and the tumor was resected and weighed. The animal experiments were approved by the Ethics Review Committee of the Second Affiliated Hospital of Soochow University (approval no. SZSH-2020-042), and were implemented strictly following the Declaration of Helsinki and the Regulations of the People's Republic of China on the Management of Laboratory Animals issued on October 31, 2017.

Immunofluorescence assay

Tumor tissue specimens were treated with 4% paraformaldehyde for immobilization and then paraffin-embedded. Tumor sections were prepared (4 μm in thickness), dewaxed using gradient alcohol, and rehydrated. Following RT sealing with bovine serum albumin (5%) for half an hour, the sections were treated with 1 h of RT incubation with anti-p-PI3K/AKT/mTOR/p38 MAPK (ab191606, ab131443, ab109268, and ab38238). After flushing with PBS, they were incubated with the Cy3- (ab98416) or fluorescein isothiocyanate-labelled goat anti-rabbit secondary antibody (ab6717) for 60 min, also at RT. All antibodies were procured from Abcam. Following nuclei labeling with 4',6-diamidino-2-phenylindole (Beyotime technology, Shanghai, China), a confocal immunofluorescence microscope (Leica LSM 800, Wetzlar, Germany) was used to visualize the images.

Statistical analyses

SPSS16.0 from SPSS Inc. (Chicago, IL, United States) was used for performing all statistical analyses, and $P < 0.05$ indicated statistical significance. Between-group differences were analyzed with unpaired, two-sided student's *t*-tests, and multi-group differences were determined by one-way ANOVA followed by Tukey's post-hoc tests. All data are described as mean ± SD.

RESULTS

Deltonin prevents GC cell proliferation and accelerates apoptosis

GES-1, AGS, HGC-27, and MKN-45 were all treated with 0-20 μM of deltonin for 24 h, after which their viability was examined using MTT assays. GC cell viability was observed to significantly decrease when the dose of deltonin exceeded 2.5 μM, while only 20 μM of deltonin exerted an inhibitory effect on GES-1 viability ($P < 0.05$ *vs* control, Figure 1A). The IC_{50} values were gauged for AGS, HGC-27, and MKN-45 cells following treatment with deltonin at different concentrations; the IC_{50} values were 3.487,

2.343, and 2.78 for AGS, HGC-27, and MKN-45 cells, respectively (Figure 1B). The GC cells were treated with 2.5 μM deltonin and then subjected to the MTT assay to examine cell viability at different time points. Deltonin inhibited GC cell viability in a time-dependent manner ($P < 0.05$ *vs* control, Figure 1C). Flow cytometry analysis revealed that deltonin treatment promoted cell apoptosis ($P < 0.05$ *vs* control, Figure 1D). And as indicated by western blotting (WB) analysis, deltonin treatment enhanced the protein levels of pro-apoptotic markers Bax, Bak, Bid, and Fas but reduced those of Rad51 and MDM2, which are associated with DNA repair processes ($P < 0.05$ *vs* control, Figure 1E). WB assays also indicated that deltonin (2.5 μM) treatment markedly lowered PI3K/AKT/mTOR and p38-MAPK protein levels in GC cells (including AGS and HGC-27), with the expression gradually decreasing with time (0, 24, 48, and 72 h) ($P < 0.05$ *vs* control, Figures 1F and G). Additionally, these proteins presented decreased expression in GC cells in a deltonin concentration-dependent manner (0, 2.5, 5, 10 μM) ($P < 0.05$ *vs* control, Figures 1H and I). The above results demonstrated the ability of deltonin to exert an inhibitory effect on GC cell growth and enhance apoptosis while inactivating p38-MAPK and PI3K/AKT/mTOR axes in these cells.

Repressing PI3K/AKT/mTOR and p38-MAPK signals suppressed the deltonin-mediated anti-tumor effects

GC cells treated with deltonin (2.5 μM) and HS-173 (0.8 nM) showed remarkably lower viability compared to the control ($P < 0.05$, Figures 3A and B). Nevertheless, deltonin + HS-173 exerted no additional influence on cell viability compared to the HS-173 alone group ($P > 0.05$, Figures 3A and B). The determination of apoptosis-related protein profiles also determined that deltonin and HS-173 individually increased the expression of Bax, Bak, Bid, and Fas, whereas co-treatment with HS-173 and deltonin barely influenced their expression levels ($P < 0.05$, Figures 3C and D). WB also showed that phosphorylated PI3K/AKT/mTOR and p38-MAPK protein levels were substantially reduced with deltonin or HS-173 treatments, whereas the administration of deltonin and HS-173 group exerted no inhibitory effect on p38-MAPK and PI3K/AKT/mTOR

axes (*vs* HS-173 group alone, $P > 0.05$, Figures 3E and F). Therefore, deltonin may repress GC cell viability by suppressing p38-MAPK and PI3K/AKT/mTOR signaling.

