Toosendanin inhibits colorectal cancer cell growth through the Hedgehog pathway by targeting Shh

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Funding information
Zhejiang Provincial Natural Science Foundation of China, Grant/Award Number: LGF20H300001; National Natural Science Foundation of China, Grant/Award Number: 81803404

Abstract
Colorectal cancer (CRC) is one of the most common gastrointestinal cancers worldwide. This complex and often fatal disease has a high mortality rate. The Hedgehog (Hh) signaling pathway is crucial in CRC. Many studies have indicated that Shh is overexpressed in cancer stem cells (CSCs), and shh overexpression is positively correlated with CRC tumorigenesis. New drugs that kill CRC cells through the Hh pathway are needed. Toosendanin (TSN), a natural triterpenoid saponin extracted from the bark or fruit of Melia toosendan Sieb. et Zucc, can inhibit various tumors. Here, we investigated the effects of TSN in CRC and explored the possible targets and mechanisms. Shh-Light II cells were treated with TSN and tested by dual luciferase reporter assays to determine the relationship with the Hh pathway. Cell Counting Kit-8 (CCK-8) assays were used to test the inhibitory effects of TSN on CRC cells. The expression of Hh components after TSN treatment was detected using western blots and quantitative reverse transcription polymerase chain reaction. Cellular thermal shift assays confirmed the targets of TSN. The same effects of TSN on xenograft tumor growth were investigated in vivo. The average weight, volume of the finally resected tumor, and the expression of Shh in the TSN-treated groups were significantly lower than those of the control group. This result strongly suggested that TSN administration inhibited CRC growth in vivo. Our research preliminarily demonstrated that the target of TSN was Shh and that TSN inhibits CRC cell growth by inhibiting the Hh pathway, identifying a new anticancer molecular mechanism of TSN in CRC.

Keywords
colorectal cancer, Hedgehog signaling pathway, HT29 cells, Shh ligand, Toosendanin

1 | INTRODUCTION

Colorectal cancer (CRC) is a heterogeneous tumor with a cell population ranging from pluripotent to differentiated cells. The latest global cancer statistics released by the World Health Organization (WHO) indicates that CRC is one of the most common malignant tumors worldwide, and the fourth leading cause of cancer-related mortality, with approximately 900,000 deaths reported every year (Dekker et al., 2019). Clinical first-line treatment for CRC, including surgical resection, radiotherapy, and chemotherapy, are the most common methods at present, but the problem of poor prognosis still exists. Accumulating evidence has indicated that it might relate to complex mechanisms of CRC. CRC undergoes transformation processes through alternative mechanisms which include Hedgehog (H. Wang et al., 2014), Wnt (Williams et al., 2015b), PI3K/Akt (Rodgers et al., 2017), Hippo (M. Sun et al., 2017), AMPK (Sansal &
Sellers, 2004), NF-κB (Sakamoto et al., 2009), MAPK (Liang et al., 2014), and JNK (Nateri et al., 2005) pathways. Moreover, the interaction of these pathways is precise and complicated, with changes in one pathway leading to changes in the other. By focusing on molecular cross-talk, it is possible to develop more effective therapeutic strategies.

The Hh signaling pathway plays a vital role in regulating the proliferation, apoptosis, angiogenesis, invasion, and metastasis of different types of cells as well as in maintaining internal homeostasis in humans (McMahon et al., 2003). Previous studies indicated that aberrant Hedgehog signaling plays a crucial role in colon cancer progression (Saif & Chu, 2010). It was reported that Hedgehog signaling-related components, Shh, PTCH1, SMO, and Gli, are observed to be overexpressed in the hyperplastic polyps, adenomas, and adenocarcinomas of colon (Berman et al., 2003; Van Den Brink & Peppelenbosch, 2006). Inhibition of the Hh pathway provides a new method and idea for the treatment of CRC. Some Hh inhibitors were practiced in preclinical (Deng et al., 2020) and clinical experiments such as cyclopamine, cabozantinib, TAK-441, Itraconazole (Goldman et al., 2015; Y. Sun et al., 2015; Varnat et al., 2009; Wu et al., 2011). However, these Hh inhibitors have limitations in treating different types of CRC (van den Brink & Hardwick, 2006). Therefore, more various and extensive research on Hh inhibitors in CRC are needed.

