Umbelliprenin isolated from *Ferula sinkiangensis* inhibits tumor growth and migration through the disturbance of Wnt signaling pathway in gastric cancer

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Abstract

The traditional herb medicine Ferula sinkiangensis K. M. Shen (F. sinkiangensis) has been used to treat stomach disorders in Xinjiang District for centuries. Umbelliprenin is the effective component isolated from F. sinkiangensis which is particularly found in plants of the family Ferula. We previously reported the promising effects of Umbelliprenin against gastric cancer cells, but its anti-migration effect remained unknown. Here we investigated the anti-migration effect and mechanism of Umbelliprenin in human gastric cancer cells. In SRB assay, Umbelliprenin showed cytotoxic activities in the gastric cancer cell lines AGS and BGC-823 in a dose-and-time-dependent manner, while it showed lower cytotoxic activity in the normal gastric epithelium cell line GES-1. During transwell, scratch and colony assays, the migration of tumor cells was inhibited by Umbelliprenin treatment. The expression levels of the Wnt-associated signaling pathway proteins were analyzed with western blots, and the results showed that Umbelliprenin decreased the expression levels of proteins of the Wnt signalling pathway, such as Wnt-2, β-catenin, GSK-3β, p-GSK-3β, Survivin and c-myc. The translocation of β-catenin to the nucleus was also inhibited by Umbelliprenin treatment. In TCF reporter assay, the transcriptional activity of T-cell factor/lymphoid enhancer factor (TCF/LEF) was decreased after Umbelliprenin treatment. The in vivo results suggested that Umbelliprenin induced little to no harm in the lung, heart and kidney. Overall, these data provided evidence that Umbelliprenin may inhibit the growth, invasion and migration of gastric cancer cells by disturbing the Wnt signaling.
Keywords: Umbelliprenin; Gastric Cancer; Wnt signaling; Metastasis; Ferula sinkiangensis K. M. Shen;

1. Introduction:

Gastric cancer is the fourth most common cancer worldwide and is one of the most prevalent cancers among men in China [Kim et al., 2015; Zheng et al., 2016], which may be related to their preference for pickled food and red meat [Zeng et al., 2015]. The clinical outcome of gastrointestinal cancer surgery is limited because of the high rates of postoperative complications, such as the systemic inflammatory response [Climent et al., 2015], which may increase tumor recurrence after surgery [Bohle et al., 2010]. Therefore, studies have focused on raising the overall survival rate for gastric cancer [Cho et al., 2016]. However, only trastuzumab and ramucirumab have been approved for the treatment of advanced gastric cancer as targeted monoclonal antibodies [Roviello et al., 2016], suggesting that less toxic and more effective therapeutic options are necessary.

Metastasis is the main cause of death in cancer patients [Tang et al., 2014]. The invasion of cancer cells is affected and modulated by many biological molecules and signaling pathways. Growing evidence indicates that there are abnormalities in the Wnt pathway in human gastric cancer [Chiurillo, 2015]. In the absence of Wnt signals, β-catenin in the cytosol is continuously degraded. On the contrary, in the presence of Wnt signals, the level of β-catenin increases, and then, β-catenin
accumulates in the cytosol and subsequently translocates to the nucleus and, binds to the T-cell factor (TCF)/lymphoid enhancer factor (LEF). These transcription factors regulate the expression of specific downstream target genes such as c-myc and Survivin, which are involved in oncogenesis [Chen et al., 2001; Dong et al., 2015]. Therefore, the components of the Wnt signalling pathway could be good molecular targets for gastric cancer therapy.

Natural products have been used as traditional medicines for gastric cancer therapy [Yang et al., 2013]. Ferula sinkiangensis K. M. Shen is a traditional folk medicine, which has been used for treating stomach disorders in Xinjiang District since the Tang Dynasty. Umbelliprenin is an effective component of F. sinkiangensis and exhibits anti-cancer effects in many cancer cells lines [Ziai et al., 2012; Mousavi et al., 2015]. Our previous studies have first explored the growth inhibition effect of Umbelliprenin in gastric cancer cells: this compound could induce apoptosis and cell cycle arrest [Zhang et al., 2015]. In the present report, we reported that Umbelliprenin can inhibit tumor growth and migration, and that the underlying anticancer mechanism of Umbelliprenin is associated with the Wnt signal pathway.