Impact of activating PI3K/AKT/mTOR and p38-MAPK on deltonin-mediated effects

Next, we treated GC cells (AGS and HGC-27) with deltonin (2.5 μM) and the PI3K activator 740 Y-P (20 μM) and found that it notably enhanced cell viability *vs* the control, wherein cell viability was inhibited by the addition of deltonin ($P < 0.05$, Figures 3A and B). Furthermore, WB showed reduced expression of apoptosis-related proteins (Bax, Bak, Bid, and Fas) in the 740 Y-P group, while the deltonin + 740 Y-P group showed increased expression of these proteins in comparison to 740 Y-P alone treatment ($P < 0.05$, Figures 3C and D). WB also indicated augmented PI3K, AKT, mTOR, and p38-MAPK phosphorylation in AGS and HGC-27 in the 740 Y-P group, whereas deltonin co-treatment suppressed this increased phosphorylation ($P < 0.05$ *vs* 740 Y-P group, Figures 3E and F). Together, these results suggest that activating PI3K/AKT/mTOR and p38-MAPK may facilitate cell proliferation and weaken the anti-cancer effects of deltonin.

Deltonin enhances GC cell chemosensitivity to cisplatin

AGS and HGC-27 were treated with 2.5 μM of deltonin or 5 μg/mL of cisplatin or cisplatin (2.5 μg/mL) + deltonin (1.25 μM). Treatment with cisplatin or deltonin considerably attenuated cell viability, whereas cisplatin + deltonin co-treatment reduced cell viability compared to cisplatin alone group ($P < 0.05$, Figures 4A and B). According to flow cytometry analysis, the apoptosis of cisplatin- or deltonin-treated cells was dramatically increased compared to the control ($P < 0.05$, Figures 4C and D), and it was further enhanced in cisplatin + deltonin group ($P < 0.05$, Figures 4C and D, *vs* cisplatin group). WB also showed elevated Bax and Bid and reduced Rad51 protein expression in cisplatin or deltonin treated cells *vs* the control. Moreover, Bax and Bid protein expression in the cisplatin + deltonin group was further increased, while Rad51 expression was considerably reduced in comparison to the expression levels in the

cisplatin alone group ($P < 0.05$, Figures 4E and F). Based on the above findings, deltonin may exert a pro-apoptotic effect and promote the chemosensitivity of GC cells to cisplatin.

Deltonin increases GC cells' chemosensitivity to cisplatin in vivo through PI3K/AKT/mTOR and p38-MAPK signal inhibition

To further verify deltonin's function and mechanism in GC cells' chemosensitivity to cisplatin, we conducted *in vivo* experiments in nude mice. The tumor-bearing mice were intervened by saline, deltonin (50 mg/kg), cisplatin (3 mg/kg), or deltonin (25 mg/kg) + cisplatin (1.5 mg/kg). Treatment with deltonin or cisplatin both reduced tumor volume and weight compared to the sham group ($P < 0.05$, Figures 5A-C), but failed to reduce mice body weight ($P > 0.05$, Figure 5D). Interestingly, the joint application of deltonin + cisplatin further mitigated the mouse tumor volume and weight in comparison to cisplatin treatment alone ($P < 0.01$, Figures 5A-C), but barely altered the body weight ($P > 0.05$, Figure 5D). We then carried out immunofluorescence assays to determine PI3K/AKT/mTOR and p38-MAPK phosphorylation levels in the tumor tissues. Both deltonin and cisplatin reduced the levels of phosphorylated p38-MAPK and PI3K/AKT/mTOR, and their combination further reduced the levels compared to the cisplatin alone group (Figures 5E-H). These findings suggest that deltonin enhanced GC cells' chemosensitivity to cisplatin by suppressing p38-MAPK and PI3K/AKT/mTOR signaling activation (Figure 6).