Toosendanin (TSN), a triterpenoid saponin extracted from the bark or fruit of Melia toosendan Sieb. et Zucc., is a traditional Chinese medicine that was used as an agricultural insecticide in ancient China (Fang & Cui, 2011; He et al., 2010). Recently, accumulating evidence has shown that TSN plays an antitumor role in various cancers, including gastric cancer (Zhou et al., 2018), osteosarcoma (T. Zhang et al., 2017), pancreatic cancer (Pei et al., 2017), hepatocellular carcinoma (Liu et al., 2016), and glioblastoma (Cao et al., 2016) through the induction of apoptosis (Tang et al., 2004) via the Wnt/β-catenin, MEK/Erk, or PI3K/AKT pathway. However, the effect of TSN on the Hh pathway in CRC has never been studied.

In this study, we investigated the anti-CRC activity of TSN in CRC cell lines HT29 and xenograft tumor model, and then found that TSN can inhibit CRC growth by inhibiting the Hh pathway targeting on Shh.

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

TSN was isolated from the bark of Melia toosendan Sieb. et Zucc and identified by NMR spectroscopy. The purity of TSN was determined to be more than 99% by high-performance liquid chromatography. Vismodegib was purchased from MedChemExpress, actinomycin D (ACD) was purchased from MedChemExpress, the mouse anti-SHH antibody (E-1, sc-365112) and mouse anti-SMO antibody (E-5, sc-166685) were purchased from Santa Cruz, and the rabbit anti-GLI1 antibody and mouse anti-GAPDH antibody were purchased from Beyotime.

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2.2 | Cell lines and cell culture

The HT-29 and NIH3T3 cell lines were purchased from the American Type Culture Collection (ATCC, American Type Culture Collection). All cells were routinely cultured according to the manufacturer’s instructions. Shh-Light II cells are NIH-3T3 cells, which were transfected with GLI-dependent Firly Luciferase and SV40-renilla Luciferase reporters.

2.3 | Dual-luciferase assays

Cells transfected with the respective luciferase plasmids and Renilla-TK construct were seeded into 48-well plates. After various treatments were performed as indicated, the luciferase activities were detected using a dual-luciferase assay kit according to the manufacturer’s instructions (Promega) with a luminometer. The firefly luciferase values were normalized to the Renilla luciferase values.

2.4 | CCK-8 assay

We used a CCK-8 assay to determine the proliferation ability of HT-29 cells. HT-29 cells were cultured and seeded into each well of 96-well plates (8 × 10⁴ cells in 100 μl/well). After incubation for 24 h in a humidified 5% CO₂ incubator at 37°C, DMSO or the test compounds at different concentrations (100 μl/well) were added to each well for 24 h. Cells in the wells were incubated with fresh RPMI 1640 medium supplemented with 10 μl/well CCK-8 working solution for 2 h at 37°C in 5% CO₂. Then, we measured the OD values at 450 nm using a microplate reader.

2.5 | Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from TSN-treated or untreated HT-29 cells using TRizol reagent (Tiangen) following the manufacturer’s protocol. A FastKing cDNA First-Strand Synthesis Kit and SuperReal PreMix Color (SYBR Green) Kit were purchased from Shanghai Sango, and the sequences were as follows:

- GAPDH-F 5′-GAGTCAACGGATTGGTCGT-3′,
- GAPDH-R 5′-GACAAGCTTCCGTCTCAG-3′,
- SMO-F 5′-GAACTGCGCTTGGTCTGA-3′,
- SMO-R 5′-GACGGTAGCGATCCAGTT-3′,
- GLI1-F 5′-AGAGCTGCGACCAATACAG-3′,
- GLI1-R 5′-ATGGCCGGAGTTGATGTA-3′,
- SHH-F 5′-GCCAGATGCTGCTGCTG-3′,
- SHH-R 5′-CCCTTCACCCCATCCGCTG-3′.
2.6 | Western blot analysis

After treatment with different concentrations of TSN for 24 h, HT-29 cells were lysed with RIPA lysis buffer containing a protease inhibitor (100×) for 1 h on ice. Cell lysates were collected and were then centrifuged for 30 min at 13,000 rpm. The protein concentrations in the lysates were quantified with a BCA kit (Beyotime), and proteins were then denatured at 100°C for 5 min in 5× SDS loading buffer. Equal amounts of proteins were loaded and separated by 8% SDS-PAGE. Proteins were transferred onto polyvinylidene fluoride membranes (Immobilon-P Transfer Membranes, USA), blocked with 5% skim milk at room temperature for 2 h and incubated at 4°C overnight with various primary antibodies. Finally, membranes were coincubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 2 h and visualized with a chemiluminescence imaging system (Amersham ImageQuant 800). Protein expression levels were semiquantitatively analyzed with ImageJ software (National Institutes of Health).

