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Original Article

N1,N3-bis(3-methoxysalicylidene) diethylenetriamine induces apoptosis in colon cancer HCT-116 cells through inhibiting Wnt1/β-catenin signaling pathway

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Abstract: Objective: The anti-cancer effect and mechanism of N1,N3-bis(3-methoxysalicylidene) diethylenetriamine (Valdien) in HCT-116 cells were assessed in this study. Methods: MTT was used to measure cytotoxicity of the Valdien. The appearance of apoptotic HCT-116 cells was detected by flow cytometry analysis. Apoptosis proteins, caspase family and Bcl-2 family were viewed by Western blotting. The Wnt1/β-catenin signaling pathway was also examined. HCT-116 cells xenograft serious combined immunodeficiency disease mice were used for the *in vivo* study. Results: Valdien greatly inhibit HCT-116 cells proliferation in a concentration and time dependent manner and found this effect was associated with apoptosis. Further analysis demonstrated that Valdien induced HCT-116 cells apoptosis by activating the death receptor pathway and regulating cyclin D1 and p21. The expression of anti-apoptotic Bcl-xL, survivin, Bcl-2 was decreased in Valdien treatment groups, whereas the expression of pro-apoptotic Bax protein was increased. Valdien downregulated expression of Wnt1, β-cateninand, c-myc, while upregulated E-cadherin expression. In addition, HCT-116 cells xenograft mouse model mice were used for the study *in vivo*. Conclusion: Valdien induced apoptosis in HCT-116 cells *in vitro* primarily *via* regulating the Wnt1/β-catenin signaling pathway and then triggering the activation of the death receptor pathway.

Keywords: Valdien, HCT-116 cells, proliferation, apoptosis, Wnt1/β-catenin signaling pathway

Introduction

Colorectal cancer (CRC) is one of the most prevalent and incident common malignant tumors, which is the third most commom cause of cancer deaths in the word [1]. In recent years, the incidence and mortality of colorectal cancer in China have increased, which has become one of the most malignant tumors that threaten the health of residents. The occurrence of colorectal cancer is closely related to diet, heredity and physical activity level [2, 3]. At present, the main treatment methods for colorectal cancer include surgery, radiotherapy, chemotherapy and targeted therapy. However, postoperative recurrence and metastasis are common reasons leading to failure of treatment. The combination of operation and chemotherapy can improve colon cancer survival and reduce the risk of recurrence and metastasis as compared with surgery only. However, clinical responses of colon cancer patients to chemotherapy vary greatly and even some patients are generally or particularly resistant to chemotherapy, which leads to different curative effects for colon cancer. Given this, there is an urgent need for research novel therapeutic strategies and new chemotherapeutic drugs to improve cancer therapy, improve the life quality and prolong life expectancy for patients with CRC [4].

In the recent years, the antitumor activity of some Schiff bases has been widely reported [5, 6]. N1,N3-bis(3-methoxysalicylidene) diethylenetriamine (Valdien, **Figure 1**), which belongs to one of Schiff bases, is more likely to be a potential antitumor agent. In view of this reason, the antitumor effect of Valdien was tested and proven by our preliminary experiment in vitro. The Valdien has been used for the synthe-

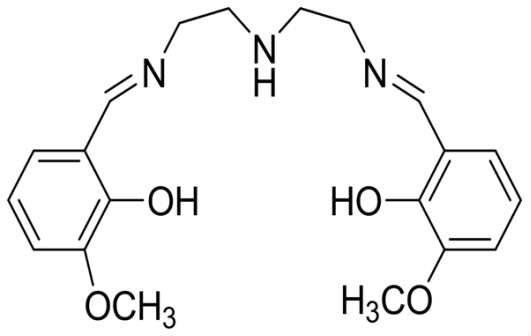

Figure 1. N1,N3-bis(3-methoxysalicylidene) diethylenetriamine (Valdien) molecular structure.

sis of magnetic and optical material in the chemical industry in the recent years [7, 8]. Valdien was synthesized by mixing diethylenetriamine (0.0125 mol) with o-vanillin (0.025 mol) in 40 mL of ethyl alcohol [9]. After being refluxed for 1 h, the yellow solution was precooled and the solvent was removed under reduced pressure to obtain one type of yellow oil. The yellow oil was left to stand overnight to form a yellow precipitate. The precipitate, Valdien, was collected by suction filtration and washed with diethyl ether.

The development of colorectal cancer is a complex multistep process involving progressive disruption of the homeostatic mechanisms that control epithelial proliferation, inflammation and differentiation [10]. Recent studies show that the Wnt signaling pathway is playing a crucial role in the regulation of cell proliferation, differentiation, apoptosis and migration. Abnormal expression and activation of Wnt signaling pathway can induce tumors [11, 12]. Wnt proteins are a family of secreted glycoproteins and signal through the frizzled (Fz) receptors in a paracrine pattern [13]. The Wnt signaling pathways could be classified into three major types. The most known one is the canonical pathway, which activates downstream genes through inhibition of GSK-3 activity, followed by accumulation of β-catenin in nucleus. Wnt1 is a member of Wnt gene family, which is the initiating factor of Wnt signaling pathway and involves in the classic Wnt signaling pathway. Many studies have shown that abnormal activation of Wnt1/β-catenin pathway is frequently involved in colorectal cancer [14]. Therefore, in the present study, we investigated the role of Wnt1/β-catenin signaling pathway in Valdien inhibited colorectal cancer cells proliferation and induced apoptosis, to investigate the effects of Valdien on colorectal cancer cell growth and plausible mechanisms.

Materials and methods

Reagents

Valdien was obtained from Dr. Wei Dou (Lanzhou University, China). Cisplatin (DDP) was obtained from Qilu pharmaceutical co., LTD, (Shandong Province, China). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cell culture medium RPMI-1640 and fluorescent dye DCFH-DA (2,7-dichlorofluorescin diacetate) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Heat-inactivated fetal bovine serum (FBS) was provided by Sijiqing Company Ltd, China. An annexin V-PE apoptosis detection kit was purchased from Beyotime Institute of Biotechnology (Nanjing, China).

The antibodies against cleaved caspase-3, caspase-8, Fas, FasL, Bcl-xL, survivin, Bcl-2, Bax, cyclin D1, β-catenin, c-Myc, and E-cadherin were purchased from Cell signaling Technology (Beverly, Mass, USA). The antibody against Wnt1 and p21 were purchased from Abcam Inc. (USA). Goat anti-rabbit or Goat anti-mouse horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary β-actin antibody was obtained from Boster Biological Technology, Ltd. (Wuhan, China). Bicinchoninic acid (BCA) protein assay reagent kit was purchased from Applygen Technologies inc., (Beijing, China).

