Original Article

# Regulation of survivin and caspase/Bcl-2/Cyto-C signaling by TDB-6 induces apoptosis of colorectal carcinoma LoVo cells

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**Background:** Colorectal carcinoma (CRC) treatment remains severe. Survivin is aberrantly overexpressed in CRC tissues and might be a potential target for CRC treatment. TDB-6 is a new taspine derivative. The purpose of this study is to investigate the inhibitory effect of TDB-6 on CRC and its underlying mechanism.

**Methods:** The MTT assay and xenograft model were utilized to investigate the inhibitory effect of TDB-6 on LoVo cells *in vitro* and *in vivo*. Hoechst staining and Annexin-V FITC/PI analysis were conducted to study the effect of TDB-6 on LoVo cell apoptosis. Mitochondrial membrane potential ($\Delta\psi m$) assay was conducted to demonstrated whether TDB-6 could induce mitochondrial-mediated apoptosis of LoVo cells. Western blotting was conducted to investigate the effect of TDB-6 on survivin protein and caspase/Bcl-2/Cyto-C signaling.

**Results:** The results indicated that TDB-6 induced mitochondria-mediated apoptosis and inhibited the proliferation and growth of LoVo cells *in vitro* and in *vivo*. Mechanistic investigation utilizing western blotting indicated that TDB-6 inhibited survivin protein expression, and the inhibitory effect was augmented by TDB-6 and YM-155 co-administration, which revealed that TDB-6 might induce apoptosis of LoVo cells by targeted regulation of survivin. TDB-6 also regulated survivin downstream signaling. It significantly increased the protein level of cleaved caspase-3, cleaved caspase-7, cleaved caspase-9, cleaved-PARP, and Cyto-C, and decreased the protein level of Bcl-2.

**Conclusions:** TDB-6 might be a promising survivin inhibitor with great potential for CRC treatment.

**Keywords:** TDB-6; colorectal carcinoma (CRC); survivin; apoptosis; signaling

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## Introduction

Colorectal carcinoma (CRC) is one of the most common tumors of the digestive system, and its morbidity and mortality rank third and fourth among all malignancies, respectively (1,2). CRC mostly occurs in the rectum and the junction of the rectum and sigmoid colon. The etiology and pathogenesis of CRC are complex. CRC is caused by a variety of factors such as genetic and environmental factors and functional molecular regulation abnormalities (3,4). A combination of surgery and radiotherapy, chemotherapy, immunotherapy, or targeted therapy is the main treatment strategy for CRC. Medication is critical for advanced CRC patients since they cannot be treated surgically because of metastasis (5-7). Erbitux, which targets epidermal growth factor receptor (EGFR), and the multiple tyrosine

 

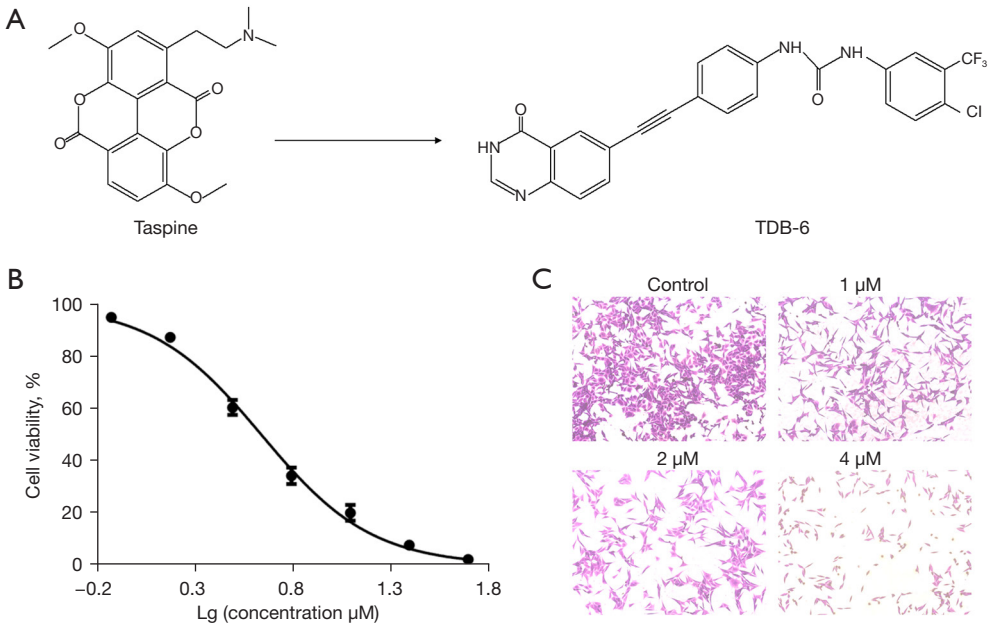


**Figure 1** TDB-6 suppressed the proliferation of LoVo cells *in vitro*. (A) Chemical structure of taspine and TDB-6; (B) MTT analysis of LoVo cells; (C) crystal violet staining of LoVo cells after TDB-6 treatment (×200).

kinase inhibitor regorafenib have been proven to improve the condition and prolong the survival of CRC patients. However, the 5-year survival rate is still low and the treatment for advanced CRC remains severe (8-10). It is of great significance to further study the pathological features of CRC and develop new anti-CRC drugs.

