

DCT015, a new sorafenib derivate, inhibits tumor growth and angiogenesis in gastric cancer models

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Abstract The objective of this study is to investigate antiproliferative activities against gastric cancer and anti-angiogenesis of DCT015, a novel sorafenib derivate, and potential mechanisms. The effects of DCT015 on proliferation and apoptosis in gastric cancer cells were evaluated by cytotoxicity assays, apoptosis analysis, flow cytometry analysis, and Western blotting assays. The *in vivo* antitumor effects were carried out in nude mice bearing gastric cancer. On the other hand, the anti-angiogenesis effects of DCT015 were measured by human umbilical vein endothelial cell (HUVEC) proliferation, migration, tube formation, and Western blotting analysis. The results showed that DCT015 inhibited the proliferation, induced the morphological changes of apoptosis, and increased the apoptosis percentage, as well as increased the “sub-G1” population in gastric cancer cells. DCT015 also significantly decreased the tumor volumes and tumor weights *in vivo* by oral administration. Immunohistochemistry assay demonstrated that DCT015 inhibited tumor growth and neovascularization. *In vitro* studies found that

DCT015 inhibited both MEK/ERK and PI3K/Akt signaling pathways by Western blotting assays. Moreover, DCT015 significantly inhibited VEGF-induced migration and tube formation in HUVECs. Western blotting analysis showed that DCT015 downregulated VEGF-induced VEGFR2 phosphorylation with the decreased phosphorylation of the downstream key proteins. Taken together, our findings highlight that DCT015 is a promising orally anticancer drug for treating gastric cancer.

Keywords DCT015 · Sorafenib · Proliferation · Angiogenesis · Apoptosis · Targeted therapy

Introduction

Gastric cancer (GC) is one of the most common gastrointestinal tumors and is the second leading cause of cancer-related mortality worldwide [1]. There were almost 1,000,000 new

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cases and over 720,000 deaths in 2012 [2]. Although first-line chemotherapy improved overall survival (OS) for patients with advanced gastric cancer (AGC), second-line chemotherapy with irinotecan or docetaxel added only ~1.5 months of OS compared with best supportive care [3, 4]. Some targeted drugs, including the vascular endothelial growth factor receptor (VEGFR) inhibitors (ramucirumab and apatinib) [3, 5] and the epidermal growth factor receptor (EGFR) inhibitor (trastuzumab) [6], have been approved for treating AGC. However, no standard third-line treatment can be used and the unmet medical need providing extended survival benefits results in the further investigation of targeted therapy, including tumor proliferation inhibition and anti-angiogenic strategies [7, 8]. Recently, the Ras/Raf/MEK/ERK pathway, PI3K/Akt pathway, and other signaling pathways have been approved to be closely related to gastric cancer [9–11]. Small molecular inhibitors targeting Raf, MEK, PI3K, and Akt are being investigated in clinical trials for the treatment of GC [8, 12].

Sorafenib (Fig. 1), an oral multiple kinase inhibitor, can inhibit the tumor growth by not only interrupting the Ras/Raf/MEK/ERK signaling cascade [13] but also targeting several other receptor tyrosine kinases, such as vascular VEGFR2, platelet-derived growth factor receptor (PDGFR), FLT3, and c-Kit [14]. These action mechanisms result in the broad anti-tumor activity [14]. Currently, sorafenib has been approved for the treatment of hepatocellular carcinoma (HCC), renal carcinoma, and thyroid carcinoma. The studies suggest that sorafenib inhibits tumor growth and disrupts tumor microvasculature through anti-proliferative and anti-angiogenic effects [15, 16]. Sorafenib has shown encouraging anti-tumor efficacy in a phase II clinical trial study in gastric cancer [17]. Therefore, it is anticipated to further develop sorafenib derivatives for increasing the anti-gastric cancer effect of sorafenib.

In the present study, in *in vitro* screening test, DCT015 was found to possess an interesting anti-tumor activity as a sorafenib derivative. Therefore, DCT015 is selected as a new candidate in order to further evaluate anti-tumor effects in gastric cancer. We also carried out some tests to investigate the

possible action mechanisms of DCT015 in gastric cancer and to support further anti-gastric cancer drug development of sorafenib derivatives.