2. Material and methods

2.1 Reagents and antibodies

Dulbecco’s Modified Eagle’s Medium (DMEM), Ham’s F12 medium, trypsin, penicillin, streptomycin and fetal bovine serum (FBS) were purchased from Gibco (CA, USA). Sulforhodamine B (SRB) and Dimethyl Sulphoxide (DMSO) were
purchased from Sigma-Aldrich (MO, USA). Transwell systems were purchased from Corning (NY, USA). Matrigel basement membrane matrix was bought from BD Biosciences (NJ, USA). M50 Super 8x TOPFlash (12457), M51 Super 8x FOPFlash (TOPFlash mutant) (12457) and pRL-SV40P (27163) were obtain from Addgene. Lipofectamine 2000 was bought from Thermo (MA, USA). The dual luciferase reporter assay kit was obtained from KeyGEN Biotech (Jiangsu, China). The nuclear and cytoplasmic protein extraction kit was purchased from Beyotime Biotechnology (Jiangsu, China). Antibodies against Wnt-2, GSK-3β, c-myc, β-catenin, p-GSK-3β, Survivin, Lamin B and β-actin were purchased from Cell Signaling Technology (MA, USA). The cECL Western Blot Kit was obtained from CoWin Biotech (Beijing, China). All the chemical reagents were of the highest grade.

2.2 Cell culture and compounds

The human gastric carcinoma cell line AGS was cultured in Ham’s F12 medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% CO₂. The human normal gastric epithelial cell line GES-1 and human gastric cancer cell line BGC-823 were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin under the same conditions. Cells were passaged at least three times before being used in experiments. Umbelliprenin was obtained from the seeds of F. sinkiangensis as previously described [Zhang et al., 2015]. Umbelliprenin was dissolved as stock solutions in dimethyl sulfoxide that were diluted with medium prior to use so that the final concentration of DMSO was
less than 0.1% (v/v).

2.3 Animals

Six-week-old male BALB/c nude mice were obtained from the Vital River Laboratories (Beijing, China) and maintained in a 12 h light/dark cycle environment (25 ± 2°C) where they received and food ad libitum. The protocol for the animal experiments was approved by the Animal Ethics Committee at the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences.

2.4 Cell viability assay

The Sulforhodamine B (SRB) assay was used to determine cell viability. Cells were seeded in 96-well plates in triplicate and cultured for 24 h at 37°C. Then, the cells were treated with Umbelliprenin in various concentrations (0, 3.125, 6.25, 12.5, 25, 50 μM). After the 24 h treatment, 50 μL of cold TCA was added to fix the cells for 1 h at 4°C. The plates were washed with water and air-dried. One-hundred microlitres of 0.4% (w/v) SRB was added and cells were stained for 15 min. After washing 4 times with acetic acid, 100 μL of Tris-base was added for 10 min, while shaking. The absorbance was measured at 540 nm using a Microplate Reader (BioTek, USA). % Cytotoxicity= (Control – Experimental) / Control*100%.

2.5 Colony Formation Assay

The colony formation assay was used to evaluate the anchorage-independent growth of gastric cancer cells. Cells were seeded in 6-well plates and treated with
Umbelliprenin (0, 6, 12, 24 μM for AGS cells and 0, 12.5, 25, 50 μM for BGC-823 cells) for 10 days. The tumor colonies were observed counted using a microscope. Then, the colonies were stained with crystal violet (1 mg/mL), and colonies larger than 200 μm were counted.

2.6 Wound healing Assay

Cell motility was detected using the wound healing assay. Cells were cultured in a 24-well plate to nearly 90% confluence. The monolayers were then carefully scratched using a sterile 200-μL pipette tip with a constant width. The cells were washed with PBS and treated with Umbelliprenin (IC_{50} values were used in both AGS and BGC-823 cells). The cells were photographed at 0, 24, 36 and 48 h after treatment to observe the distances that the cells had migrated. The cell motility was calculated as follows: Cell motility = (distance at 0 h - distance at 24, 36 or 48 h) / distance at 0 h*100%.