2.7 | Cellular thermal shift assay (CETSA)

CETSA was performed to confirm the interaction between SHH and TSN in vitro by western blot analysis. The CETSA technique is based on ligand-induced stabilization of target proteins. In brief, HT29 cells cultured to 90% confluence in six-well plates were treated with medium containing DMSO or TSN (1 µM) for 12 h. After treatment, cells were isolated with trypsin, collected by centrifugation (1000 rpm, 5 min), and resuspended in phosphate-buffered saline (PBS). The cell suspension was divided equally into six PCR tubes and heated in a temperature gradient from 40 to 65°C over 3 min. The lysates were quantified with a BCA kit (Beyotime), and proteins were then denatured at 100°C for 5 min in 5× SDS loading buffer. Equal amounts of proteins were loaded and separated by 8% SDS-PAGE. Proteins were transferred onto polyvinylidene fluoride membranes (Immobilon-P Transfer Membranes, USA), blocked with 5% skim milk at room temperature for 2 h and incubated at 4°C overnight with various primary antibodies. Finally, membranes were coincubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 2 h and visualized with a chemiluminescence imaging system (Amersham ImageQuant 800). Protein expression levels were semiquantitatively analyzed with ImageJ software (National Institutes of Health).

2.8 | Stability test of mRNA

ACD, a transcription inhibitor, inhibits DNA transcription by binding DNA at the transcription initiation site. ACD has been used in many experiments to study the stability of mRNA (Egyhazi, 1974; Toku et al., 1983). HT29 cells cultured to 90% confluence in 24-well plates were treated with treated with either 0.1 µM actinomycin D alone or a combination of 1 µM TSN and 10 µM actinomycin D (Y. Wang et al., 2005). Total RNA was extracted at 0, 4, 8, 12, 16, and 24 h. Then, the total RNA was performed on qRT-PCR analysis.

2.9 | BODIPY-cyclopamine (BC) competitive binding assays

HEK293T cells were cultured and seeded into each well of 24-well plates (1 × 10^5 cells in 500 μl/well). At 80%–90% confluence, the cells were transfected with a Human Flag-6 × His-tagged Smo expression vector (1 μg/well) using Lipofectamine® 3000 Reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions. After 48 h, the cells were treated with 12.5 nM BC and various concentrations of the test compounds before incubation at 37°C for 2–4 h. The cells in each well were washed twice with PBS (0.2 ml/well), fixed with 4% paraformaldehyde for 15 min at room temperature, washed two more times with PBS, stained with 0.1 μg/ml DAPI solution for 15 min (0.1 ml/well), and finally observed and imaged with an IX-73 microscope (Olympus). The data are expressed as the percentage of BC incorporation compared with that observed with BC alone by ImageJ software (Gao et al., 2021; Infante et al., 2016; Zhu et al., 2019).

2.10 | Animals and in vivo tumor xenograft studies

Female BALB/c/nu/nu nude mice weighing about 18 g were obtained from Zhejiang Province Experimental Animal Center. All animal experiments were approved by the Animal Ethics and Research Committee of Zhejiang. HT29 cells (5 × 10^5) were injected subcutaneously into the right flank of mice (n = 16). When the tumor volume reached around 50 mm3, mice were randomly divided into two groups: control (mice receiving saline; n = 6) and TSN (1 mg/kg; n = 6).

The saline and TSN were intraperitoneally given once daily for 20 days. When the treatment began, the mean tumor volumes were calculated every day with a caliper, using the formula: volume = (length × width^2)/2. The mice were killed 24 h after the final dose and tumors were resected aseptically for weight and volume calculation. Besides, the tumors were fixed and subjected to immuno-histochemical analysis.

2.11 | Statistical analysis

Data are presented as means ± SDs. Student’s t-test or one-way ANOVA was used for statistical analysis to compare the different groups in this study. Differences with p ≤ .05 were considered statistically significant.