Materials and methods

Drug and reagents

DCT015 and sorafenib were provided by Nanjing Luye Sike Pharmaceuticals Co., Ltd. (Fig. 1). Drugs were prepared initially as a 40-mmol/L stock solution in dimethyl sulfoxide (DMSO) and diluted in appropriate media for all *in vitro* assays. The DMSO concentration was kept below 0.05 % in the cell cultures in order to keep no detectable effects on the cells. DCT015 or sorafenib was suspended in aqueous 0.5 % carboxymethylcellulose sodium (CMC-Na) (*w/v*) and stored at 4 °C for all *in vivo* tests.

Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), and Roswell Park Memorial Institute (RPMI) 1640 medium were obtained from Gibco-BRL (Gaithersburg, MD, USA). Propidium iodide (PI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Co. (USA). The primary antibodies p-VEGFR2 (no. 3817S), PI3K (no. 4257), p-PI3K p85 (4228S), MEK (no. 8727S), p-MEK (Ser217/221) (no. 9154S), caspase-3 (no. 9665), Bax (no. 2772), Mcl-1 (no. 5453S), β -actin (no. 49702), p-Akt (Ser474) (no. 4060), Akt (no. 4691), Ki67 (8D5, no. 9449s), and CD31 (PECAM-1, no. 3528 s) were purchased from Cell Signaling Technology (Beverly, MA, USA). The primary antibodies ERK1/2 (SC-135900) and p-ERK1/2 (Thr202) (SC-101760) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibody VEGFR2 (ab39256) was obtained from Abcom Company (Cambridge, MA, USA) and Bcl-2 (p-Ser70) from Novus Biological (CO, USA). The horseradish peroxidase (HRP)-conjugated secondary antibodies were from Beyotime Biotechnology (Qingdao, China). VEGF (lot 113913012) was purchased from R&D Systems (Minneapolis, MN, USA). Matrigel Matrix (Lot 4342007) was purchased from Corning Life Sciences (Bedford, MA, USA). GT Vision III Detection System was provided by GeneTech Company Limited (Shanghai, China).

Cell lines and cell cultures

Human gastric carcinoma cell lines (AGS, BGC-823, MNK45, and SGC7901), human prostate cancer cell lines (PC3, LNCaP, and DU145), human colon cancer cell lines (HCT-116 and SW480), human melanoma cell line (A375), human cervical carcinoma cell line (SiHa), and

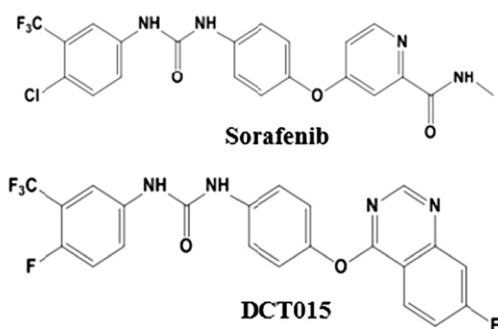


Fig. 1 The chemical structure of sorafenib and DCT015

human umbilical vein endothelial cell (HUVEC) were obtained from the Cell Culture Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. All cells were maintained in DMEM or RPMI 1640 supplemented with 10 % (v/v) heat-inactivated FBS in a humidified 5 % CO₂ atmosphere at 37 °C.

Animals

Male Balb/c nu/nu nude mice (5–6 weeks old; purchased from Vital River Laboratory Animal Technology Co., Ltd.) were used for in vivo experiments. Animals were maintained under controlled environment at 25 °C on a 12-h light/dark cycle, which was free access to food and water in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80-23). All animal protocols were in compliance with the Ethics Committee of Yantai University.

Cancer cells growth inhibition assay

To determine the cell viability of DCT015 or sorafenib on cancer cell lines, cell survival was measured by MTT assay. Cells were plated in 96-well plate with a density of 5×10^3 cells/well. After 24 h incubation, the cells were exposed to DCT015 or sorafenib at concentrations ranging from 5 to 60 μ M and cultured at 37 °C in a humidified atmosphere for 48 h. Twenty microliters of MTT (5 mg/ml) was added to each well and incubated for an additional 4 h at 37 °C. The medium was subsequently discarded, and 150 μ L of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using a Molecular Devices SpectraMax M5 (Molecular Devices, USA). The 50 % inhibitory concentration (IC₅₀) values were calculated using the GraphPad Prism v. 5.0.