Cell lines and cell culture

Human colon carcinoma HCT-116 cells, human colorectal cancer HT-29 and Lovo were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells of HT-29, HCT-116 and Lovo were all cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin in a CO_2 incubator with a humidified atmosphere of 5% CO_2 at 37°C. The cells in logarithmic growth phase were used in all experiments.

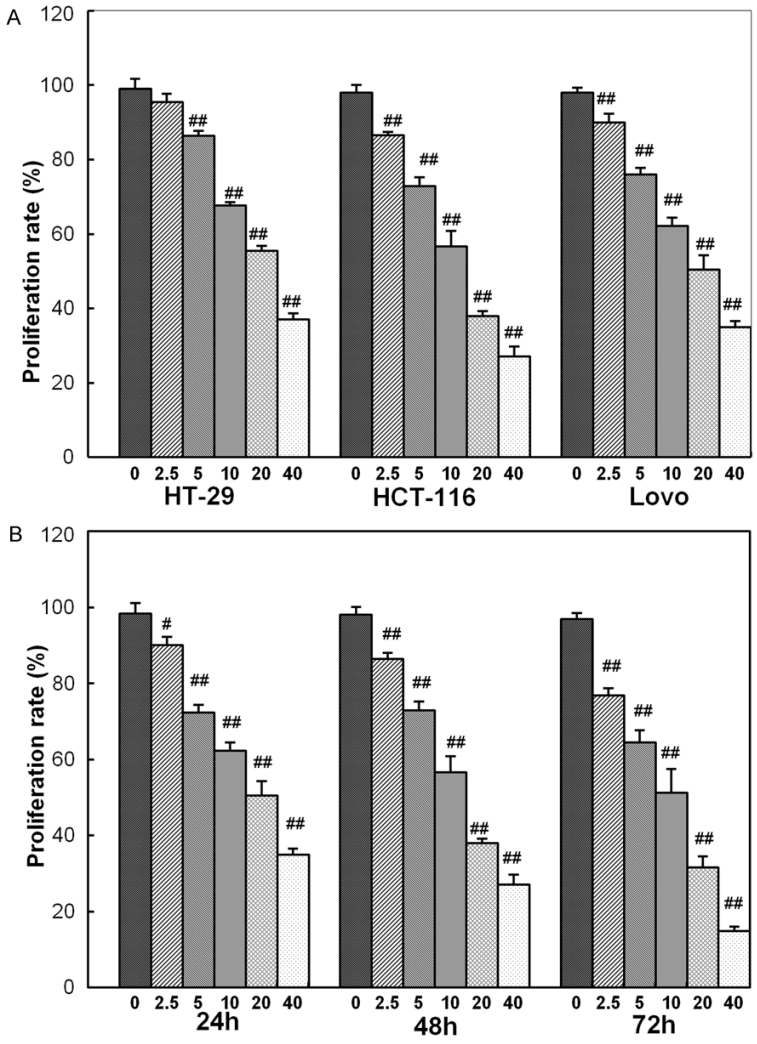

Figure 2. Effect of Valdien on the proliferation of HCT-116 cells. A. Valdien inhibits proliferation of HT-29, HCT-116, and Lovo cells in a concentration-dependent manner. Cells were cultured in 96-well plate and treated with different concentrations of Valdien for 48 hours. B. Valdien inhibits proliferation of HCT-116 cells in a dose-dependent manner and a time-dependent manner. Cells were cultured in 96-well plate and treated with different concentrations of Valdien (2.5-40 μg/mL) for 24, 48 and 72 hours, respectively. MTT assay was used to evaluate the cells population proliferation. Each datum indicated mean ± SD of 3 independent experiments. $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ versus control.

MTT assay

The inhibition effects of Valdien on HT-29, HCT-116 and Lovo cells proliferation were assessed by MTT assay. The cells were seeded in 96-well plates with 100 μl of the appropriate medium at a density of 1×10^5 cell/well. After 24 h of incubation, the cells were incubated with different concentrations of Valdien (2.5, 5, 10, 20 and 40 μg/mL) for a further 48 hours. For the time course assay, the incubation time with Valdien was 24, 48, and 72 hours, respectively. Subsequently, 20 μL of MTT solution (5 mg/mL) was added to each well at a final concentration of 0.5 mg/mL and incubated at 37°C with 5% CO_2 for another 4 h. Then, the supernatants were discarded, and 100 μL DMSO were added to each well to dissolve purple crystals of formazan. The plate was shaken for 10 minutes to allow complete solubilization and the absorbance was read at 570 nm using an ELISA microplate reader (EL800, BioTek Instruments Inc.). The IC_{50}, which is the concentration that caused 50% inhibition of cancer cell growth, was calculated using the logit method [15].

Cell cycle analysis

To examine whether Valdien inhibited cell cycles, we analyzed cell cycle by the flow cytometry. Briefly, HCT-116 cells were seeded in 6-well plates for 24 h, and then they were cultured with Valdien (20 and 40 μg/mL) for 24 h. Following incubation, the cells were collected and fixed with 70% ethanol at 4°C overnight. The samples were analyzed with a flow cytometry.

Hoechst fluorescent staining

For Hoechst fluorescent staining, HCT-116 cells from exponentially growing cultures were seeded in 12-well culture plates and treated with Valdien at 20 and 40 μg/mL for 48 h. The HCT-116 cells were harvested and fixed in 4% paraformaldehyde and incubated at room temperature for 30 min. The fixative was removed and the HCT-116 cells were washed twice with phosphate-buffered saline (PBS), resuspended in 50 μL of PBS containing 16 μg/mL bisbenzimide trihydrochloride (Hoechst 33258), and incubated for

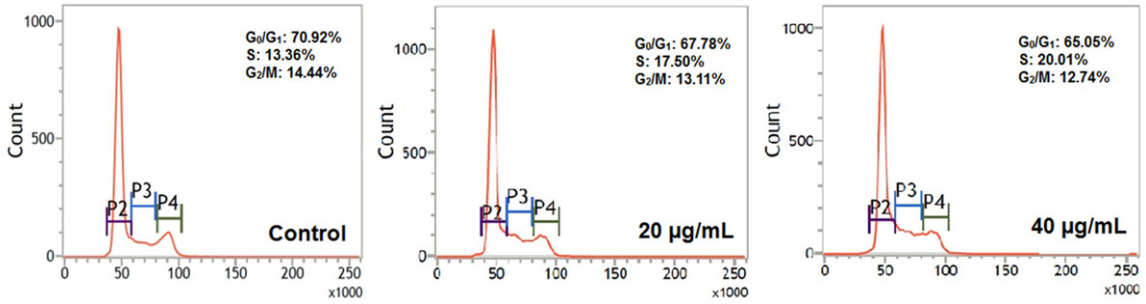

Figure 3. Cell cycle distribution of HCT-116 cells treated with Valdien detected by flow cytometry. Cells were cultured in 6-well plate for 24 h and than treated with different concentrations of Valdien (20 and 40 μg/mL) for 24 hours.