3 | RESULTS

3.1 | TSN inhibited the Hh pathway

To investigate the effect of TSN on Hh signaling pathway activity, we first evaluated its activity by dual luciferase reporter assays using a reporter cell line, Shh-Light II, which is an NIH 3T3 cell line containing a stably integrated Gli-luciferase reporter. After treatment with varying concentrations of TSN ranging from 0.156 to 10 nM, the firefly luciferase intensities, which correlated with the inhibitory activity of Hh, were initially measured (Figure 1). Firefly luciferase intensities were then normalized to the corresponding intensities of Renilla luciferase, which was stably cotransfected into cells to
evaluate the transfection efficiency and exclude false positive results caused by general cytotoxicity. The bioactivity results demonstrated that TSN displayed better inhibitory activity, with an IC\textsubscript{50} value of 0.827 nM. Furthermore, this result demonstrated that the anticancer activity of TSN is closely related to the Hh pathway.

3.2 | TSN inhibited CRC cell growth

To explore the effects of TSN on HT-29 CRC cells, the antiproliferative activity of TSN was evaluated. The HT-29 cell line was incubated for 24 h after treatment with different concentrations of TSN, and CCK-8 assays were then carried out. Figure 2 shows that the viability of TSN-treated cells decreased in a concentration-dependent manner and that the IC\textsubscript{50} value of TSN in HT-29 cells was 0.346 µM.

3.3 | TSN regulated the mRNA levels of Hh signaling pathway components in HT-29 cells

To explore the mechanism by which TSN inhibits the proliferation of HT-29 cells through the Hh signaling pathway, the mRNA levels of related Hh signaling pathway components were examined by qRT-PCR. In response to treatment with TSN for 24 h at concentrations ranging from 0.1 to 3 µM, the mRNA levels in the Hh signaling pathway were decreased in a dose-dependent manner (Figure 3). These results suggest that TSN might suppress HT-29 cell growth by inhibiting Hh signaling pathways.

3.4 | TSN suppressed the expression of the corresponding proteins in the Hh signaling pathway

Having demonstrated that TSN-induced inhibition of HT29 colon cancer cell growth is closely related to the Hh pathway via regulation of the mRNA levels of the key signaling components, we then performed western blot analysis to investigate the expression levels
of the corresponding proteins. TSN significantly inhibited the protein expression of SHH, SMO, and GLI1 in a concentration-dependent manner (Figure 4).

3.5 | TSN inhibited Hh pathway signaling at the level of SHH

Our previous experiments showed that the inhibitory effect of TSN on HT-29 cell growth was closely related to the Hh pathway, with significantly altered regulation of SHH expression. We thus speculated that TSN inhibits the canonical Hh pathway by downregulating SHH. To further explore the mechanism which TSN inhibits HT29 cell growth, we conducted CETSA, analyzed mRNA half-lives after inhibition of transcription, and performed an assay of competitive binding to SMO to verify the target of TSN.

3.5.1 | TSN-SHH binding was measured by CETSA

CETSA, which is based on ligand-induced stabilization of target proteins, was carried out to confirm the interaction between SHH and TSN in vitro by western blot analysis (Langebäck et al., 2019; Li et al., 2018). The thermal stability of SHH in HT29 cells was tested in the temperature range of 40–65°C after exposure to TSN for 12 h. The control cells were treated with DMSO. SHH was still clearly detectable in cells exposed to TSN but not in control cells (Figure 5a,b). Thus, the thermal stability of SHH in TSN-pretreated cells was higher than that in control cells. Collectively, these results showed the specific binding of TSN to SHH in HT29 cells.

3.5.2 | TSN reduces the stability of SHH mRNA

We evaluated the effect of TSN on the stability of SHH mRNA in HT-29 colon cancer cells by indirectly analyzing the mRNA half-life after inhibition of transcription with ACD. Treatment with TSN obviously decreased the SHH mRNA half-life (Figure 6), suggesting that TSN decreases the SHH mRNA level in CRC cells by promoting its degradation (Shen et al., 2021).