Morphology analysis by Hoechst staining in AGS cells after DCT015 treatment

To identify if DCT015 or sorafenib induces cell apoptosis, we analyzed the apoptosis cells by Hoechst 33258 staining. Hoechst 33258 is a blue fluorescent dye that can penetrate the cell membrane and is commonly used in cell apoptosis detection. Briefly, AGS cells were cultured in 96-well plates with a density of 8000 cells/well and treated with DCT015 or sorafenib at different concentrations for 24 h. The cells were stained with Hoechst 33258 at room temperature for 20 min, followed by phosphate buffered saline (PBS) washing. Nuclear morphology of apoptotic cells were then visualized and analyzed using Cellomics ArrayScan II image analysis system (Cellomics, Pittsburgh, PA, USA).

Effects of DCT015 on sub-G1 cell population in AGS cells by flow cytometry analysis

Flow cytometry was used to investigate the induction of a sub-G1 cell population, a hallmark of apoptosis. For sub-G1 cell population analysis, AGS cells were incubated with DCT015 or sorafenib for 24 h. The cells were made into a single cell suspension with trypsin digestion method first. Then, the cells were harvested and washed with PBS twice and fixed in 800 μ L of ice-cold 80 % ethanol at –20 °C overnight. After being washed with PBS, the cell pellets were collected by centrifugation and re-suspended in 1 mL of hypotonic buffer (0.5 mg/mL RNase) with PI (50 mg/mL) and incubated at 37 °C in the dark for 30 min. The stained cells were analyzed by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Cell cycle distribution was detected with ModFit software (Verity Software House, Topsham, ME, USA).

Effects of DCT015 on signaling pathways in AGS or BGC-823 cells by Western blotting

Synchronized gastric cancers cells (AGS or BGC-823) were incubated with DCT015 or sorafenib at different concentrations for 48 h. Treated cells were washed twice with PBS and then lysed in a lysis buffer consisting of radioimmunoprecipitation assay (RIPA) and 1 % of phenylmethanesulfonyl fluoride (PMSF) at 4 °C for 30 min. After centrifugation at 12,000g for 10 min, the protein content of the supernatant was determined using a protein assay reagent from Beyotime Biotechnology. The samples (30 μ g of protein) were mixed with 5 \times loading buffer. The mixtures were boiled at 100 °C and were subjected to 10 or 12 % SDS-polyacrylamide gels. Proteins on the gel were electrotransferred onto polyvinylidene difluoride (PVDF) with transfer buffer. The membrane was blocked with 5 % non-fat milk in Tris-buffered saline containing 0.1 % Tween-20. The blots were incubated overnight with primary antibodies against MEK, p-MEK, ERK, p-ERK, PI3K, p-PI3K, Akt, p-Akt, Mcl-1, Bax, caspase-3, or Bcl-2. Then, blots were incubated with 1:2000 dilution of HRP-conjugated secondary antibody and washed three times with PBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence detection kit. The densities of the bands were quantified using ImageJ software.

HUVEC proliferation assay

We used the MTT method to test the anti-proliferation effect of DCT015 or sorafenib on HUVECs. HUVECs were seeded in 96-well plates with a density of 6×10^3

cells per well overnight. The medium was replaced by DMEM medium (10 % FBS) containing DCT015 or sorafenib at concentrations ranging from 0.3 to 30 μM . After 24 h, MTT solution (5 mg/mL) was added to each well, and incubation continued for 4 h. DMSO was added to dissolve the MTT formazan product, and the absorbance was measured at 570 nm using a Molecular Devices SpectraMax M5.

Migration assay in HUVECs

Cell migration assay was performed using scratch wound healing assay. Briefly, HUVECs (3×10^4 cells/well) were seeded in six-well plates. The cells grown to 75–80 % confluence were treated with serum starvation overnight to achieve synchronization. A straight line was scratched across the culture with a pipette tip. Following scratching, cells were washed with PBS three times and were treated with DCT015 or sorafenib in the presence of VEGF (30 ng/mL). The plate was incubated at 37 °C and 5 % CO_2 for 24 h. Images were taken by a computer-assisted microscope (Leica, DMIRB, Germany) at $\times 100$ magnification. The wound width was determined with Image-Pro Plus software, and the percentage of inhibition was expressed using untreated cells at 100 %.