DISCUSSION

GC is a prevalent internal gastrointestinal malignancy with a high clinical fatality rate[24]. The current methods are ineffective for early GC diagnosis, owing to which GC is often diagnosed at the end stage when it is accompanied by distant metastasis and chemotherapy resistance. Moreover, surgical treatment and drug chemotherapy display poor efficacy[25]. Cisplatin is a frequently used chemotherapy drug for many malignant tumor diseases and is also extensively adopted in the context of GC[26,27]. Regarding the

primary mechanism of cisplatin in cancer treatment, it triggers DNA damage in tumor cells. Unfortunately, cisplatin treatment can easily contribute to the drug resistance of tumor cells and influence the function of chemotherapy[28]. Hence, probing the drug action mechanisms in GC has great clinical implications for its treatment. Here, we discovered that deltonin hinders p38-MAPK and PI3K/AKT/mTOR signal activation to boost GC cell apoptosis and promote their chemosensitivity to cisplatin.

Deltonin is known as an anti-tumor drug that curbs tumor cell angiogenesis to restrain tumor growth and facilitate apoptosis[29]. Deltonin inhibits AKT and p38-MAPK signaling pathway activation to further inhibit mouse colon cancer cell proliferation and bolster tumor cell apoptosis[18]. Furthermore, the intake of deltonin significantly suppresses colon cancer C26 cell proliferation in tumor-bearing mice, restricts tumor angiogenesis, and elicits cell apoptosis, thus prolonging the life cycle of the mice[30]. All the above studies confirm that deltonin enhances cancer cell apoptosis and represses cancer in a multitude of tumor diseases, which aligns with the observations in this study. Here, we demonstrated that deltonin considerably inhibits proliferation, boosts apoptosis, and dampens DNA repair in GC cells.

Chemotherapy is a prevailing method for GC, effectively extending patients' life. Cisplatin is a typical drug used in GC chemotherapy. Nonetheless, GC resistance is a leading contributor to chemotherapy failure[31,32]. Many studies have evaluated drug resistance in GC, including the most complicated molecular and drug mechanisms[33]. For instance, ten-eleven translocation-2 (TET2), a DNA demethylase, modulates interleukin (IL)-6 levels in the tumor microenvironment *via* histone acetylation, thus influencing cell resistance, and TET2 overexpression notably mitigates cisplatin resistance in GC cells[34]. Curcumin also augments GC cells' sensitivity to adriamycin and other chemotherapy drugs by down-regulating the nuclear factor-kappaB (NF-κB) axis in human GC SGC-7901 cells and a downstream anti-apoptotic target gene of NF-κB[35]. Most of the prior studies have investigated the tolerance of chemotherapeutic drugs in GC from the aspect of molecular and drug mechanisms. Here, we unveiled that deltonin efficaciously augmented GC cells' chemosensitivity to cisplatin and

thereby boosted the anti-tumor function of cisplatin *via* eliciting apoptosis and DNA damage.

PI3K/AKT/mTOR and p38-MAPK signals were initially considered as factors that could regulate inflammation and immune response and affect inflammatory reactions, cell proliferation, differentiation, apoptosis, and other cellular processes[36,37]. Recent evidence has also demonstrated the pro-oncogenic functions of p38-MAPK and PI3K/AKT/mTOR in several tumors[38,39]. For instance, an *in vitro* experiment on GC cells has revealed that blocking PI3K/AKT/mTOR signal activation augments GC cells' resistance to paclitaxel and promotes their apoptosis[40]. Afatinib dampens p38-MAPK and PI3K/AKT/mTOR signal activation, thereby eliciting GC cell apoptosis and bolstering their resistance to chemotherapy[41]. All these conclusions align with the outcomes of our current study findings. Here, we discovered that deltonin significantly hinders p38-MAPK and PI3K/AKT/mTOR signal activation, thereby bolstering GC cell apoptosis and attenuating the cells' resistance to cisplatin.

CONCLUSION

In summary, through a series of experiments, we uncovered that treating GC cells (AGS, HGC-27, and MKN-45) with deltonin results in reduced proliferation ability and increased apoptosis rate; of these, HGC-27 cells exhibited the best proliferation capability and the lowest apoptosis rate. Therefore, we exploited AGS and HGC-27 for further experiments and analyses. Our experiments demonstrated the ability of deltonin to promote GC cell apoptosis and chemosensitivity to cisplatin by lowering PI3K/AKT/mTOR and p38-MAPK-associated protein levels, offering novel insights into the mechanism of drug action. Nevertheless, further investigations are required to understand how deltonin represses these two axis, and *in vivo* experiments should be conducted using both male and female nude mice and other GC cell lines.

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