Apoptosis reduction is a pivotal feature of tumors and induction of apoptosis has gradually become a promising anti-tumor treatment (11,12). Abnormal regulation of mitochondrial-mediated endogenous apoptotic pathways plays an important role in tumorigenesis. The induction of mitochondrial-mediated apoptosis will not only lead to the rapid decrease of mitochondrial membrane potential and electron transport decoupling, but also cause the increase of mitochondrial membrane permeability, which further induces the release of cytochrome-C (Cyto-C) (13-15). In the cytoplasm, Cyto-C can bind to non-activated caspase-9 to produce apoptotic bodies and activate caspase-9, thus promoting the apoptosis of tumor cells (16). Inhibitor of apoptosis protein (IAP) is a natural caspase inhibitor, and survivin is a member of the IAP family, which directly or indirectly suppresses the activity of caspase-3 and caspase-7. Survivin can also interfere with the activity of caspase-9 and block mitochondria-mediated apoptosis (17,18). Previous studies indicated that survivin was widely expressed in gastric, liver, lung, breast, and prostate cancers, but it was not expressed or showed low expression in adjacent tissues, making survivin a potential tumor marker (19). Specific inhibition of survivin could induce apoptosis and suppress the proliferation of hepatocellular carcinoma (HCC), and similar effects were observed in CRC, leukemia, and breast cancer, among others. Therefore, survivin might be a promising target for tumor inhibition (20,21).

Natural products are a kind of valuable compound library, containing rich medicinal resources. Many natural products and their chemical derivatives have been found to be effective in tumor suppression (22). Taspine, isolated from the medicinal plant Radix Aconitum, is a natural product with extensive pharmacological activities, including the inhibition of bacteria, angiogenesis, and malignant tumors (23,24). TDB-6 is a new taspine derivative synthesized in our laboratory, the lipid solubility and bioavailability of which were significantly improved compared to that of taspine. In this study, the anti-CRC effect of TDB-6 (*Figure 1A*) was investigated *in vitro* and *in vivo*. We mainly studied the inhibitory effect of TDB-6 on the proliferation and apoptosis of CRC cells, and preliminarily investigated the underlying mechanism. We present the following article in accordance with the ARRIVE reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-780/rc).

 

## Methods

### *Chemicals and reagents*

TDB-6 was obtained from the Research and Engineering Center for Natural Medicine, Xi'an Jiaotong University (Xi'an, China). Dulbecco's modified Eagle's medium (DMEM) was purchased from HyClone (Logan, UT, USA). Phosphate-buffered saline (PBS) and fetal bovine serum (FBS) were obtained from ExCell Bio (Shanghai, China). Trypsin and antibiotics were obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Dimethyl sulfoxide (DMSO), Rhodamine 123, and 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-2H-tetrazolium bromide (MTT) powder were obtained from Sigma-Aldrich (St. Louis, MO, USA). The Annexin V FITC/PI cell apoptosis reagent kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). The BCA protein assay reagent kit was obtained from Pierce Biotechnology (Waltham, MA, USA). RIPA lysis buffer was purchased from Pioneer Biotechnology Co., Ltd. (Shaanxi, China). Protease and phosphatase inhibitors were obtained from Roche Tech. (Basel, Switzerland). Tween-20 was obtained from Amresco (Pennsylvania, USA). Sodium carboxylmethyl cellulose (CMC-Na) was purchased from Jinyi Biology Co., Ltd. (Henan, China). Bcl-2, survivin, Bid, cleaved caspase-3, cleaved caspase-7, cleaved caspase-9, cleaved caspase-PARP, and Cyto-C rabbit mAb were from Protein Technology Group (Chicago, Illinois, USA). HRP-conjugated goat anti-rabbit IgG and β-actin rabbit mAb were obtained from ABclonal (Boston, MA, USA).

### *Cell line and cell culture*

The human CRC cell line LoVo was obtained from the Shanghai Institute of Cell Biology at the Chinese Academy of Sciences. The cells were cultured in DMEM supplemented with 10% FBS and 5% antibiotics. The cells were incubated in 95% air and 5% $CO_2$ in a humidified atmosphere at 37 ℃.

### *Animals and xenograft model*

Male BALB/c nude mice (4 to 6 weeks old, 18–22 g) were housed at the Laboratory Animal Center of Xi'an Jiaotong University with a 12-h light-dark cycle. The animals were permitted free consumption of a standard chow diet and water. All the animal studies were conducted according to regional authority guidelines. To establish the xenograft model of colon cancer, 12 mice received 200 μL LoVo cell suspension ($2\times10^7$ cells/mL) per mouse by subcutaneous inoculation. When the LoVo cells developed as a palpable tumor, the tumor volume was measured once every 2 days. The mice were randomly divided into 3 groups (4 mice per group) when the tumor volume reached 100 $mm^3$. The grouped mice received TDB-6 (40 or 80 mg/kg) or 0.5% CMC-Na solution every day by intragastric administration. The dose of TDB-6 (40 or 80 mg/kg) was determined according to the pre-experiment result, including the acute toxicity study and pharmacodynamic investigate the body weight of each mouse was recorded daily. After 2 weeks, the mice were sacrificed by the cervical dislocation method. The tumors and spleens were removed and weighed. Animal experiments were performed under a project license (No. 2022-1434) granted by the Research Ethics Committee of Xi'an Jiaotong University, in compliance with the Chinese Academy of Sciences Animal Care and Use Committee guidelines for the care and use of animals. A protocol was prepared before the study without registration.