Tube formation assay in HUVECs

The formation of capillary tube-like structures by HUVECs was evaluated on a 96-well plate coated with Matrigel. Matrigel was thawed at 4 °C overnight. Sixty microliters of precooled Matrigel was transferred to a 96-well plate and left to polymerize at 37 °C for 1 h. HUVECs (1×10^4 cells/well) were seeded in 2 % serum-complete medium. Drugs were diluted to the required concentration in cell culture media. HUVECs were treated with DCT015 or sorafenib in the presence of VEGF and incubated for 24 h. The results were recorded by photographing with a computer-assisted microscope at $\times 50$ magnification. Tubular structures were quantified by calculating the ratio of confluent area of network with ImageJ software. The confluent area in the control group was designated as 100 %.

Effects of DCT015 on VEGF-induced VEGFR-2 signaling pathway

HUVECs were starved with 2 % serum-complete DMEM medium for 24 h and then treated with different concentrations of DCT015 or sorafenib for 24 h. After being induced with VEGF (30 ng/mL) at 37 °C for 2 h, cells were treated for analysis of VEGFR-2, p-VEGFR-2, MEK, p-MEK, ERK, p-ERK, AKT, p-AKT, and β -actin,

respectively. Cells were washed with PBS and lysed in RIPA and PMSF-mixed lysis buffer, prior to analysis of activation of the relevant signaling pathways by Western blotting, as described above. The densities of the bands were quantified using ImageJ software.

Human gastric cancer xenograft models

AGS tumors models were established by injecting 3×10^6 cells mixed with Matrigel into the dorsal area of male nude mice. After 10 days, mice bearing tumors around 100 mm^3 were selected and randomly divided into four groups (six/each group). Mice were administrated with DCT015 at 30 and 60 mg/kg or sorafenib at 60 mg/kg by oral gavage once a day. Control mice were given the same volume of CMC-Na. Body weights and tumor dimensions were measured twice a week during the treatment. Tumor volumes were calculated according to the following formula: volume (mm^3) = $0.5 \times \text{length (mm)} \times \text{width (mm)} \times \text{width (mm)}$. The inhibition rate (IR) of tumor growth was calculated by the following formula, IR (%) = $[(A - B) / A] \times 100$, where A and B were the mean tumor weight in the control and treatment groups, respectively.

Immunohistochemistry staining

The animals were sacrificed after the last treatment, and tumors were harvested and fixed in 4 % formalin for

Table 1 Effects of DCT015 on 12 lines of cancer cell viability were determined by MTT assay

Cell lines	IC ₅₀ (μM)	
	Sorafenib	DCT015
AGS	32.09 \pm 2.27	21.94 \pm 2.96
BGC-823	39.43 \pm 4.01	22.89 \pm 2.08
MNK-45	46.28 \pm 1.50	22.34 \pm 3.27
SGC-7901	48.69 \pm 3.90	37.60 \pm 3.60
SIHA	20.98 \pm 4.05	22.94 \pm 3.09
A375	29.48 \pm 1.38	25.04 \pm 1.01
PC3	26.38 \pm 1.19	24.59 \pm 1.20
LNCAP	21.69 \pm 1.27	17.26 \pm 2.41
DU145	20.05 \pm 3.92	20.68 \pm 3.54
SW480	38.89 \pm 3.53	21.22 \pm 1.30
HCT116	36.93 \pm 3.39	18.19 \pm 1.87
H460	47.88 \pm 1.60	34.02 \pm 2.40

Note: Tumor cells were plated in 96-well plate with a density of 5×10^3 cells/well. After 24 h incubation, the cells were exposed to DCT015 or sorafenib from 5 to 60 μM and cultured for 48 h. MTT assay was used, and the absorbance was measured at 570 nm. IC₅₀ values were calculated using GraphPad Prism v. 5.0. The results were presented as mean \pm S.D.

immunohistochemistry (IHC) assay. After paraffin embedding, the tumor sections were cut into 4- μ m-thick slices. The deparaffinized tissue slices were immersed in boiling citrate buffer (pH 6.0) for antigen retrieval. The endogenous peroxidase activity was blocked with 3 % hydrogen peroxide for 15 min under dark condition. The slices were incubated with anti-CD31 or anti-Ki67 antibody for 2 h at room temperature, followed by GT Vision III Detection System as secondary antibody for 30 min at room temperature. 3,3-Diaminobenzidine (DAB) was used to visualize positive immune reaction [18]. The images were captured with a digital camera (Olympus DP25).