3.5.3 | Competitive binding ability of TSN to SMO

To determine whether the inhibitory potency of TSN against the Hh pathway is due to its targeting of SMO, we used BC, a cyclopamine derivative with a fluorescent label, to analyze the direct interaction of TSN with SMO. First, HEK293T cells were transfected with the wild-type SMO expression vector. The binding ability of TSN to SMO was tested at concentrations of 0.1, 0.3, 1, and 3 μM, based on the effective concentration determined in the previous experiment and the concentrations that were

![Figure 4](image-url)  
**Figure 4** HT-29 cells were stimulated with TSN for 24 h, and the protein expression levels of SHH, SMO, and GLI1 were determined using western blot analysis. Each treatment was performed in triplicate. The data are shown as the mean ± SD values. *p < .05, **p < .01, and ***p < .001 compared with the control group. TSN, toosendanin.
proven to be noncytotoxic considering the results of the MTT assay in HEK 293T cells. The fluorescence intensity associated with BC binding to SMO was used to evaluate the ability of TSN to act on SMO. HEK293T cells overexpressing SMO and BC were used as negative controls. Compared with the findings in the negative control and positive drug control (vismodegib 500 nM) cells, the findings in the TSN-treated cells indicated that TSN could not competitively bind with SMO (Figure 7). These results suggest that TSN does not directly affect SMO.

3.6 | Effects of TSN administration on xenograft tumor growth

To further evaluate the in vivo antitumor effects of TSN, a nude mouse xenograft model was used for our animal study. HT29 cells were subcutaneously inoculated into nude mice. Mice were injected with vehicle, control (mice receiving saline; \( n = 6 \), i.p.), TSN (1 mg/kg; \( n = 6 \), i.p.) for 20 days (S. Zhang et al., 2019). As shown in Figure 8, TSN significantly suppressed the tumor weight of the HT29 xenografts compared with control, which demonstrates that TSN could effectively inhibit the growth of CRC cells in a xenograft model. At the same time, the tumor tissues were subjected to hematoxylin and eosin staining and immunohistochemical analysis. The protein expression level of SHH in animal groups treated with TSN was significantly lower than those in other groups (Figure 8). Thus, TSN strongly sensitizes CRC cells towards SHH expression in vivo.

4 | DISCUSSION

Colon cancer is one of the most common malignancies worldwide. Its pathogenesis is complex and requires accumulated alterations in multiple genes and pathways. Previous studies indicated that Wnt, PI3K/Akt, Hedgehog, ErbB, Notch, NF-κB, and MAPK can promote the carcinogenesis of CRC. BMP, AMPK, and Hippo can inhibit the development and progression of CRC (Alvarez-Medina et al., 2008; Irshad et al., 2017; Pernicova & Korbonits, 2014; Williams et al., 2015a). However, RHOA and JNK may play dual roles in CRC (Figure 9). The Hh signaling pathway plays multiple roles in the formation of CRC. Activation of the Hh signaling pathway has also
been reported to be associated with Gli1-induced lymphangiogenesis and tumor cell regeneration in CRC, which is associated with metastatic capacity and resistance to CRC chemotherapy. Studies have shown that the formation of CRC is positively correlated with high expression of SHH in the Hh pathway (Bian et al., 2007; Tiwari et al., 2018a). In addition, SHH promotes the progression from adenoma to adenocarcinoma. A large number of studies have also shown that the downstream components of the Hh signaling pathway are key to the pathogenesis of CRC, and the synergistic effect of Smo and Gli plays the most important role in Hh regulation of CRC. Moreover, the interactions among these pathways are precise and complicated. These complexities also lead to difficulties in drug treatment of CRC. Chemotherapy, such as fluoropyrimidine (5-FU) based, is associated with certain limitations, such as existing systemic toxicity, unsatisfying response rate, unpredictable innate and acquired resistance, and low tumor-specific selectivity (Vera et al., 2015). Meanwhile, only a few pathways in which experimentally identified targeted agents can be proved to be efficient in clinical studies, and a large group of targeted drugs remain in preclinical status or Phase I trials (Krishnamurthy & Kurzrock, 2018; Tiwari et al., 2018b). To solve this problem, on the one hand, deeper research on the mechanism of known anti-CRC compounds is needed; on the other hand, it is very necessary to find and discover new products.