Table 1. Cell cycle ratio of HCT-116 cells treated with Valdien detected by flow cytometry ($\bar{x} \pm s$, n=3, 24 h)

Group	G_1 (%)	S (%)	G_2 (%)
0	70.01 ± 2.05	13.27 ± 0.89	14.35 ± 1.19
20 μg/mL	68.19 ± 1.32	18.42 ± 1.28##	13.42 ± 1.22
40 μg/mL	65.51 ± 1.01#	21.75 ± 1.51##	12.81 ± 0.96

Results are expressed as means ± SD, #P < 0.05 or ##P < 0.01 versus control.

10 min at room temperature. Ten-microliter aliquots of the cells were placed on glass slides, and triplicate samples of 500 cells each were counted and scored for the incidence of apoptotic chromatin condensation by using a Zeiss fluorescent microscopy.

Transmission electron microscopy

To gain further insight into the anti-proliferative effects of Valdien on HCT-116 cells, transmission electron microscopy (TEM) was used to obtain ultrastructural information. HCT-116 cells (1×10^5) were treated with Valdien (20 and 40 μg/mL) at 37°C for 48 h and then collected for electron microscopy [16]. The pellet was fixed in 5% glutaraldehyde for 30 min and then placed in 1% osmium tetroxide in 0.1 mol/L sodium cacodylate (PH 7.4) for 1 h. The HCT-116 cells were desiccated in graded series of acetone and embedded with EPON-812. Ultrathin sections were prepared and observed under a Hitachi transmission electron microscope with ×8000 magnifications after double staining with uranium and plumbum. The untreated cells were used as a control.

Flow cytometric analysis of apoptosis

For apoptosis assay, the HCT-116 cells were seeded in 12-well plates at a density of 1×10^5 cell/well under standard culture conditions and kept over at 37°C humidified incubator with 5% CO_2. After incubation with Valdien (20 and 40 μg/mL) for 48 h, the HCT-116 cells were harvested and washed twice with cold PBS. Apoptotic cell death was identified by double supravital staining with recombinant FITC-conjugated Annexin V and PE, using the apoptosis detection kit by the instructions of the manufacturer. Flow cytometric analysis was performed immediately after supravital staining.

Western blotting analysis

The HCT-116 cells (1×10^5 cells/well) were treated with different concentrations of Valdien (2.5-40 μg/mL) for 48 hours. For Western blot analysis, the cells were harvested, washed with cold PBS, and then lysed in RIPA buffer (Beyotime, China) [8, 9]. Protein concentrations were measured by the BCA method. For western blotting analysis, 30 μg of protein from each sample was separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 0.5% BSA in TBST (pH 8.0) for 1.5 h and then incubated overnight at 4°C with suitably diluted primary antibodies. Then the membranes were incubated in horseradish peroxidase (HRP)-linked anti-biotin and the appropriate secondary antibody in TBST with 0.5% BSA for 1 h at room temperature. The blots were detected using the ECL reaction. Quantification of protein bands was achieved by densitometric analysis using Image-Pro Plus software (Media Cybernetics, Inc. USA).

Xenograft model of colon carcinoma

A total of 32 female athymic BALB/c nude mice, aged 6 weeks and weight 18-22 g were provid-

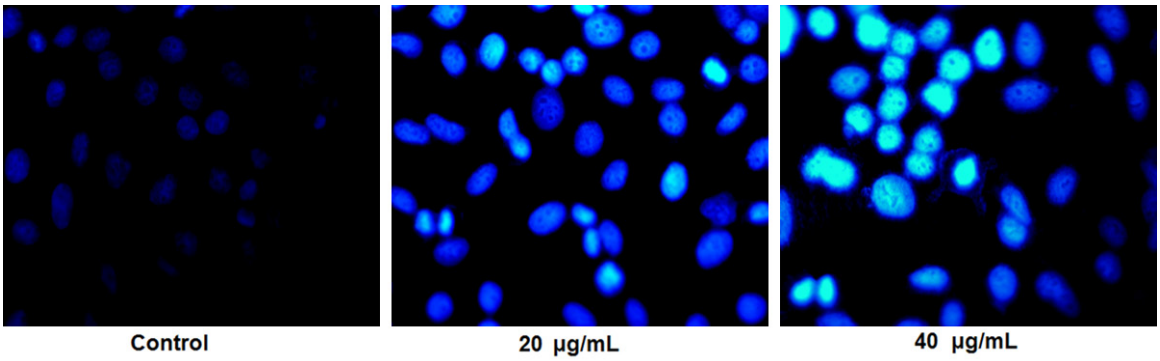

Figure 4. Photomicrographs of HCT-116 cells stained with bisbenzimide trihydrochloride (Hoechst 33258) to evaluate apoptosis. HCT-116 cells from exponentially growing cultures were seeded in 12-well culture plates and treated with Valdien at 20 and 40 μg/mL for 48 h.