Statistical analysis

All data are presented as mean \pm SD. Statistical analyses were performed using one-way ANOVA, followed by LSD post

hoc test in SPSS software (v. 16.0). $p < 0.05$ was considered statistically significant.

Results

DCT015 inhibited tumor cells growth in vitro

The proliferative inhibitory ability of DCT015 on tumor cells was investigated among a series of types of cancer cell lines. The results of IC₅₀ were shown in Table 1. In general, DCT015 was more active against most of the tested cancer cell lines compared to sorafenib. According to these results, gastric cancers were found to be more sensitive to DCT015 than sorafenib and were selected for further investigation of the possible mechanisms of action of DCT015.

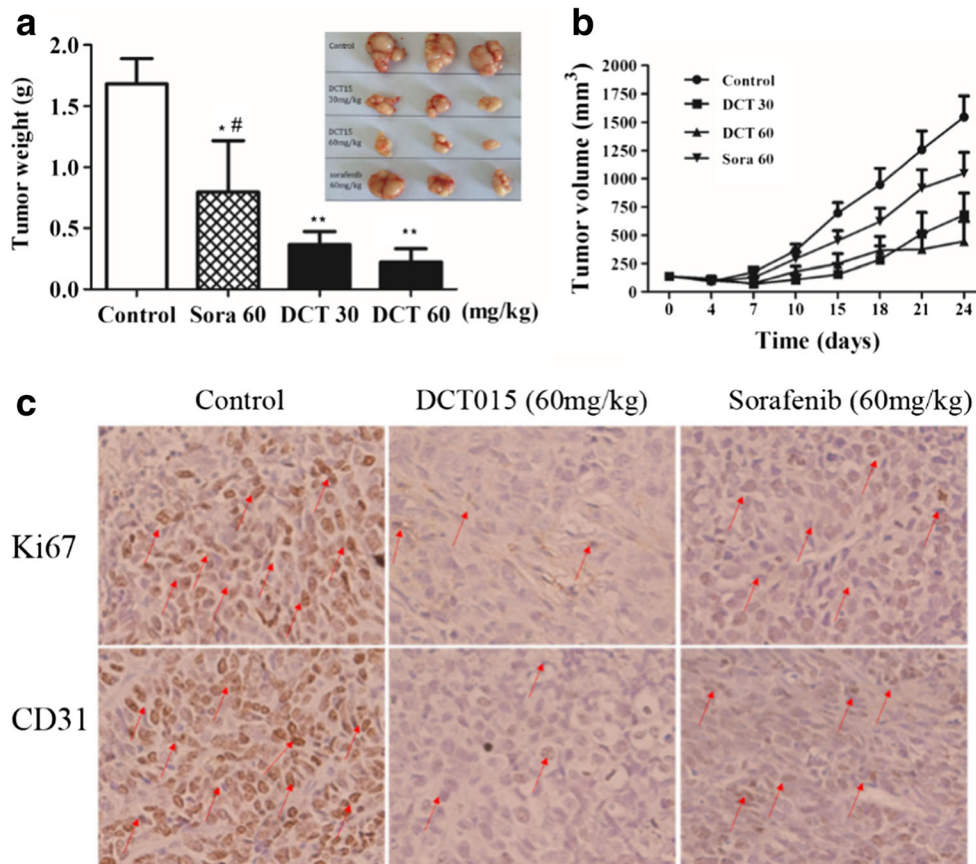


Fig. 2 DCT015 suppressed tumor growth in AGS tumor xenograft model. **a** AGS tumor-bearing mice were treated orally with DCT015 at 30 and 60 mg/kg or sorafenib at 60 mg/kg once daily for 24 days. DCT015 and sorafenib produced robust efficacy against AGS tumor xenograft models. **b** Tumor volumes were measured twice a week. DCT015 had satisfactory inhibition effects against AGC tumor growth compared with sorafenib at 60 mg/kg. **c** The tumor tissues were fixed in 4 % formalin for immunohistochemistry (IHC) assay. The deparaffinized tissue slices were immersed in boiling citrate buffer for antigen retrieval.