### *Cell viability assay*

LoVo cells were trypsinized and re-plated into a 96-well plate with a total cell suspension volume of 180 μL per well. Following incubation for 24 h, an increasing gradient of TDB-6 (0.39–50 μM) was added into the plate, with a total volume of 20 μL per well. After 48 h, the medium with TDB-6 was replaced by MTT solution dissolved in serum free medium. Following incubation for 4–6 h, the supernatant was removed and 150 μL DMSO was added per well. The plate was subsequently shaken 15 min for thorough dissolution and measured at 490 nm.

### *Cell apoptosis assay*

The cells were seeded in a 6-well plate at proper density for 24 h. For the TDB-6 treatment experiment, cells were subsequently exposed to TDB-6 (0, 1, 2, 4 μM). For YM-155 and TDB-6 treatment experiments, the cells were exposed to YM-155 (100 nM), TDB-6 (4 μM), or both. At 48 h later, the treated cells were trypsinized and resuspended in binding buffer, followed by incubation with annexin V-FITC for 15 min and Propidium Iodide (PI) for 5 min in the dark. Finally, the stained cells were detected by a NovoCyte 2040R flow cytometer (ACEA, San Diego, CA, USA).

 

### *Hoechst staining*

LoVo cells were cultured in a 96-well plate at proper density for 24 h. For the TDB-6 treatment experiment, cells were subsequently exposed to TDB-6 (0, 1, 2, 4 μM). For YM-155 and TDB-6 treatment experiments, the cells were exposed to YM-155 (100 nM), TDB-6 (4 μM), or both. After 48 h incubation, the treated cells were washed 3 times with PBS and subsequently fixed with 4% paraformaldehyde for 15 min. Following washing again with PBS, the cells were incubated with hoechst 33258 for 15 min. Images were obtained using an inverted fluorescence microscope.

### *Mitochondrial membrane potential (Δψm) assay*

LoVo cells were seeded in a 6-well plate at proper density for 24 h. After treatment with TDB-6 (0, 1, 2, 4 μM) for 48 h, the cells were rinsed with DMEM, followed by incubation with Rhodamine 123 (1 mM) for 30 min at 37 ℃ in the dark. The fluorescently labeled cells were rinsed again and analyzed using a NovoCyte 2040R flow cytometer (ACEA, San Diego, CA, USA).

### *Western blot assay*

Treated cells or tumor tissues were placed at 4 ℃ in RIPA buffer supplemented with a protease and phosphatase inhibitor cocktail. After centrifuging at 12,000 r/min for 10 min, the precipitate was discarded and the supernatant was collected for western blotting. A BCA Protein Quantification kit was utilized to determine the protein concentrations of cells or tissues samples. In order to separate the proteins, equivalent amounts of samples were electrophoresed on SDS-polyacrylamide gels. Then the proteins were transferred to polyvinylidene difluoride membranes, following by blocking with 5% non-fat milk for 2 h and incubating with primary antibodies overnight. After washing 4 times with TBST for 5 min each, the membranes were incubated with secondary antibodies (HRP-conjugated) at 37 ℃ for 1 h, after which they were washed 4 times with TBST for 5 min each. After detection with an ECL kit, protein bands were visualized by a Tanon 5200 automatic chemiluminescence image analysis system (Tanon, Shanghai, China).

### *Statistical analysis*

All data are expressed as the mean ± SD. Statistical analysis was performed by SPSS (version 16.0; IBM, Armonk, NY, USA). Statistical differences between different treated groups was analyzed by ANOVA. A Student's *t*-test was used to compare one group with the control group. Significance was set at $P<0.05$.

## Results

### *TDB-6 suppressed the proliferation of LoVo cells*

The effect of TDB-6 on the viability of LoVo cells was investigated by the MTT assay and the results are shown in *Figure 1B*. The viability of LoVo cells was significantly inhibited by TDB-6 in a dose-dependent manner, with an $IC_{50}$ value of 4.49±0.33 μM. The crystal violet staining assay was conducted on LoVo cells treated with different concentrations of TDB-6 (1, 2, and 4 μM). The results in *Figure 1C* demonstrated that the proliferation of LoVo cells was obviously suppressed by TDB-6.

### *TDB-6 inhibited the tumor growth of the xenograft model in nude mice*

A xenograft model was utilized to investigate the anti-tumor effect of TDB-6 on LoVo cells *in vivo*. The results indicated that TDB-6 significantly delayed tumor growth at the indicated concentrations (*Figure 2A-2C*). The inhibitory rate of TDB-6 on tumor growth was more than 50% (56.91%) at the concentration of 80 mg/kg (*Figure 2D*). Spleen index (spleen weight/body weight) is closely associated with immunity, and can reflect side effects of drugs. As shown in *Figure 2E,2F*, the spleen weight and body weight showed no significant difference between the TDB-6 treated groups and control group, indicating no visible damage was caused by TDB-6 on BALB/c nude mice.