2.7 Transwell-migration / invasion assays

Cell migration was analyzed in a 24-well transwell plate with 8-μm pore size polyvinylidene filter membrane. Cells were seeded into the upper chamber (1×10^5) in serum-free Ham’s F12 medium and treated with Umbelliprenin (0, 6, 12, 24 μM for AGS cells and 0, 12.5, 25, 50 μM for BGC-823 cells). Ham’s F12 medium with 10% serum was added to the lower chamber. After incubation for 24 h, the cells on the upper side of the filter were removed with cotton swabs, and the filters were fixed.
with 4% formaldehyde for 10 min at room temperature, and stained with crystal violet for 15 min, the number of cells in five random fields of each triplicate filter was counted under the light microscope. The cell invasion assay was conducted under similar procedure, except that 80 μL of Matrigel was used to coat the upper chamber for 12 h before the cells were seeded.

2.8 β-Catenin / TCF Transcription Reporter Assay

The TCF-reporter plasmids (TOPFLASH and the negative control FOPFLASH) and Renilla luciferase (pRL-SV40P) plasmid were obtained from Addgene. Cells were seeded in 6-well plates and the reporter plasmids containing TCF binding sites (TOPFLASH, 500 ng/well) or mutant, inactive TCF binding sites (FOPFLASH, 500 ng/well) were transiently transfected into the cells for 6 hours using Lipofectamine 2000. The cells were co-transfected with the Renilla luciferase (pRL-SV40P, 5 ng/well) plasmid to normalize the transfection efficiency with the internal control. Then, the medium was renewed and different concentrations of Umbelliprenin were added. After the 24 h treatment, TCF-mediated gene transcription was expressed by the ratio of TOPFLASH: FOPFLASH luciferase activity, and each value was normalized to the Renilla luciferase activity.

2.9 Protein isolation and western blot

Fractionated nuclear and cytosolic proteins were obtained according to the manufacturer’s instructions. Briefly, cells were treated with Umbelliprenin (0, 6, 12,
24 μM for AGS cells and 0, 12.5, 25, 50 μM for BGC-823 cells) for 24 h, harvested
and washed twice with ice-cold PBS. 500 μL of cytoplasmic extract agent A and B
were added and the suspension was centrifuged at 12,000 rpm for 5 min. The
supernatant contained cytosolic proteins. The nuclear residue was mixed with 200
μL of the nuclear extract agent. The mixture was vortexed for 30 min and then
centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant contained nuclear
proteins.

To obtain whole-cell lysates, cells were lysed in lysis buffer after treatment with
Umbelliprenin (0, 6, 12, 24 μM for AGS cells and 0, 12.5, 25, 50 μM for BGC-823
cells) for 24 h and the protein concentrations were determined by the BCA method.
Protein samples were separated by SDS-PAGE and transferred onto PVDF
membranes. After blocking for 1 h in the 5% non-fat milk solution, the membranes
were incubated with the primary antibody overnight at 4°C. Then the primary
antibody was washed with TBST for 3 times and incubated with secondary antibody
for 1 h at room temperature. Protein bands were detected using ECL and the levels of
β-actin were used to ensure equal loading of proteins.

2.10 Immunofluorescence staining to detect the nuclear translocation of
β-catenin

Cells were cultured in plates and treated with Umbelliprenin (0, 6, 12, 24 μM for
AGS cells and 0, 12.5, 25, 50 μM for BGC-823 cells) for 24 h. The treated cells were
fixed with 4% paraformaldehyde for 10 min and washed twice with PBS. These cells
were blocked with 5% bovine serum albumin in PBS for 60 min, and then incubated with the primary antibody (diluted 1:200 in PBS containing 3% BSA) overnight at 4°C. After washing twice with PBS, the cells were co-incubated with the IgG PE-conjugated secondary antibody (diluted 1:400 in PBS containing 3% BSA) and DAPI for 1 h at room temperature. The cells were examined with the Image Xpress system (Molecular Devices, USA).

2.11 Safety of Umbelliprenin in tumor xenograft models

Animal experiments were conducted according to the previous description [Zhang et al., 2015]. Briefly, mice were inoculated subcutaneously with the human gastric cancer cells BGC-823 (1.0 × 10⁶) on the right flank. The mice were randomized to six groups and their conditions were observed every day. Umbelliprenin were diluted in 200 μL 0.9% NaCl solution and administered to each mouse at a dose of 10 mg/kg or 20 mg/kg twice daily for 12 days. Mice were then euthanized and the lungs, livers, hearts and kidneys were fixed and preserved in formalin for hematoxylin-eosin staining.