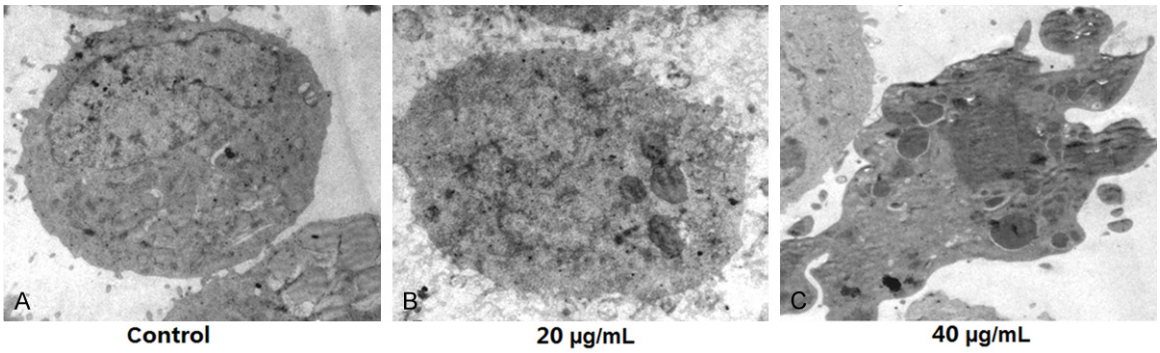

Figure 5. Effects of Valdien on ultrastructure of HCT-116 cell demonstrated by transmission electron microscope of ultra-thin section (48 h ×8000). A. Cells from control sample with intact cell wall; B and C. Cells were incubated with Valdien (20 and 40 μg/mL) at 37°C for 48 h. All images are shown at the same magnification.

ed by Beijing Weitonglihua Laboratory Animal Co., Ltd. (Beijing, China; animal quality license, SCXK (Jing) 2013-0021). The specific pathogen free (SPF) nude mice were reared under the sterile conditions at a constant temperature of 22-24°C, and 50-55% humidity with a 12 h light/dark cycle. The nude were freely fed with standard laboratory forage and water. The HCT-116 cells were injected subcutaneously into the right front armpit of each nude mouse at the density of 1×10^8 cells/mL. After transplantation, the nude mice were randomly divided into five groups: model control group (n=7), positive control group (Cisplatin group, DDP) (n=6) and Valdien low dose (Valdien-L, 5 mg/kg, n=6), medium dose (Valdien-M, 10 mg/kg, n=6), and high dose (Valdien-H, 20 mg/kg, n=7) treatment group. Valdien was injected intraperitoneally at dose of 5, 10 and 20 mg/kg per day for 21 days. DDP group mice were injected at dose of 5 mg/kg every three days, for 4 times. The weights of the mice and the volume of the tumor size were recorded every two days until the animals were sacrificed. All nude mice were sacrificed with isoflurane inhalation anesthesia and cervical dislocation method after treatment with Valdien for 21 d and tumors were quickly excised for further analysis. The tumor growth inhibition rate was counted by the following formula: Inhibition rate (IR) (%) = $(1-W_t/W_c)\times100$, where Wt and Wc represent tumor weight of treatment and control groups. This study was performed in strict accordance with the Animal Research Institute Committee guidelines of Gansu provincial Hospital (Lanzhou, China). The animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Gansu provincial Hospital. All of the animal experimental procedures were carried out in the experimental animal center of

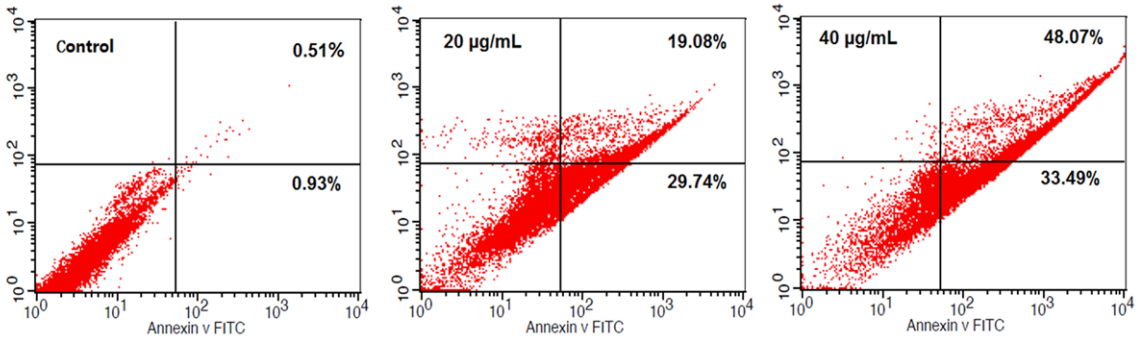

Figure 6. Valdien promotes apoptosis of HCT-116 cells. HCT-116 cells were seeded in 12-well-plates overnight, and then were treated with different concentrations of Valdien (20 and 40 μg/mL) for 48 hours. Annexin-V/PE stain was used to measure apoptosis populations. Apoptosis populations are measured by flow cytometry. The results are representative of three separate experiments.

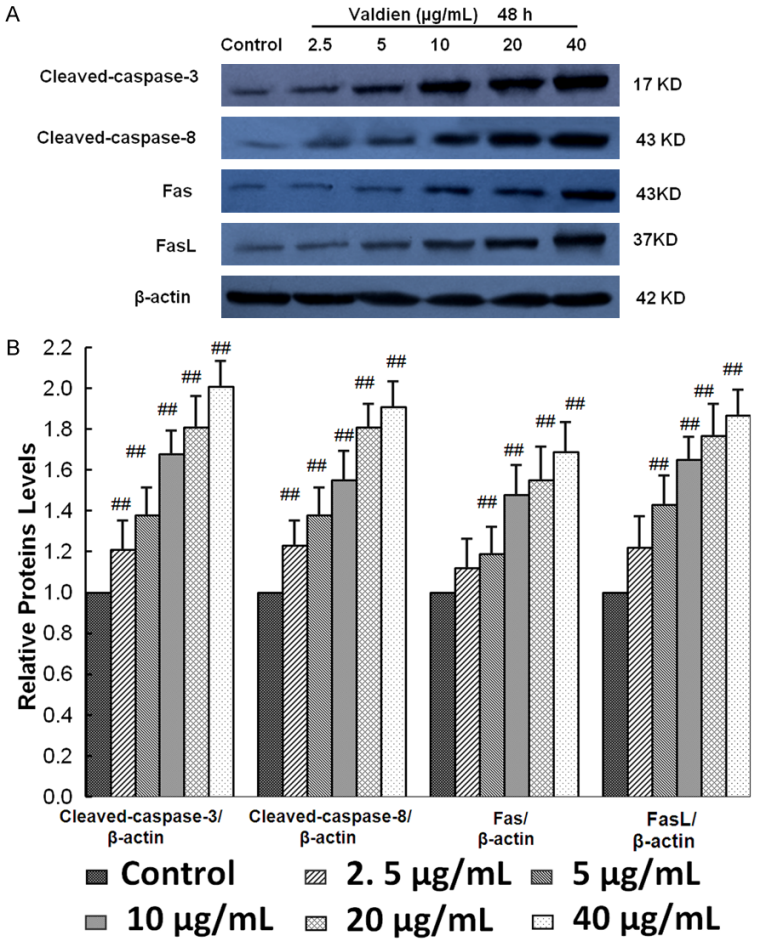

Figure 7. Apoptosis triggered by Valdien was mediated via the caspase cascades. A. Valdien induced dose-dependent activation of caspase-3, -8, Fas and FasL. B. Columns represent the ratios of Cleaved-caspase-3, -8, Fas and FasL to β-actin after treatment with Valdien 2.5-40 μg/mL) for 48 h. The proteins were subjected to Western blot analysis using the respective antibodies as indicated. Data are representative of three separate experiments. $^{\#\#}P < 0.01$ versus control.