Natural bioactive products may be key resources for new drug candidates. TSN, a naturally occurring triterpenoid saponin derived from the bark or fruit of Melia toosendan Sieb. et Zucc, was shown to have promising antitumor efficacy both in vitro and in vivo. Two targets of TSN on CRC have been previously reported. Wang's studies on TSN in colon cancer prove that TSN acted on the κ-opioid receptor and inhibited its activity, thereby inhibiting Wnt/β-catenin signaling and inducing CRC cell apoptosis. At the same time, Wang's studies proved that TSN inhibited the activity of β-catenin through suppressing p-AKT, activating GSK-3β, and then inducing degradation of β-catenin (G. Wang et al., 2015; H. Wang et al., 2020). These two studies suggest that the anti-CRC mechanism and target of TSN may not be unique. As we introduced, the Hh signaling pathway plays an important role in CRC, and there are no reports about TSN on the Hh signaling pathway at present. Therefore, in this experiment, we studied the regulation of TSN on the Hh signaling pathway in CRC cell line HT29 (Figure 10).

In our study, we first used a screening approach in Shh-Light II cells to demonstrate that TSN can regulate the Hh pathway. Based on these findings, we speculated that the anticancer mechanisms of TSN are related to the Hh pathway, a previously unreported relationship. Our data obviously suggested that TSN can suppress the growth of HT29 CRC cells and revealed that TSN regulates the mRNA and protein levels of the Hh signaling pathway components in HT-29.
Therefore, we determined that the inhibitory effect of TSN on CRC cell growth was closely related to the Hh pathway. Our data suggested that among the evaluated proteins, Shh showed the most obvious decreasing trend in expression under TSN treatment. Shh ligand is a type of Hh ligand and is the initial point of the Hh signaling pathway. Therefore, according to the results of western blot and qRT-PCR, we inferred that TSN inhibited the canonical Hh signaling pathway by acting on the upstream part of the pathway and then regulated the whole way step by step (Salaritabar et al., 2019). Therefore, we hypothesized that Shh may be a target of Hh pathway regulation and further carried out CETSA to prove our hypothesis. CETSA, a biophysical assay that allows the measurement of target engagement (TE) in intact cells and tissues can directly assess drug binding at the target protein level (the protein reports) by applying the critical heating step while cells are still intact and the target protein is in its proper cellular environment. The CETSA results suggested that TSN can bind to Shh directly. Moreover, the BC competitive binding assay and the SHH mRNA stability assay also helped us to confirm that TSN inhibits CRC cell growth through the Hh pathway by targeting Shh. Many studies have proved that Shh is expressed in CSCs. And tumor masses showed a positive correlation between Shh overexpression and CRC tumorigenesis (Douard et al., 2006; H. Wang et al., 2012). The results of the clinical trial conducted by Yoshikawa indicated that Shh can contribute to the process of adenoma-adenocarcinoma aggravation and induce hyperplastic polyps (Yoshikawa et al., 2009). It is a very significant discovery that TSN might be one kind of Shh inhibitor. The same effects of TSN on xenograft tumor growth were investigated in vivo. During the whole xenograft tumor growth period, the tumor volume was measured. The average weight, volume of the finally resected tumors, and the expression of shh in the TSN-treated groups were significantly lower than those of the control group. This result strongly suggested that TSN administration inhibited CRC growth in vivo.

**FIGURE 8** Inhibition of the growth of xenograft tumors derived from HT29 cells by TSN administration. (a) The image shows the difference of tumor volume between the TSN-treated groups and the control group on Day 20. (b) The differences of tumor volume between the TSN-treated groups and the control group were statistically significant. (c) The differences of tumor weight between the TSN-treated groups and the control group were statistically significant (*p < .05). (d) After treatment, the tumors were removed and subjected to hematoxylin and eosin staining analysis. TSN, toosendanin.

**FIGURE 9** Pathways offering potential sites for targeted therapy.

![Pathways offering potential sites for targeted therapy](image)
Collectively, the results of the current study indicated that TSN might exert its anti-CRC effects by targeting on SHH in the Hh pathway. These findings highlight a potentially valuable application for TSN in treating CRC. However, whether TSN could still regulate other multipathways which need to validate and further investigate.

**AUTHOR CONTRIBUTIONS**

Meng Zhang: experiments, investigation, methodology, software, formal analysis, drafted the first version of the manuscript and revised; Zhongyi Tao, Lijuan Gao, Wenkang Huang, and Shifang Xu: investigation, methodology; Xiaoyu Li and Yiping Ye: experiments design. Fengyang Chen: revised the manuscript. All authors read and approved the final manuscript.