2.12 Statistical analysis

All data were analyzed by the IBM SPSS statistics 19 software. All tests were conducted at least three times. Statistical significance was defined as *p < 0.05 and **p < 0.01. The results were expressed as mean ± SD which represent three independent tests.
3. Results:

3.1 Umbelliprenin reduced the viability in AGS and BGC-823 human gastric cancer cells, but not in GES-1 cells.

The chemical structure of Umbelliprenin isolated from the seeds of *F. sinkiangensis* is shown in Figure 1A. Because Umbelliprenin appeared to be most effective in gastric cancer cells among other common cancer cells, we studied its anti-proliferative effects in the two human gastric cancer cell lines: AGS and BGC-823 as well as in the human gastric epithelial cell line GES-1. The IC\(_{50}\) values for the three cancer cell lines varied. The results showed that Umbelliprenin inhibited the growth in both the AGS and BGC-823 gastric cancer cell lines with an IC\(_{50}\) of 11.74 \(\mu\)M and 24.62 \(\mu\)M, respectively, the IC\(_{50}\) for AGS was lower than that for the BGC-823 cells (Figure 1C and Figure 1D). In addition, Umbelliprenin was less cytotoxic in GES-1 cells (IC\(_{50}\): 97.55 \(\mu\)M) compared with AGS and BGC-823 cells (Table 1). Based on the IC\(_{50}\) values, we chose 12 \(\mu\)M and 24 \(\mu\)M Umbelliprenin, as well as their multiples or fractions, as the concentrations for treating AGS and BGC-823 cells, respectively. Furthermore, as the colony formation ability is closely related to tumorigenesis *in vivo*, we detected the impact of Umbelliprenin on the growth of gastric cancer cells. The results showed that Umbelliprenin significantly inhibited the colony forming ability of AGS cells in a dose-dependent manner, while it was less effective in BGC-823 cells (Figure 1B). Together, these data indicate that Umbelliprenin may decrease the proliferation and tumor forming ability in AGS and BGC-823 gastric cancer cells.
3.2 The effects of Umbelliprenin on the invasion and migration in AGS and BGC-823 cells

Umbelliprenin was effective in reducing cellular migration in gastric cancer cells.

We performed a wound healing assay, and images representing the migration capability of the cells were taken at different time points, at the same site and magnification. Forty-eight hours after scratching, in the presence of Umbelliprenin at its IC$_{50}$ value, the wound was healed approximately by 77.4% and 64.2% for AGS and BGC-823 cells compared with the control group, respectively (Figure 2C). The migration assay using the transwell-migration system also showed that Umbelliprenin effectively inhibited the migration of AGS and BGC-823 cells (Figure 2A and Figure 2B). Furthermore, the inhibitory effect of Umbelliprenin on the invasion of gastric cancer cells was examined in a transwell assay using Matrigel-coated filters. Compared with the control group, fewer AGS and BGC-823 cells penetrated the filters after Umbelliprenin treatment. Additionally, Umbelliprenin decreased the invasive potential of cells in a dose-dependent manner (Figure 2A and Figure 2B). Therefore, both the migration and invasion of gastric cancer cells were significantly suppressed after Umbelliprenin treatment.

3.3 Umbelliprenin inhibited the transcriptional activity of β-catenin/TCF in the human gastric cancer cell lines AGS and BGC-823

To explore whether Umbelliprenin plays a role on the dysregulation of the
Wnt/β-catenin pathway in gastric cancer cells, we examined the transcriptional activity of β-catenin/TCF after Umbelliprenin treatment. For the purpose, we transfected the TOPFLASH or FOPFLASH plasmids into AGS and BGC-823 gastric cancer cells. The transfection efficiency was normalized with Renilla luciferase. As shown in Figure 3A and Figure 3B, after 24 h of treatment Umbelliprenin reduced the TCF-dependent luciferase activity (TOPflash) in a dose-dependent manner in both gastric cancer cell lines, while it did not change the activity of the FOPflash control plasmid. In AGS cells, the transcriptional activity of TCF was 62.93%, 31.54%, and 18.19% relative to the control group at concentrations of 6, 12 and 24 μM of Umbelliprenin, respectively (P<0.05). In BGC-823 cells, the transcriptional activity of TCF was 52.91%, 43.50%, and 21.15% relative to the control group at concentrations of 12.5, 25 and 50 μM of Umbelliprenin, respectively (P<0.05). This indicates that the transcriptional activity of β-catenin/Tcf could be inhibited by Umbelliprenin. Thus, Umbelliprenin suppresses the Wnt/β-catenin signaling pathway in the two gastric cancer cell lines.