The endogenous peroxidase activity was blocked with 3 % hydrogen peroxide. The slices were incubated with anti-CD31 or anti-Ki67 antibody for 2 h, followed by GT Vision III Detection System as secondary antibody. DAB was used to visualize positive immune reaction, and the images were captured with a digital camera. DCT015 and sorafenib strongly decreased Ki67-positive cells and inhibited the expression of CD31 in tumor tissues. The results in Fig. 2a, b were presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, compared to the control group. # $p < 0.01$, compared to the sorafenib group

Table 2 The effects of DCT015 and sorafenib in gastric cancer xenograft models

Group	Dosage (mg/kg/day)	No. of animals (<i>n</i>) Begin/end	Body weight (g)		Tumor weight (g)	IR (%)
			Begin	End		
Control	–	6/6	24.84±2.95	25.35±3.47	1.68±0.21	–
DCT015	30	6/6	25.71±1.32	24.51±1.82	0.36±0.11*	78.34 %
	60	6/6	23.67±3.84	23.33±2.31	0.22±0.11**#	86.77 %
Sorafenib	60	6/6	24.15±1.37	24.59±1.42	0.79±0.22*	52.70 %

Note: * $p < 0.05$ and ** $p < 0.01$, compared to the control group. # $p < 0.05$, compared to the sorafenib group

DCT015 strongly inhibited AGS tumor growth in vivo

We further examined the in vivo anti-cancer activities of DCT015 in nude mice bearing human AGS gastric cancer xenografts. The mice were treated once daily for 24 days with DCT015 or sorafenib. We found that DCT015 at 30 and 60 mg/kg and sorafenib at 60 mg/kg inhibited the tumor growth on AGS xenograft (Fig. 2a, b, Table 2). Both drugs caused partial tumor regression compared to the control group. On inhibiting tumor volumes and tumor weights, DCT015 displayed to be more potent than sorafenib at 60 mg/kg. However, the animals in DCT015 and sorafenib groups only showed slight weight changes and no deaths were found (Table 2). These data suggested that DCT015 had more

significant inhibition effects against AGS tumor growth than sorafenib did with similar side effects.

The nuclear protein Ki67 is a proliferation index, which is expressed only by dividing cells. Angiogenesis provides necessary nutrients and oxygen for tumor development, growth, and metastasis. To further explore whether DCT015 inhibits tumor growth and angiogenesis in vivo, IHC anti-Ki67 and anti-CD31 staining were conducted. As shown in Fig. 2c, DCT015 and sorafenib could strongly decrease Ki67-positive cells and inhibit the expression of CD31 in tumor tissues compared to the control group. These results suggested that DCT015 could significantly suppress gastric tumor growth through inhibiting cell proliferation and angiogenesis.