### *TDB-6 induced the apoptosis of LoVo cells by regulating survivin*

The effect of TDB-6 on LoVo cell apoptosis was investigated by flow cytometry. The results of Annexin-V FITC/PI analysis in *Figure 3A* showed that TDB-6 increased the percent of apoptotic cells in a dose-dependent manner compared to the control group. Hoechst staining was also conducted on LoVo cells and the result is shown in *Figure 3B*. TDB-6 could induce condensed bright blue apoptotic nuclei in LoVo cells, demonstrating its apoptosis induction effect. The result of the Δψm assay indicated

 

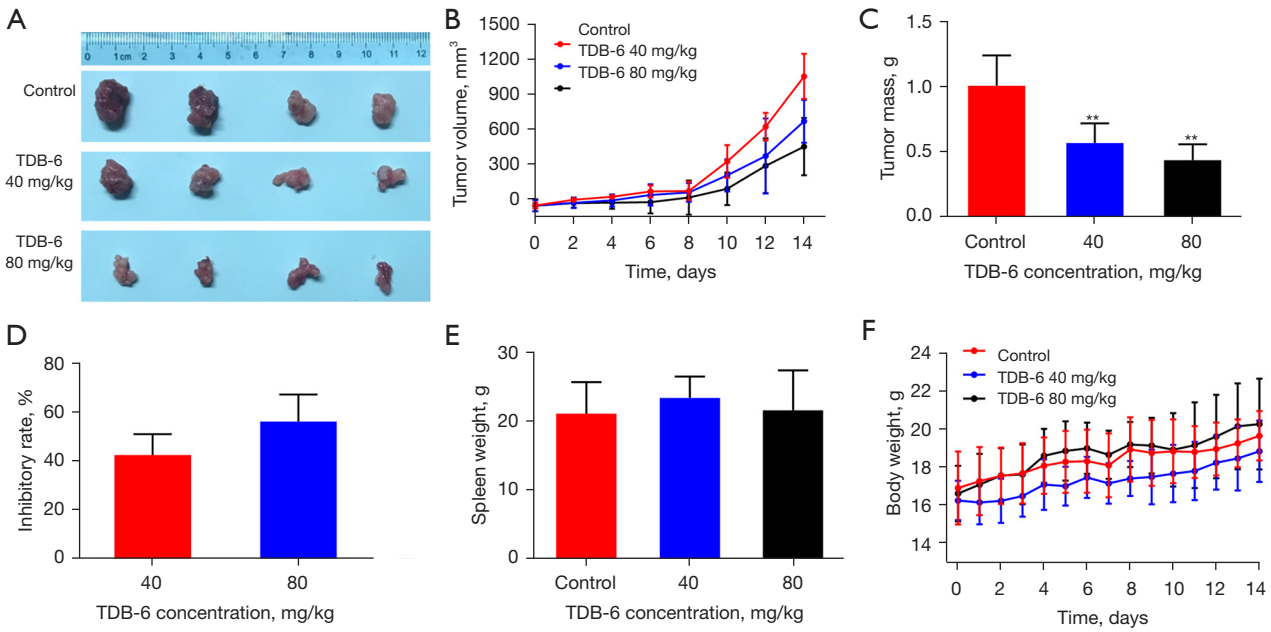


**Figure 2** TDB-6 inhibited the growth of LoVo cell xenografts in BALB/c nude mice. (A) Images of tumors in the control and TDB-6 treated group. (B) Tumor volume change throughout the study. (C) Effect of TDB-6 on tumor mass. (D) Tumor inhibitory rate of TDB-6 treated groups (40 and 80 mg/kg) compared to the non-treated group. (E) Effect of TDB-6 on spleen weight. (F) Body weight change throughout the study. Values are presented as mean ± SEM; n=4. **P<0.01 *vs.* the control group.

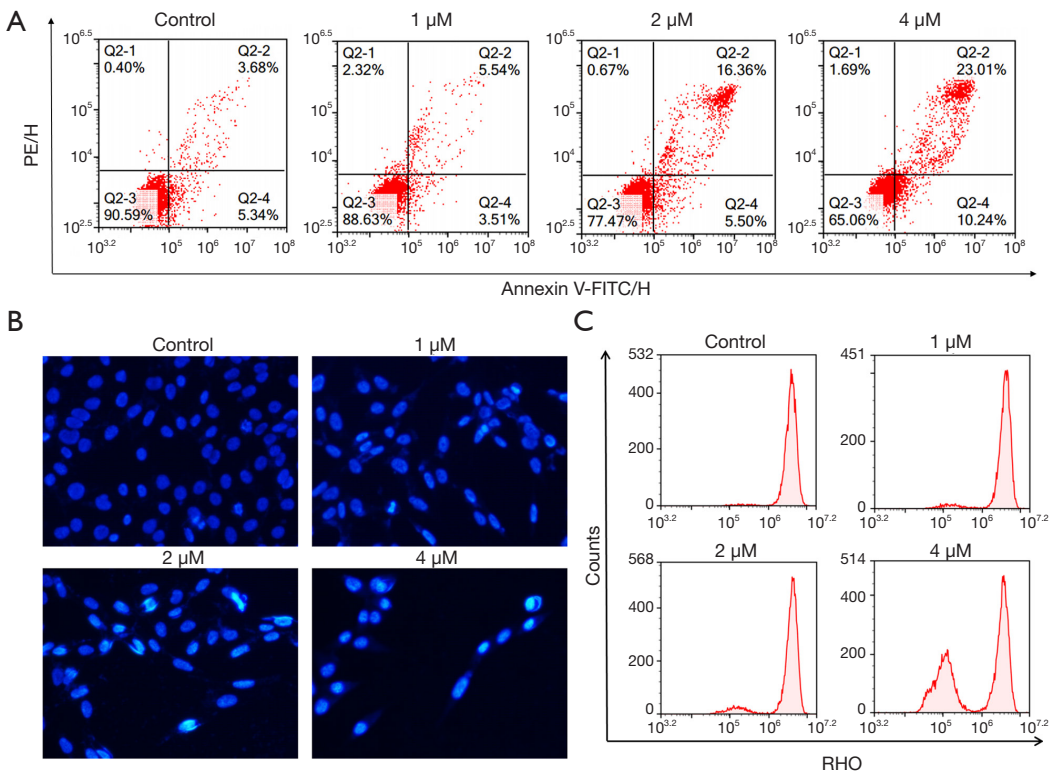


**Figure 3** TDB-6 induced the apoptosis of LoVo cells. (A) The effect of TDB-6 on LoVo cell apoptosis determined by flow cytometry; (B) hoechst staining assay of LoVo cells (×200); (C) mitochondrial membrane potential assay of LoVo cells by Rhodamine 123 (RHO) staining.