**ACKNOWLEDGMENTS**

This study was supported by the Zhejiang Provincial Natural Science Foundation of China (Grant No. LGF20H300001) and the National Natural Science Foundation of China (Grant No. 81803404).

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**DATA AVAILABILITY STATEMENT**

All authors ensure that all data and materials as well as software applications and custom code support their published claims and comply with field standards.

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**REFERENCES**

Alvarez-Medina, R., Cayuso, J., Okubo, T., Takada, S., & Marti, E. (2008). Wnt canonical pathway restricts graded Shh/Gli patterning activity through the regulation of Gli3 expression. *Development*, 135(2), 237–247. [https://doi.org/10.1242/dev.012054](https://doi.org/10.1242/dev.012054)

Berman, D. M., Karhadkar, S. S., Maitra, A., Montes De Oca, R., Gerstenblith, M. R., Briggs, K., Parker, A. R., Shimada, Y., Eshleman, J. R., Watkins, D. N., & Beachy, P. A. (2003). Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. *Nature*, 425(6960), 846–851. [https://doi.org/10.1038/nature01972](https://doi.org/10.1038/nature01972)

Bian, Y. H., Huang, S. H., Yang, L., Ma, X. L., Xie, J. W., & Zhang, H. W. (2007). Sonic Hedgehog-Gli1 pathway in colorectal adenocarcinomas. *World...
model. Medical Science Monitor, 21, 2316–2321. https://doi.org/10.12659/MSM.893590

Tang, M. Z., Wang, Z. F., & Shi, Y. L. (2004). Involvement of cytochrome c release and caspase activation in toosendanin-induced PC12 cell apoptosis. Toxicology, 201(1-3), 31–38. https://doi.org/10.1016/j.tox.2004.03.023

Tiwari, A., Saraf, S., Verma, A., Panda, P. K., & Jain, S. K. (2018a). Novel targeting approaches and signaling pathways of colorectal cancer: an insight. World Journal of Gastroenterology, 24(39), 4428–4435. https://doi.org/10.3748/wjg.v24.i39.4428

Tiwari, A., Saraf, S., Verma, A., Panda, P. K., & Jain, S. K. (2018b). Novel targeting approaches and signaling pathways of colorectal cancer: an insight. World Journal of Gastroenterology, 24(39), 4428–4435. https://doi.org/10.3748/wjg.v24.i39.4428

Toku, S., Nabeshima, Y., & Ogata, K. (1983). Binding of actinomycin D to mRNA in vivo and in vitro. Journal of Biochemistry, 93(2), 361–366. https://doi.org/10.1093/oxfordjournals.jbchem.a134188

Varnat, F., Duquet, A., Malerba, M., Zbinden, M., Mas, C., Gervaz, P., & Ruizi Altaba, A. (2009). Human colon cancer epithelial cells harbour active HEDGEHOGL-GLI signalling that is essential for tumour growth, recurrence, metastasis and stem cell survival and expansion. EMBO Molecular Medicine, 1(6-7), 338–351. https://doi.org/10.1002/emmm.200900039

Vera, R., Alonso, V., Gállego, J., González, E., Guillén-Ponce, C., Pericay, C., Rivera, F., Safont, M. J., & Valladares-Ayebes, M. (2015). Current controversies in the management of metastatic colorectal cancer. Cancer Chemotherapy and Pharmacology, 76(4), 659–677. https://doi.org/10.1007/s00280-015-2808-6

Wang, G., Feng, C. C., Chu, S. J., Zhang, R., Lu, Y. M., Zhu, J. S., & Zhang, J. (2015). Toosendanin inhibits growth and induces apoptosis in colorectal cancer cells through suppression of AKT/GSK-3beta/beta-catenin pathway. International Journal of Oncology, 47(5), 1767–1774. https://doi.org/10.3892/ijo.2015.3157

Wang, H., Ke, F., & Zheng, J. (2014). Hedgehog-glioma-associated oncogene homolog-1 signaling in colon cancer cells and its role in the celecoxib-mediated anti-cancer effect. Oncology Letters, 8(5), 2203–2208. https://doi.org/10.3892/ol.2014.2439