Gansu provincial Hospital. All efforts were made to minimize animal suffering.

Immunohistochemistry

After mice were sacrificed, quickly excised tumor tissues and fixed in 10% formalin. After routine deparaffinization, antigen retrieval, and block endogenous peroxidase activity, the paraffin-embedded tumor sections were then incubated with antibodies against Bcl-2 (1:100) or Bax (1:100). According to the standard avidin-biotin complex (ABC) kits instructions, visualized by exposing to diaminobenzidine (DAB) substrate, the slides were counterstained with Mayer's hematoxylin before undergoing dehydration, clearing and mounting steps.

Statistical analysis

All data were expressed as mean ± SD of three independent experiments and each experiment included triplicate sets. Statistical analysis was performed by one-way ANOVA followed by Dunnett's test, with the level of $P < 0.05$ considered to be significant.

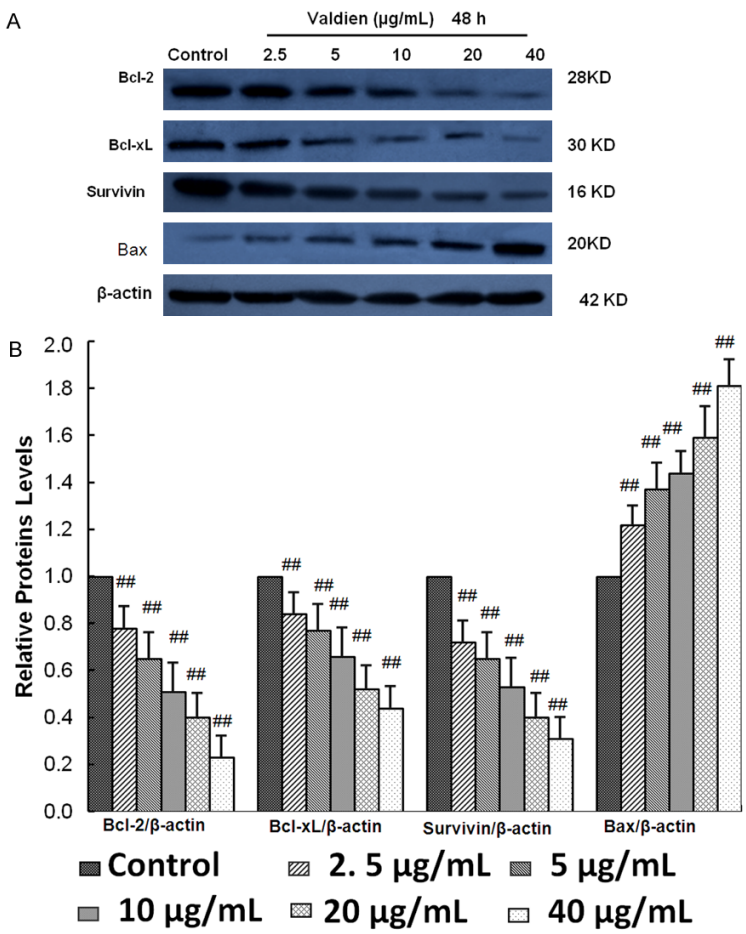

Figure 8. Effect of Valdien on the expressions of Bcl-xL, survivin, Bcl-2 and Bax in HCT-116 cells. A. Valdien significantly decreased the expression of Bcl-xL, survivin and Bcl-2 in a dose-dependently, whereas markedly increased the expression levels of Bax. B. Columns represent the ratios of Bcl-xL, survivin, Bcl-2 and Bax to β-actin. Protein was extracted from HCT-116 cells treated with Valdien (2.5-40 μg/mL) for 48 h. Data are representative of 3 separate experiments. Parallel blotting was performed with β-actin antibody. $^{\#\#}P < 0.01$ versus control.

Results

Valdien inhibits HCT-116 cells proliferation

The MTT assays were used to examine the effects of Valdien on cells proliferation of colorectal cancer cells lines. After treatment 48 h, Valdien (2.5, 5, 10, 20, and 40 μg/mL) significantly reduced the proliferation of HT-29, HCT-116 and Lovo cells in a concentration-dependent manner (**Figure 2A**). The results also demonstrated that treatment with Valdien caused inhibition of HCT-116 cells proliferation in a concentration-dependent and time-dependent manner (**Figure 2B**). The IC_{50} values of Valdien for the HCT-116 cells at 24, 48 and 72 h were 19.84, 12.6 and 9.60 μg/mL, respectively.

Valdien induces S-phase arrest in HCT-116 cells

As we have observed a significant growth-inhibitory effect of Valdien on HCT-116 cells, we further investigated whether Valdien could inhibit the cell cycle progression. To examine whether Valdien inhibited colon cancer cell HCT-116 growth through blocking cell cycle progression, we treated HCT-116 cell with concentrations of Valdien (20 and 40 μg/mL) for 24 h and subjected to flow cytometric analysis. As shown in **Figure 3** and **Table 1**, the cell number in S phase was significantly increased, accompanying with a decrease in G_1 and G_2/M phase when compared with the control cells after treating the cells with Valdien for 24 hours. The data suggests that the inhibition of cell proliferation by Valdien is associated with the induction of S-phase arrest.