 

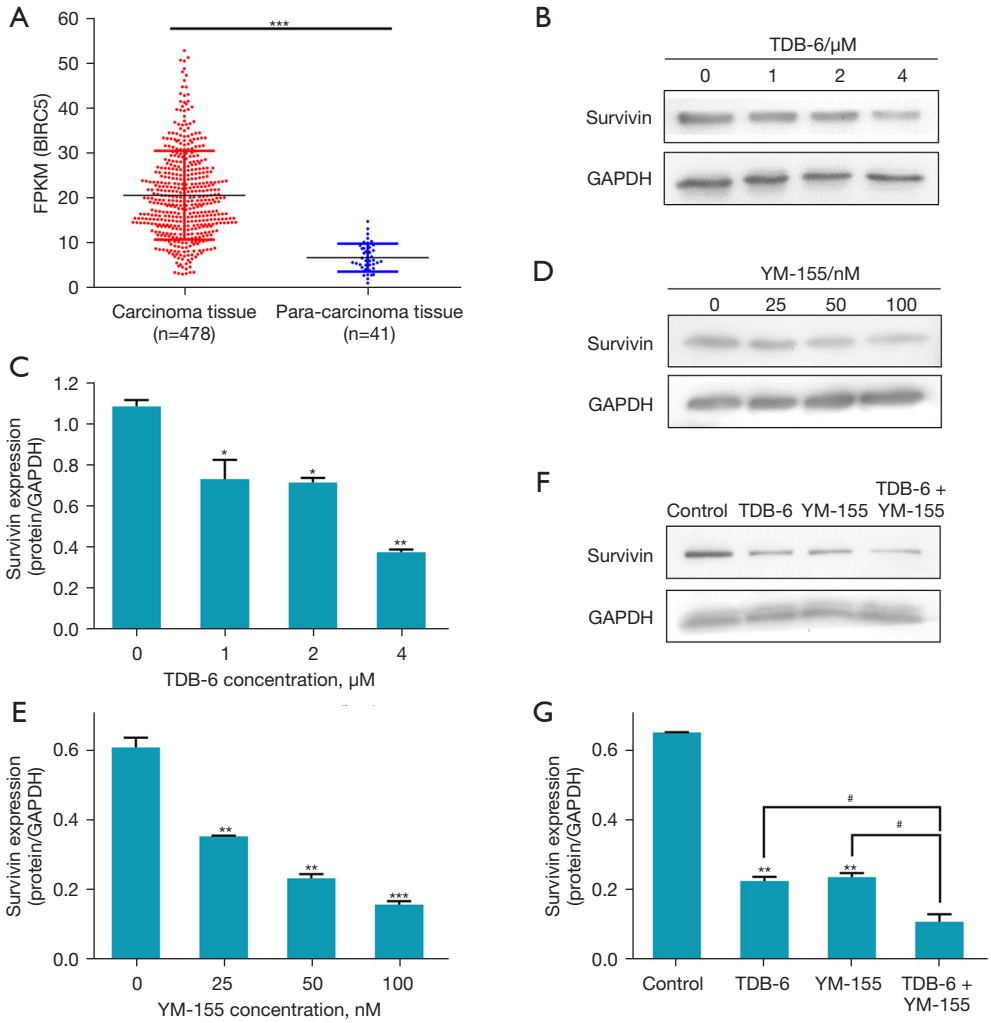


**Figure 4** The suppression of survivin protein expression by TDB-6. (A) Survivin mRNA analysis of CRC patients in TCGA database. (B,D,F) Western blot analysis of survivin after TDB-6 (B), YM-155 (D), and combined (F) treatment, respectively. (C,E,G) Quantification of (B), (D), and (F). *P<0.05, **P<0.01, ***P<0.001 *vs.* the control group; #P<0.05 *vs.* the TDB-6 + YM-155 group. CRC, colorectal carcinoma; TCGA, The Cancer Genome Atlas.

that the fluorescence intensity of Rhodamine 123 in LoVo cells decreased significantly after TDB-6 treatment, which indicated the decrease of mitochondrial membrane potential (*Figure 3C*). These results demonstrated that TDB-6 could induce mitochondrial-mediated apoptosis of LoVo cells in a dose-dependent manner.