Wang, H., Li, Y. Y., Wu, Y. Y., & Nie, Y. Q. (2012). Expression and clinical significance of hedgehog signaling related components in colorectal cancer. Asian Pacific Journal of Cancer Prevention, 13(5), 2319–2324. https://doi.org/10.7314/apjcp.2012.13.5.2319

Wang, H., Wen, C., Chen, S., Wang, F., He, L., Li, W., Zhou, Q., Yu, W. K., Huang, L., Chen, J., Liu, R., Li, W., Yang, X., & Liu, H. (2020). Toosendanin-induced apoptosis in colorectal cancer cells is associated with the kappa-opioid receptor/beta-catenin signaling axis. Biochemical Pharmacology, 177, 114014. https://doi.org/10.1016/j.bcp.2020.114014

Wang, Y., Hacker, A., Murray-Stewart, T., Fleischer, J. G., Woster, P. M., & Casero, R. A. Jr. (2005). Induction of human spermine oxidase SMO (PACH1) is regulated at the levels of new mRNA synthesis, mRNA stabilization and newly synthesized protein. Biochemical Journal, 386(Pt 3), 543–547. https://doi.org/10.1042/BJ20041084

Williams, C. S., Bernard, J. K., Demory Beckler, M., Almohazey, D., Washington, M. K., Smith, J. J., & Frey, M. R. (2015a). ERBB4 is over-expressed in human colon cancer and enhances cellular transformation. Carcinogenesis, 36(7), 710–718. https://doi.org/10.1093/carcin/bgv049

Williams, C. S., Bernard, J. K., Demory Beckler, M., Almohazey, D., Washington, M. K., Smith, J. J., & Frey, M. R. (2015b). ERBB4 is over-expressed in human colon cancer and enhances cellular transformation. Carcinogenesis, 36(7), 710–718. https://doi.org/10.1093/carcin/bgv049

Wu, J. Y., Xu, X. F., Xu, L., Niu, P. Q., Wang, F., Hu, G. Y., Wang, X. P., & Guo, C. Y. (2011). Cyclopamine blocked the growth of colorectal cancer SW116 cells by modulating some target genes of GLI1 in vitro. Hepato-gastroenterology, 58(110-111), 1511–1518. https://doi.org/10.5754/hge10765

Yoshikawa, K., Shimada, M., Miyamoto, H., Higashijima, J., Miyatani, T., Nishioka, M., Kurita, N., Iwata, T., & Uehara, H. (2009). Sonic hedgehog relates to colorectal carcinogenesis. Journal of Gastroenterology, 44(11), 1113–1117. https://doi.org/10.1007/s00535-009-0110-2

Zhang, S., Cao, L., Wang, Z. R., Li, Z., & Ma, J. (2019). Anti-cancer effect of toosendanin and its underlying mechanisms. Journal of Asian Natural Products Research, 21(3), 270–283. https://doi.org/10.1080/10286020.2018.1451516

Zhang, T., Li, J., Yin, F., Lin, B., Wang, Z., Xu, J., Wang, H., Zuo, D., Wang, G., Hua, Y., & Cai, Z. (2017). Toosendanin demonstrates promising antitumor efficacy in osteosarcoma by targeting STAT3. Oncogene, 36(47), 6627–6639. https://doi.org/10.1038/onc.2017.270

Zhou, Q., Wu, X., Wen, C., Wang, H., Wang, H., Liu, H., & Peng, J. (2018). Toosendanin induces caspase-dependent apoptosis through the p38 MAPK pathway in human gastric cancer cells. Biochemical and Biophysical Research Communications, 505(1), 261–266. https://doi.org/10.1016/j.bbrc.2018.09.093

Zhu, M., Wang, H., Wang, C., Fang, Y., Zhu, T., Zhao, W., Dong, X., & Zhang, X. (2019). L-4, a well-tolerated and orally active inhibitor of Hedgehog pathway, exhibited potent anti-tumor effects against medulloblastoma in vitro and in vivo. Frontiers in Pharmacology, 10, 89. https://doi.org/10.3389/fphar.2019.00089

How to cite this article: Zhang, M., Tao, Z., Gao, L., Chen, F., Ye, Y., Xu, S., Huang, W., & Li, X. (2022). Toosendanin inhibits colorectal cancer cell growth through the Hedgehog pathway by targeting Shh. Drug Development Research, 83, 1201–1211. https://doi.org/10.1002/ddr.21951