3.4 Inhibition of nuclear translocation of β-catenin by Umbelliprenin

To determine whether Umbelliprenin suppressed β-catenin mediated transcription by interfering with the nuclear translocation of β-catenin, we investigated the β-catenin subcellular localization in Umbelliprenin -treated AGS and BGC-823 cells by immunofluorescence staining. As shown in Figure 4A, β-catenin preferentially accumulated in the nucleus in the control group. By contrast, after treatment with
Umbelliprenin for 24 h, the localization of β-catenin decreased in the nucleus but increased in the cytoplasm and at the plasma membrane. This result was confirmed by Western blot analysis in which β-catenin was increased in the cytoplasmic protein fraction and decreased in the nuclear protein fraction of the treated groups (Figure 4B). The relative expressions of cytoplasmic and nuclear proteins were shown in Figure 4C. Therefore, Umbelliprenin may mediate its effect by inhibiting the translocation of β-catenin to the nucleus.

3.5 Umbelliprenin inhibits Wnt signaling by decreasing the phosphorylation of GSK-3β and reducing the downstream effectors of Wnt signaling

Wnt signaling substantially impacts gastric tumorigenesis and prognosis [Bohle et al., 2010]. To further determine the mechanisms by which Umbelliprenin inhibits cellular proliferation, migration and invasion, we studied the Wnt signaling. The expression levels of Wnt signaling-associated proteins in both AGS and BGC-823 cells were measured by western blot. Wnt-2, β-catenin, and GSK-3β, potential modulators of Wnt signaling, as well as the downstream targets of Wnt signaling Survivin and c-myc, were significantly reduced in Umbelliprenin treated cells compared to the control group (Figure 5A and Figure 5C). The relative expressions were shown in Figure 5B and Figure 5D.

3.6 The safety of Umbelliprenin in BGC-823 tumor xenograft models

Previous studies showed that Umbelliprenin could effectively inhibit the growth of
tumor in vivo. Thus, we evaluated the safety of Umbelliprenin in BGC-823 tumor xenograft models. All mice tolerated the treatment procedure well and did not show toxic symptoms or signs. The analysis of biochemical markers for the liver, as ALT and AST, showed no significant change between the different groups (Table 2). In addition, no histological abnormality was shown in the lungs, liver, heart, and kidneys of mice between the Umbelliprenin treatment groups and control group at the end of the treatment (Figure 6). Together, these data suggest that Umbelliprenin effectively inhibits tumor growth, and does not cause obvious drug-induced adverse effects.

4. Discussion

Natural products with potential for gastric cancer treatment have received much attention. Umbelliprenin has been reported to possess the promising effect of inducing apoptosis in a variety of cancer cell types such as leukemia, breast cancer and bladder carcinoma [Mousavi et al., 2015; Gholami et al., 2013; Haghighi et al., 2014] and exhibits biological effects in multiple signal transduction pathways. For example, a study showed that Umbelliprenin could activate both the intrinsic and extrinsic pathways of apoptosis in the Jurkat T-CLL cell line, which accounted for the inhibition of cellular proliferation and apoptosis induction [Gholami et al., 2013]. Therefore, further studies on the anti-gastric cancer effects of Umbelliprenin are necessary. Our previous results showed that Umbelliprenin among seven compounds isolated from the seeds of F. sinkiangensis, exhibits the strongest antitumor effect
in vivo and in vitro. Based on these results, we performed further research on Umbelliprenin. In the present study, we demonstrated the anti-metastatic and anti-proliferative effects of Umbelliprenin on the AGS and BGC-823 gastric cancer cell lines in vitro and its safety in vivo. These results indicated that Umbelliprenin might be an effective inhibitor of tumor migration, and were obtained using wound healing, transwell migration and invasion assays. We also demonstrated that the mechanism of action of Umbelliprenin include the inhibition of the Wnt signaling pathways and the decrease of c-myc and Survivin.