As a member of the IAP family, survivin can suppress the apoptosis of tumor cells. The Cancer Genome Atlas (TCGA) database was used to analyze the survivin mRNA levels in HCC and non-cancerous colon tissues, and the result in *Figure 4A* indicated that survivin mRNA levels were markedly higher in carcinoma tissues than in para-carcinoma tissues. Western blotting was conducted to investigate whether TDB-6 could suppress the expression of survivin. The result showed that TDB-6 obviously decreased the protein expression of survivin in a dose-dependent manner (*Figure 4B,4C*). YM-155 is a specific inhibitor of survivin and also decreased the survivin protein level at the indicated doses (*Figure 4D,4E*). Moreover, TDB-6 and YM-155 were used in combination on LoVo cells to investigate the targeted regulation of survivin by TDB-6. The results in *Figure 4F,4G* indicated that combined use of TDB-6 and YM-155 could suppress the protein expression of survivin more effectively compared to the single use of

 

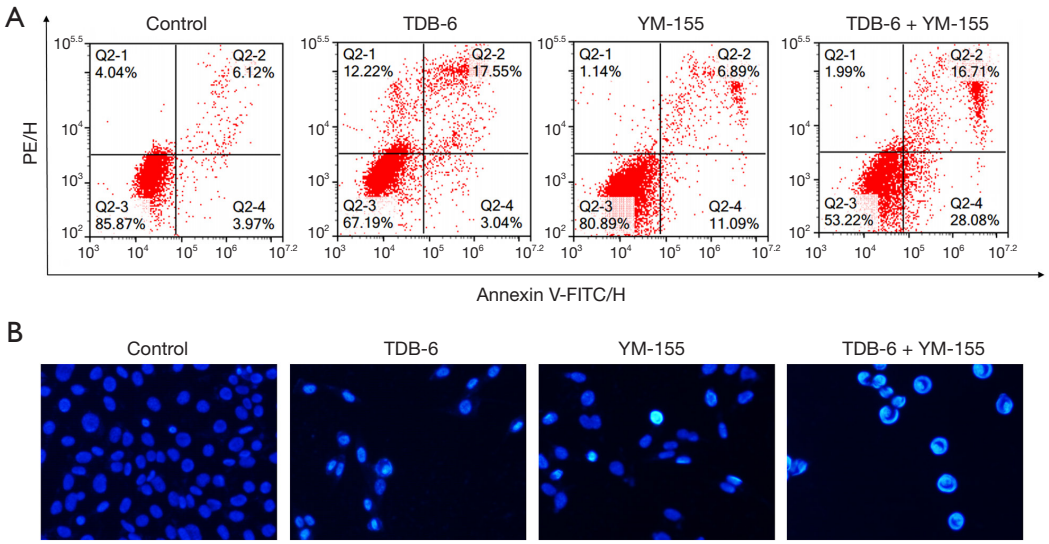


**Figure 5** TDB-6 and YM-155 induced the apoptosis of LoVo cells synergistically. (A) The effect of TDB-6, YM-155, and TDB-6 + YM-155 on LoVo cell apoptosis determined by flow cytometry. (B) Hoechst staining assay of LoVo cells after treatment with TDB-6, YM-155, and TDB-6 + YM-155 (×200).

either reagent. These results preliminarily indicated that the induction of apoptosis in LoVo cells by TDB-6 correlated with its targeted suppression of survivin protein.

The apoptosis-induction effect of TDB-6 and YM-155 co-administration on LoVo cells was also investigated by Annexin-V FITC/PI analysis and hoechst staining. The result in *Figure 5A* showed that combined use of TDB-6 and YM-155 increased the percent of apoptotic cells more effectively than the single use of either reagent. A similar result was gained in the hoechst staining assay in *Figure 5B*. More condensed bright blue dots could be observed in the TDB-6 and YM-155 co-administration group compared to the single use of either reagent. These results indicated that TDB-6 could induce the apoptosis of LoVo cells by targeted regulation of survivin.

### *TDB-6 regulated the downstream signaling of survivin*

The downstream signaling of survivin was further investigated by western blot analysis after TDB-6 treatment, and the results are shown in *Figure 6*. The expression of the anti-apoptotic protein Bcl-2 was significantly inhibited by TDB-6 in a dose-dependent manner (*Figure 6A,6B*). TDB-6 obviously increased the protein level of cleaved caspase-3, cleaved caspase-7, and cleaved caspase-9 (*Figure 6A,6B,6D*). The protein levels of cleaved-PARP and Cyto-C were also up-regulated by TDB-6.

The *in vivo* regulation of TDB-6 on survivin and its downstream apoptosis related proteins was also evaluated by western blotting. TDB-6 could significantly inhibit the expression of survivin at the indicated doses (*Figure 7A,7B*). As an anti-apoptotic protein, Bcl-2 was also obviously suppressed by TDB-6 (*Figure 7A,7B*). After the inhibition of survivin by TDB-6, the protein levels of cleaved caspase-3, cleaved caspase-7, cleaved caspase-9, cleaved-PARP, and Cyto-C significantly increased (*Figure 7A,7C,7D*). These results indicated that TDB-6 could suppress the expression of survivin and its downstream anti-apoptotic protein and increase the expression of pro-apoptotic proteins in LoVo cells *in vitro* and *in vivo*.

## Discussion

In this study, we proved that TDB-6 could significantly suppress the proliferation and xenograft growth of LoVo cells *in vitro* and *in vivo*, and no visible damage was observed in BALB/c nude mice, suggesting that TDB-6 might be an effective and safe choice for the treatment of CRC.