Valdien induces apoptosis in HCT-116 cells

Hoechst was used to understand whether Valdien inhibited HCT-116 cells proliferation by induced apoptosis. As showed in **Figure 4**, HCT-116 cells treated with Valdien (20 and 40 μg/mL) 48 h display condensation of chromatin and appearance of apoptotic bodies. Transmission electronic microscopy (TEM) was used to evaluate morphological ultrastructural. Apoptotic cells observed in response to Valdien for 48 h were characterized by nuclear fragmentation and strong condensation of chromatin (**Figure 5**). Valdien-induced apoptosis in HCT-116 cells was further confirmed using Annexin V/PE staining assays. As shown in **Figure 6**, treatment of HCT-116 with Valdien for 48 h

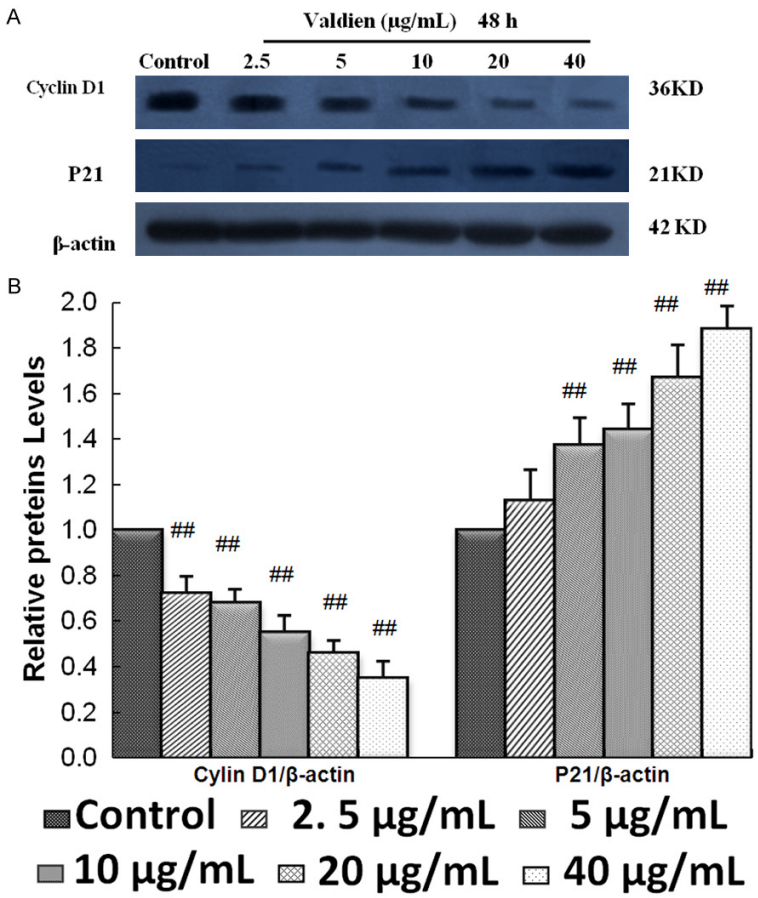

Figure 9. Effect of Valdien on the expressions of cell cycle protein cyclin D1 and p21 in HCT-116 cells. A. Valdien significantly decreased the protein expression of cyclin D1 and up-regulated p21. B. Columns represent the ratios of cyclin D1 and p21 to β-actin. Protein was extracted from HCT-116 cells treate 2.5-40 μg/mL Valdien for 48 h. Data are representative of 3 separate experiments. Parallel blotting was performed with β-actin antibody. $^{\#\#}P < 0.01$ versus control.

increased the percentage of apoptotic cells in a dose-dependent manner.

Effect of Valdien on the apoptosis-related protein expression in HCT-116 cells

In order to investigate the potential mechanism of Valdien-induced apoptosis in HCT-116 cells, we detected the expression level of apoptosis-related protein. Our result showed that HCT-116 cells incubated with Valdien (2.5-40 μg/mL) for 48 h markedly increased Fas, FasL, cleaved caspase-3 and caspase-8 in a concentration-dependent manner (**Figure 7**). To further examine the mechanism of Valdien-induced apoptosis in HCT-116 cells, we used the western blotting to assess the expression level of antiapoptosis proteins (such as Bcl-xL, survivin and Bcl-2) and proapoptotic proteins (Bax). As showed in **Figure 8**, Valdien significantly decreased the protein expression level of Bcl-xL, survivin and Bcl-2 in a dose-dependently, whereas markedly increased the levels of pro-apoptotic Bax.

Effect of Valdien on the expression of cell cycle protein cyclin D1 and p21

As shown in **Figure 9**, Valdien decreased cyclin D1 and up-regulated p21 protein expression, which participate in cell cycle regulation. These results indicate Valdien could induce cell cycle arrest at S phase by regulating cyclin D1 and p21.

Valdien regulated Wnt1/β-catenin signaling pathway in HCT-116 cells

Recent studies showed that Wnt signaling pathways of abnormal activation play a key role in the process of cells carcinogenesis, tumorigenesis and tumor invasion, especially the abnormal activation of Wnt1/β-catenin signaling pathway is one of the important reasons for the occurrence of colon cancer. To examine the mechanism responsible for the observed effects of Valdien, we further tested the protein expression levels of the factors related to Wnt1 signaling pathway by western blotting. We demonstrated that Valdien had significantly inhibited the expression of Wnt1, β-catenin, c-Myc protein levels in a dose-dependent manner, whereas markedly increased the expression levels of E-cadherin (**Figure 10**).

Valdien inhibits HCT-116 cells growth in vivo

To investigative whether Valdien could inhibit colon cancer growth *in vivo*, we examined the effects of Valdien on the growth of xenograft