The activation of the Wnt/β-catenin signaling is found approximately 30% to 50% of gastric cancer tissues and in many gastric cancer cell lines [Ooi et al., 2009]. β-catenin is a multifunctional protein that was found as an E-cadherin-binding protein involved in the regulation of cell-to-cell adhesion and works as a transcriptional regulator in the Wnt signaling pathway [Kikuchi et al., 2011]. It accumulates in the cytoplasm and translocates to the nucleus, where it behaves as transcriptional coregulatory factor by interacting with the TCF/LEF complex and activates target oncogenes, such as c-myc and Survivin [Eastman et al., 1999; Zhang et al., 2014]. Our results suggest that Umbelliprenin down-regulates Wnt, resulting in the decrease of phosphorylated GSK-3β and reduction of Wnt downstream effectors, such as β-catenin, Survivin, c-myc, MMP2 and MMP9. Furthermore, Umbelliprenin treatment caused the inhibition of the nuclear translocation of β-catenin and the reduction of the activity of the TCF-reporter. Although results suggest that Umbelliprenin interferes with the Wnt/beta-catenin signaling and therefore inhibits
the activity of the TCF reporter, reduction of the reporter activity might be partially caused the Umbelliprenin-induced migration and invasion. Our results are consistent with those previous studies showing that decreased Wnt expression is associated with decreased phosphorylation of GSK-3β; decreased expression of β-catenin, c-myc and Survivin; lower activity of the TCF reporter; and reduced migration and invasion [Luo et al., 1999].

Taken together, for the first time, we showed strong evidence that Umbelliprenin isolated from the seeds of *F. sinkiangensis* can inhibit cell growth, migration and invasion, at least in part, through the Wnt signaling pathway. Other reports have shown that the Wnt antagonists inhibit the growth of HCC cells *in vitro* and *in vivo* through inhibition of the formation of the TCF-4/β-catenin complex and its transcriptional activity and downregulation of the β-catenin/TCF-4 target genes c-myc and Survivin [Wei et al., 2010]. By contrast, we found that Umbelliprenin inhibits the Wnt pathway by decreasing nuclear translocation of β-catenin rather than disrupting the TCF-4/β-catenin complex. Therefore, treatments that combine Umbelliprenin with an antagonist of TCF-4/β-catenin may lead to promising effects on suppressing the activation of the Wnt signaling pathway.

In conclusion, our data indicate that Umbelliprenin has anti-migration effects in AGS and BGC-823 gastric cancer cells, targets the Wnt signaling pathway, and exhibits good safety during the treatment in the BGC-823 xenograft *in vivo* model: indeed, no abnormalities in regard to body weight, daily diet, liver function and histological characteristics of lung, spleen, heart, kidney and liver tissue were
observed. Therefore, Umbelliprenin could be a promising approach for gastric cancer
treatment; however, further investigations are necessary.

Conflict of interest

The authors declare that they have no competing interests

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Sciences.

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The cytotoxic activities of 7-isopentenyloxy coumarin on 5637 cells via induction of apoptosis and cell cycle arrest in G2/M stage. DARU. 22:3.


Figure Legends

Figure 1: Chemical structure and inhibition effects on cell viability of Umbelliprenin.

A. Chemical structure of Umbelliprenin isolated from the seeds of Ferula sinkiangensis. B. Colony formation assay. AGS or BGC-823 cells were grown for 10 days after incubation with different concentrations of Umbelliprenin. C. The effects of Umbelliprenin on the viability of AGS human gastric cancer cells. D. The effects of Umbelliprenin on the viabilities of human gastric cancer cells BGC-823. AGS and BGC-823 cells were exposed to various concentrations of Umbelliprenin (0, 3.125, 6.25, 12.5, 25, 50 μM) for 24 h, 48 h or 72 h, followed by the Sulforhodamine B (SRB) assay. The data represent the mean value of three independent experiments and are expressed as the mean ± SD. **p < 0.01, *p < 0.05 were considered statistically significant.

Figure 2: Umbelliprenin inhibits the migration and invasion of gastric cancer cell lines.

A. The migration or invasion of cells was measured by transwell or matrigel-coated filter transwell assays, respectively. Cells were incubated with Umbelliprenin for 24 h, and the migrated or invaded cells were fixed and stained with crystal violet. B. The migration and invasion assays data are expressed as the means ± SD and represent the mean value of three independent experiments. **p < 0.01, *p < 0.05. C. The migration
of AGS and BGC-823 cells was examined in a wound healing assay. Cells were scratched and treated with Umbelliprenin. Images were obtained using a microscope at 0 h, 24 h, 36 h and 48 h. Data are presented as the mean ± SD and represent three independent experiments. **p < 0.01, *p < 0.05 were considered statistically significant.