Inducing apoptosis is an important part of anti-tumor research, and the effect of TDB-6 on LoVo cell apoptosis was investigated. Annexin-V FITC/PI analysis and hoechst staining revealed that TDB-6 could increase the percent of apoptotic LoVo cells in a dose-dependent manner. Moreover, the Δψm assay demonstrated that TDB-6

 

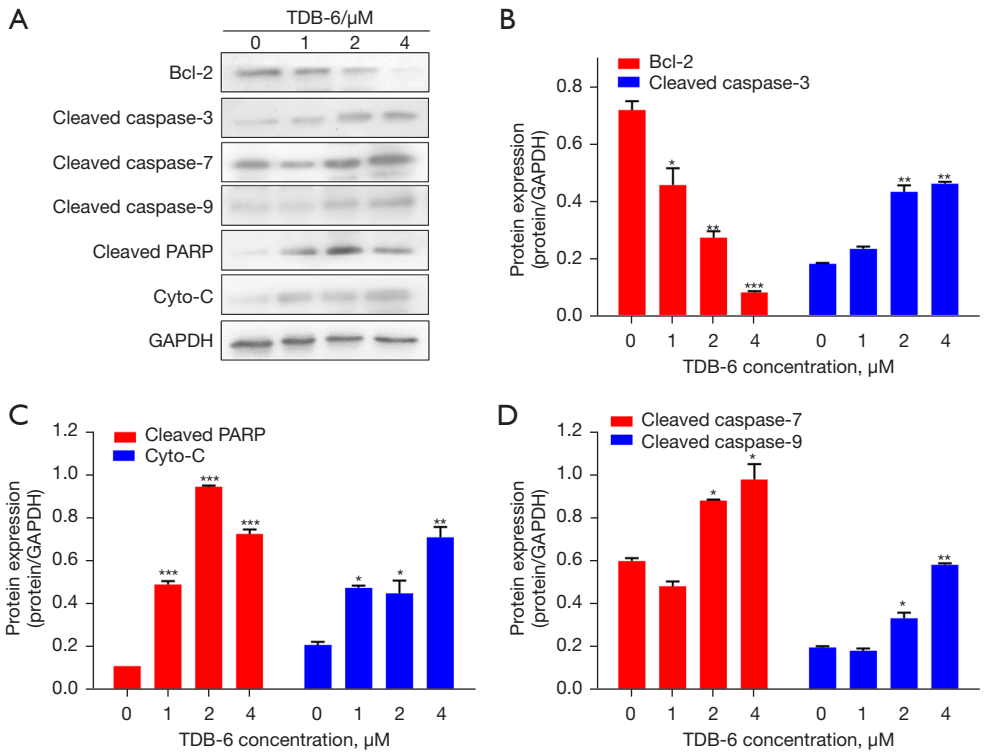


**Figure 6** TDB-6 regulated survivin downstream caspase/Bcl-2/Cyto-C signaling *in vitro*. (A) Western blot analysis of survivin downstream caspase/Bcl-2/Cyto-C signaling after TDB-6 treatment at the indicated doses. (B-D) Quantification of (A). *P<0.05, **P<0.01, ***P<0.001 *vs.* the control group.

reduced the mitochondrial membrane potential of LoVo cells, indicating the occurrence of mitochondrial-mediated apoptosis.

Survivin is the smallest member of the IAP family and is overexpressed in many human cancers, including cancers of the lung, breast, stomach, esophagus, pancreas, prostate, liver, and ovary (25). The dramatic dysregulation of expression between normal adult tissues and cancers makes survivin an attractive and perhaps ideal target for cancer drug discovery. In this study, we found that the survivin mRNA level was almost 3-fold higher in carcinoma than in para-carcinoma tissues according to the analysis of TCGA database. Western blotting was further conducted to investigate the inhibitory effect of TDB-6 on survivin expression, and the results indicated that TDB-6 reduced the protein expression of survivin in a dose-dependent manner. Moreover, co-administration of TDB-6 and YM-155 could decrease the protein expression of survivin more effectively compared to the single use of either reagent, indicating a synergistic inhibitory effect. These results demonstrated targeted suppression of TDB-6 on survivin protein. In order to prove whether TDB-6 and YM-155 could induce the apoptosis of LoVo cells synergistically, Annexin-V FITC/PI analysis and hoechst staining were conducted, and the results showed that combined use of TDB-6 and YM-155 significantly enhanced the percent of apoptotic LoVo cells compared to the single use of either reagent. In conclusion, TDB-6 could induce the apoptosis of LoVo cells by targeted inhibition of survivin protein.

Caspases are a group of proteases with similar structures that participate in cell growth, differentiation, and apoptosis regulation. As a negative apoptosis regulator, survivin inhibits the activation of caspase molecules including caspase-3, caspase-7, and caspase-9 (26). Poly ADP-ribose polymerase (PARP) is a DNA repair enzyme and plays an important role in DNA damage repair and apoptosis. PARP can be cleaved by caspase-3 and caspase-7, leading to the loss of enzyme function and cell instability (27). Activation of caspase-3 and caspase-7 will suppress the anti-apoptotic protein Bcl-2 (18), which causes the increase of mitochondrial membrane permeability and the release of Cyto-C, promoting the apoptosis of tumor cells (*Figure 7E*). In this

 