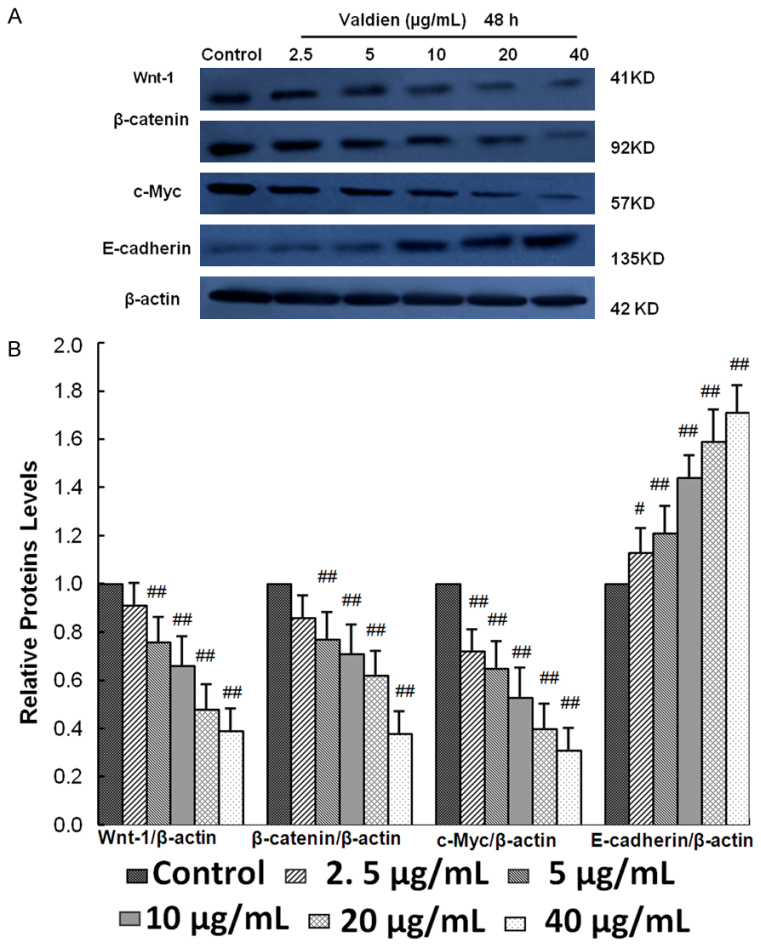

Figure 10. Effects of Valdien on the Wnt1/β-catenin signaling pathway. A. HCT-116 cells were treated with 2.5-40 μg/mL Valdien for 48 h. Valdien significantly reduced the protein expression levels of Wnt1, β-catenin, c-Myc, whereas markedly upregulated the expression levels of E-cadherin. B. Columns represent the ratios of Wnt1, β-catenin, c-myc, and E-cadherin to β-actin. Data are representative of 3 separate experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus control.

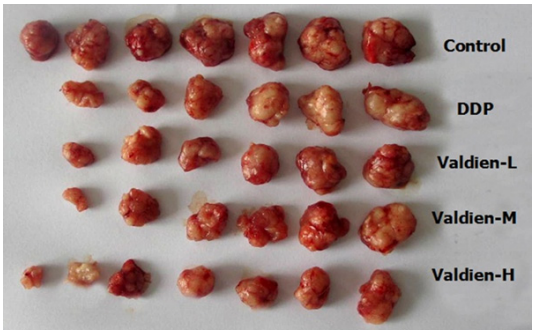

Figure 11. Valdien could significantly reduce the xenograft tumor volumes. Tumors of control mice, PDD and different dose of Valdien treated mice on d 21 are shown. Valdien-L, 5 mg/kg, Valdien-M, 10 mg/kg, and Valdien-H, 20 mg/kg.

colonic tumors in athymic BALB/c nude mice. As shown in **Figure 11**, Valdien obviously suppressed the tumor growth as compared with control group. The average tumor volume and tumor weight were remarkably reduced in Valdien treated groups, suggesting Valdien strongly inhibited tumor growth in xenograft human colonic tumor model (**Table 2**). Moreover, our results showed that the mimic of Valdien treated mice have little effect on body weight at the tested concentration, suggesting little toxicity of this compound (**Table 2**).

We used immunohistochemical staining to study the proteins expression of Bcl-2 and Bax in Valdien-treated and control tumor samples. Our results showed that the percentage of Bax positive cells was obviously increased in Valdien-treated groups as compared with the control group (**Figure 12**). The apoptotic promoting effect of this mimetic complex was further confirmed by the results generated from Bcl-2 immunohistochemical staining. As showed form **Figure 12**, majority of tumors from control group displayed strong to moderate Bcl-2 staining intensity but treated with different doses of Valdien (5, 10 and 20 mg/kg) showed weak or undetectable Bcl-2 expression. The results indicated that MnSODm suppress tumor growth may be through promoting apoptosis, in line with down-regulation of Bcl-2 and activation of Bax.

Discussion

Colon cancer is one of the most common malignant tumors, and its incidence is increasing year by year. However, the high toxicity and low selectivity of most currently used antitumor drugs compromise the beneficial treatment effects of these agents [17, 18]. Therefore,

Table 2. Effect of Valdien on the inhibition of the xenograft tumor volumes on HCT-116 cells in mice ($\bar{x}$ ± s)

Group	n	Begin body weight (g)	End body weight (g)	Tumor weight (g)	Inhibition Rate (IR%)
Control	7	19.4 ± 1.9	23.3 ± 2.1	1.79 ± 0.33	-
DDP	6	19.5 ± 1.8	21.6 ± 2.4	1.01 ± 0.49#	43.48
Valdien-L, 5 mg/kg	6	19.8 ± 2.1	22.2 ± 2.5	1.07 ± 0.50#	40.43
Valdien-M, 10 mg/kg	6	19.3 ± 1.8	22.8 ± 2.6	0.99 ± 0.56#	44.58
Valdien-H, 20 mg/kg	7	19.2 ± 2.3	22.6 ± 2.2	0.70 ± 0.36##	60.92

HCT-116 cells (1×10^7 cells/animal) were inoculated subcutaneously into the right front armpit of mice. Animals were divided into five groups and treated with different dose of Valdien and DDP 48 h after inoculation. Valdien was administered intraperitoneally at dose of 5, 10, 20 mg/kg per day for 21 days. DDP was administered intraperitoneally at dose of 5 mg/kg every three days for once. Control mice received normal saline. 24 h after the last treatment, all the animals were sacrificed and tumors were extirpated. Results are expressed as means ± SD, #$P < 0.05$ or ##$P < 0.01$ versus control. IR (%) = $(1\text{-}W_t/W_c)\times100$, where W_t and W_c represent tumor weight of treatment and control groups.

there is an urgent need for developing new chemotherapeutic agents with relatively high selectivity and low toxicity to prevent and treat colon cancer. Whether Valdien has antitumor effects on colon cancer and what are the pharmacological mechanisms and molecular targets are poorly understood. This study was designed to investigate the mechanism by which Valdien mediates its antitumor effects in colon cancer both *in vitro* and *in vivo*.

As is commonly known, uncontrolled cell proliferation is the main mechanism for neoplastic progression [19]. The present study indicated that that Valdien remarkably inhibited cell proliferation in HT-29, Lovo and HCT-116 cell lines in a concentration-dependent manner. The results also showed that Valdien inhibition was against the HCT-116 cells proliferation in a time-dependent manner. In addition, Valdien displayed more sensitive to HCT-116 cells than HT-29 and Lovo cell lines, so we chose HCT-116 cells for the following studies. Cell proliferation was controlled by the progression of the cell cycle [20]. To investigate how Valdien inhibited cell proliferation, we used the flow cytometry to assess the effect of Valdien on cell cycle distribution. The result showed Valdien induced cell cycle arresting at S phase by regulating cyclin D1 and p21, which played the important role in cell cycle regulation.