Figure 3: Umbelliprenin treatment decreases the activity of the TCF reporter in gastric cancer cells.

A. and B. AGS and BGC-823 cells were transfected with the TOPFLASH or FOPFLASH plasmid together with the Renilla plasmid as control. Cells were then treated with Umbelliprenin (0, 6, 12, 24 μM for AGS cells or 0, 12.5, 25, 50 μM for BGC-823 cells) for 24 hours. The luciferase activity was measured and the results were normalized. The data represent the mean value of three individual experiments. *p < 0.05 and **p < 0.01 were considered statistically significant.

Figure 4: Umbelliprenin inhibits the nuclear translocation of β-catenin in gastric cancer cells.

A. The translocation of β-catenin was examined by immunostaining. Cells were treated with UM (0, 6, 12, 24 μM for AGS cells or 0, 12.5, 25, 50 μM for BGC-823 cells) for 24 h, stained with the anti-β-catenin antibody and analysed using the Image Xpress Micro imaging system (MD, USA). B. Protein expressions were detected by western blot. After treatment with Umbelliprenin (0, 6, 12, 24 μM for AGS cells or 0,
12.5, 25, 50 μM for BGC-823 cells) for 24 h. Cellular fractionation was carried out to determine the cellular localization of β-catenin. Lamin B and β-actin were used as controls for nuclear fraction and cytoplasmic fraction, respectively. C. The relative expression of cytoplasmic and nuclear proteins was analyzed. The results obtained from a representative experiment are shown (n=3). Statistical significance was **p < 0.01. 

Figure 5: Umbelliprenin downregulates the Wnt signal pathway, and Survivin and c-myc protein expression levels.

The expression of the regulatory proteins of the Wnt signal pathway, Survivin and c-myc protein were determined by western blot. A. and C. AGS or BGC-823 cells were treated with Umbelliprenin (0, 6, 12, 24 μM for AGS cells or 0, 12.5, 25, 50 μM for BGC-823 cells) for 24 h. β-actin was used to confirm equal protein loading. B. and D. The relative expression levels of proteins are shown. All tests were performed in triplicate. *p < 0.05 and ** p<0.01 were considered statistically significant. 

Figure 6: Low toxicity of Umbelliprenin in vivo.

The results showed representative hematoxylin-eosin staining to investigate the potential toxicity of Umbelliprenin in lung, heart, kidney and liver of Umbelliprenin-treated and control mice on day 12 (n = 8).
Table 1. Cytotoxicity of Umbelliprenin isolated from the seeds of *Ferula sinkiangensis* in three cell lines.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC50 (μM)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>AGS</td>
</tr>
<tr>
<td>Umbelliprenin</td>
<td>11.74±1.33</td>
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</tbody>
</table>

<sup>a</sup> IC<sub>50</sub> is the concentration of compound causing 50% growth inhibition for each cell line after 24 h treatments. The results represent the mean values of three independent tests.
Table 2. Serum analysis for liver function (mean ± SD, n = 8)

<table>
<thead>
<tr>
<th>Group</th>
<th>AST(U/L)</th>
<th>ALT(U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>202.6±27.9</td>
<td>31.8±2.3</td>
</tr>
<tr>
<td>Umbelliprenin (10mg/kg)</td>
<td>190.4±33.8</td>
<td>30.3±4.1</td>
</tr>
<tr>
<td>Umbelliprenin (20mg/kg)</td>
<td>172.9±17.1*</td>
<td>32.1±3.1</td>
</tr>
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*p < 0.01, *p < 0.05 were considered statistically significance compared with control group. And the biochemical markers ALT and AST were shown no significance between different groups.

Blood samples were collected and centrifuged at 12,000 rpm for 15 min to obtain the serum. Liver function was evaluated based on the serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). All biochemical parameters were evaluated by an automated biochemical analyzer (Beckman).
Figure 2
Figure 4
Figure 3

(A) AGS

(B) BGC-823

Relative luciferase activity (%)

Umbelliprenin (μM)

0 6 12 24

TOP FOP

0 12.5 25 50

TOP FOP

**

Figure 3
Figure 5
Figure 6