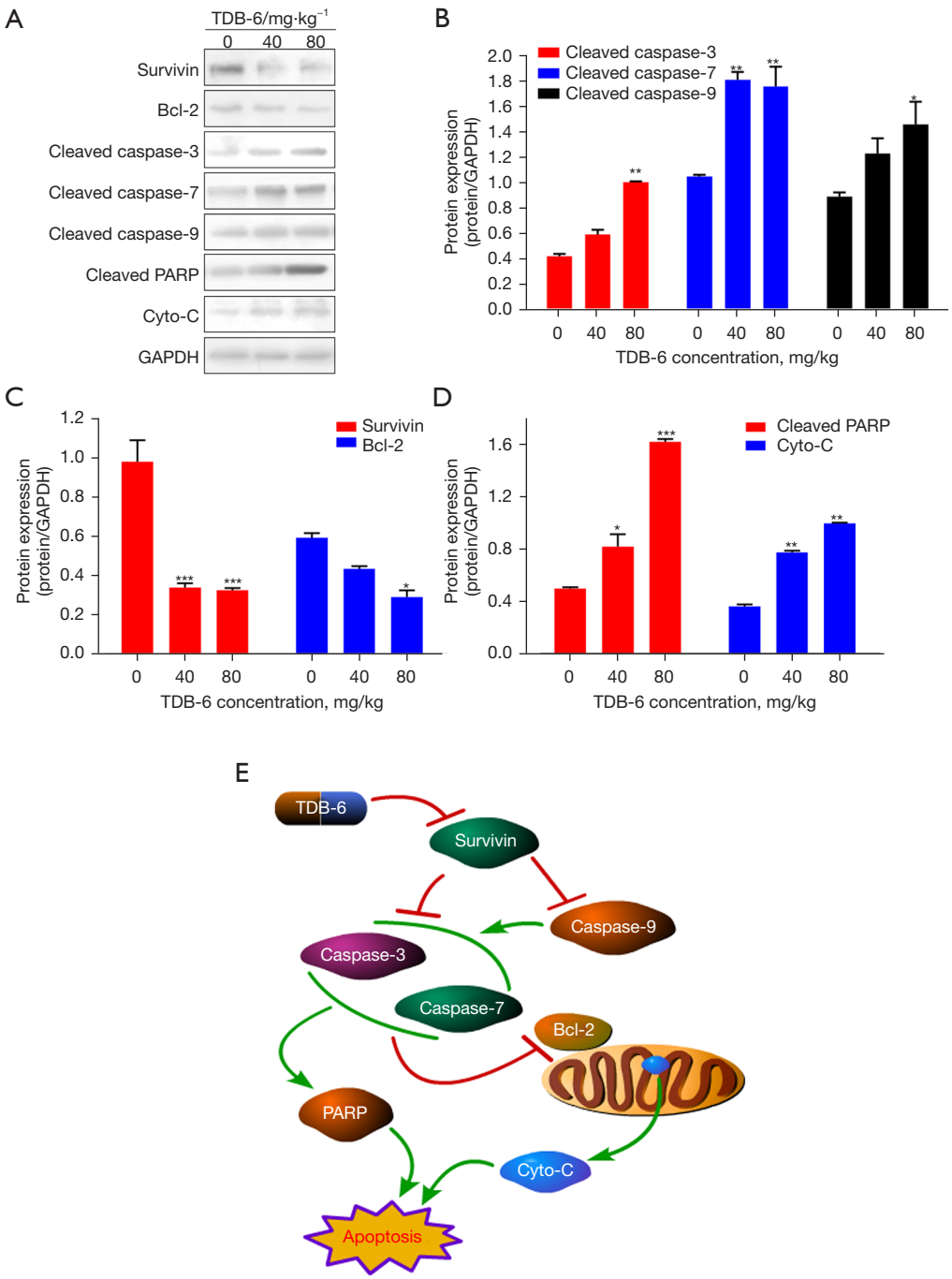


**Figure 7** TDB-6 regulated survivin and its downstream caspase/Bcl-2/Cyto-C signaling *in vivo*. (A) Western blot analysis of survivin and its downstream caspase/Bcl-2/Cyto-C signaling after TDB-6 treatment at the indicated doses. (B-D) Quantification of (A). (E) Potential demonstration of the inhibitory effect of TDB-6 on survivin protein and downstream caspase/Bcl-2/Cyto-C signaling in the suppression of LoVo cell growth. *P<0.05, **P<0.01, ***P<0.001 *vs.* the control group.

 

study, we proved that TDB-6 could suppress the expression of survivin protein and increase the protein level of cleaved caspase-3, cleaved caspase-7, and cleaved caspase-9 in LoVo cells. The protein level of Bcl-2 decreased significantly but the expression of cleaved-PARP and Cyto-C proteins increased obviously after TDB-6 treatment at the indicated doses in LoVo cells. Similar results were observed in the LoVo xenograft model. These results indicated that TDB-6 could regulate survivin downstream caspase/Bcl-2/Cyto-C signaling to induce mitochondria-mediated apoptosis of LoVo cells.

In conclusion, the obtained data indicated that TDB-6 could induce mitochondria-mediated apoptosis and inhibit the proliferation and growth of LoVo cells *in vitro* and *in vivo* via targeted regulation of survivin and its downstream caspase/Bcl-2/Cyto-C signaling. To sum up, TDB-6 might be a promising candidate for the inhibition of CRC.

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## Footnote

*Reporting Checklist:* The authors have completed the ARRIVE reporting checklist. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-780/rc

*Data Sharing Statement:* Available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-780/dss

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-780/coif). All authors report that this work was supported by the Key Scientific Research Project on Inheritance and Innovation of Traditional Chinese Medicine and Development of Qin Medicine (No. 2021-02-zz-015), the Natural Science Foundation of Shaanxi Province (No. 2022JM-605), and the Talent Support Program in Shaanxi Provincial People's Hospital (No. 2021JY-29). The authors have no other conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under a project license (No. 2022-1434) granted by the Research Ethics Committee of Xi'an Jiaotong University, in compliance with the Chinese Academy of Sciences Animal Care and Use Committee guidelines for the care and use of animals.



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