Apoptosis is an important phenomenon in cytotoxicity induced by antitumor drugs. The execution of apoptosis, or programmed cell death [21], is associated with characteristic morphological and biochemical changes mediated by a series of gene regulation and cell signaling pathways. To unveil the underlying mechanism of Valdien inhibition cell proliferation, we examined HCT-116 cells treated with Valdien for evidence of apoptosis. After treated with Valdien, the HCT-116 cells displayed clearly morphological signs of apoptosis in microscopic observation. In addition, the percentage of apoptotic evidenced by annexin V-PE staining was gradually increased after being treated with different concentrations of Valdien in HCT-116 cells.

Apoptosis is a form of programmed cell death that typically leads to caspase activation through two major routes, the extrinsic death receptor and the intrinsic mitochondrial pathways [22]. Fas is a cell surface protein that belongs to the death receptor family, and has a pivotal role in apoptosis of various cancer cells included colorectal [23] via activation by its natural ligand, FasL. The death-inducing signaling complex (DISC) is rapidly formed after Fas stimulation, which consists of oligomerized Fas, FADD and procaspase-8. After binding to the DISC, pro-caspase-8 homodimers undergo a conformational change, and autocatalytic processing induces the generation of active caspase-8, leading to the activation of caspase-3. This caspase cascade leads to DNA damage and cell apoptosis [24-27]. In our study, we found that treatment of HCT-116 cells with Valdien resulted in increased the expression of Fas, FasL, cleaved caspase-8 and caspase-3. This indicated that Valdien triggers apoptosis through the death receptor pathway.

In addition, apoptosis is regulated by a complex process that is tightly regulated by the balance of several antiapoptotic proteins (such as Bcl-xL, survivin and Bcl-2) and proapoptotic proteins (such as Bax and Bak) [28]. Our studies suggested that Valdien induced apoptosis of HCT-116 cells is associated with down-regulation the expression levels of Bcl-xL, Bcl-2 and

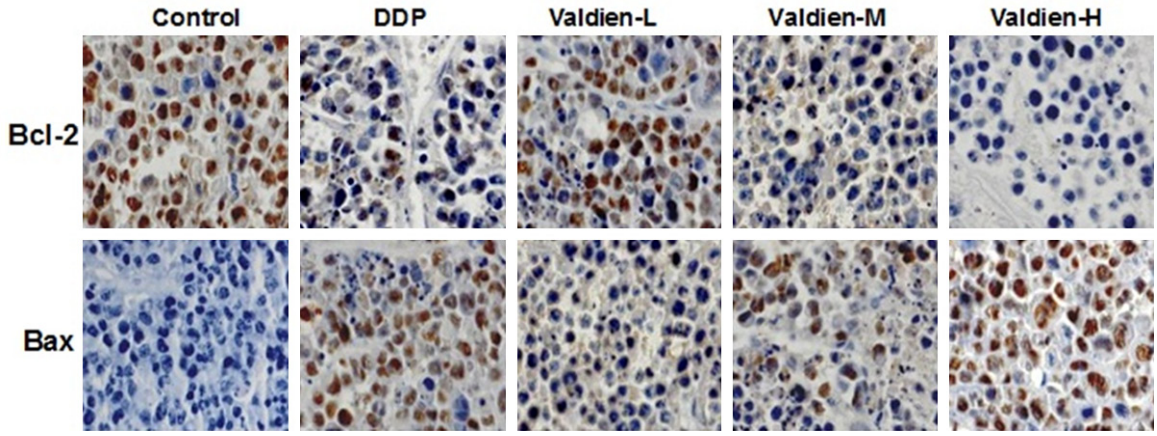

Figure 12. Effect of Valdien on HCT-116 tumor cell apoptosis *in vivo*. Immunohistochemical staining was carried out on the Valdien-treated and control tumors using antibodies against Bcl-2 and Bax. Photos were taken under ×100 magnification.

survivin while markedly increased the expression levels of Bax.

Wnt signaling pathway is a kind of highly conservative signal transduction pathway in the process of biological evolution. As an important member of the Wnt family, Wnt1 has been reported as a proto-oncogene which has the ability to activate the Wnt/β-catenin signaling pathway [29]. Studies have indicated that Wnt1 could regulate the progression of cancer because it promotes cell proliferation, migration and prolongs cancer cell survival [30, 31]. Wnt1 binds to specific Frizzled (FZD) surface receptors of target cells to activate distinct intracellular pathways, resulting in the accumulation and nuclear localization of downstream molecule β-catenin protein. β-catenin protein, as a key component of Wnt pathway controlling proliferation and cell cycle, is responsible for cellular adhesion since it forms a complex with E-cadherin [32]. Changes of membrane β-catenin level may be caused by the decrease of membrane E-cadherin. Wnt antagonist remarkable inhibited cancer cells invasion and induced the expression of Wnt/β-catenin transcriptional target gene E-cadherin [29]. C-Myc gene, as the downstream target gene of Wnt/β-catenin signaling pathway, is over-expressed in many human malignant tumors. Some clinical studies have reported that abnormal activation of Wnt/β-catenin pathway is frequently involved in colon cancer [33, 34]. To explore the role and mechanism of Valdien in colon cancer, we investigated the expression levels of Wnt1 and the downstream molecules β-catenin, c-Myc and enforced E-cadherin in Wnt signaling. The down-regulation of Wnt1 expression by Valdien inhibited the constitutive expression of β-catenin, c-Myc and enforced E-cadherin levels. Taken together, these data indicated that the inactivation of the Wnt1/β-catenin signaling pathway played an important role in Valdien induced HCT-116 cell death.

Xenograft colonic tumors in athymic BALB/c nude mice were used to investigate the antitumor effect of Valdien on colon cancer *in vivo*. The result showed that Valdien could effectively suppress tumor growth *in vivo* and without any influence on the body weight of mice. Our study also showed that the anticancer effect on HCT-116 cells xenografted mice may be due to Valdien induced apoptosis.

In summary, this study provides evidence that Valdien inhibited the proliferation of HCT-116 *in vitro*. The mechanisms of Valdien inhibited the proliferation and induced apoptosis of Valdien in this study may be partially due to regulating the Wnt1/β-catenin signaling pathway involving the activation of the death receptor pathway. Moreover, the *in vivo* antitumor activity of Valdien is demonstrated in the human colon cancer cells line HCT-116 xenografted model. The significance of this study lies in that it examines the functions of Valdien in human colon cancer for the first time. The findings obtained here may be helpful for understanding the properties of Valdien as a candidate of antitumor drugs.

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Disclosure of conflict of interest

None.

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