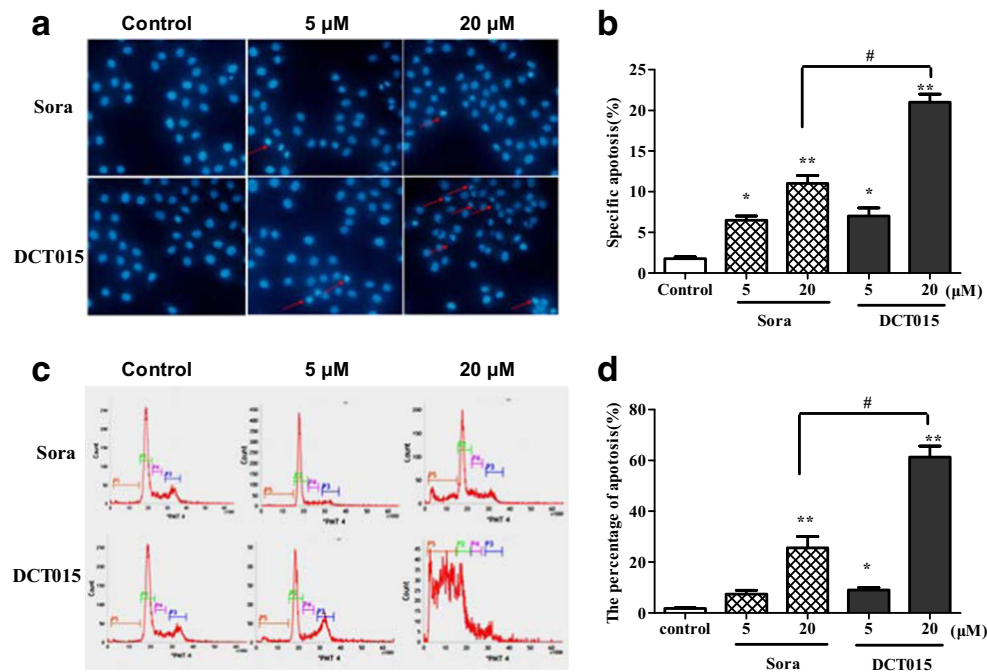


Fig. 3 DCT015 induced apoptosis in AGS cells. **a, b** Hoechst 33258 staining. Cells were treated with DCT015 or sorafenib at 5 and 20 μM for 24 h. The cells were stained with Hoechst 33258 at room temperature for 20 min. Specific apoptosis, including both nuclear condensation and/or nuclear fragmentation, was observed and analyzed by using Cellomics ArrayScan II image analysis system. DCT015 showed more potent apoptosis-induced effects than sorafenib did. **c, d** Cell cycle

distribution. Cells were treated with DCT015 or sorafenib for 24 h and were stained with PI. Cell cycle distribution was assessed using flow cytometry and analyzed by ModFit software. Cell cycle analysis showed a more significant increase in the “sub-G1” population with DCT015 than with sorafenib treatments. The results were presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, compared to the control group. # $p < 0.01$, compared to the sorafenib group

DCT015 induced apoptosis of gastric cancer cells

Because DCT015 showed anti-tumor activities against gastric cancer cells *in vitro*, cell apoptosis analysis was carried out by Hoechst and PI staining assays. Hoechst 33258 staining was conducted after treatment for 24 h in AGS cells; DCT015 was more potent than sorafenib in the morphological changes of apoptosis (nuclear condensation and/or nuclear fragmentation) and the increased amounts of apoptosis cells (Fig. 3a, b). Cell cycle analysis by flow cytometry showed a more significant increase in the “sub-G1” population with DCT015 than with sorafenib treatments, while both drugs produced less effects on cell cycle distribution in AGS cells (Fig. 3c, d). These results indicated that the proliferative inhibition of DCT015 was partially due to drug-induced apoptosis in gastric cancer cell lines, and the potency of DCT015 was higher than sorafenib.

Effects of DCT015 on expression of cell apoptosis-related protein in gastric cancer cells

The Bcl-2 and caspase families are the key executioners of apoptosis. Bcl-2, caspase-3, Mcl-1, and Bax protein levels were determined to understand whether they functioned in the mechanism of DCT015-induced apoptosis in AGS and BGC823 gastric cancer cells. After a 48-h treatment at 5, 10, and 20 μM , Western blotting analysis (Fig. 4) showed that DCT015 decreased the expression

of the anti-apoptotic proteins Mcl-1 and Bcl-2 and increased the expression of the pro-apoptotic proteins Bax and caspase-3. These data suggested that DCT015-induced apoptosis may be dependent on the downregulation of the anti-apoptotic proteins Bcl-2 and Mcl-1 and the upregulation of pro-apoptotic proteins Bax and caspase-3.

DCT015 inhibited the MEK/ERK and PI3K/Akt signaling pathways in gastric cancer cells

To gain further insights into the molecular mechanism of the inhibitory effects of DCT015 on gastric cancer cells, we evaluated the effects of DCT015 on the key proteins in the MEK/ERK and PI3K/Akt signaling pathways in BGC-823 and AGS cells 48 h after treatment. The results showed that DCT015 and sorafenib dramatically decreased the expression of phosphorylated MEK, ERK, PI3K, and Akt, whereas the total protein levels of each protein were not significantly changed (Fig. 5). Moreover, though there were no significant differences in the phosphorylation levels of MEK, ERK, PI3K, and Akt between DCT015 and sorafenib in BGC-823 and AGS cells, the densities of the bands in DCT015 group appeared lower than the corresponding levels in sorafenib group at 20 μM . Therefore, the anti-cancer activities of DCT015 were attributed to the dual inhibition of MEK/ERK and PI3K/Akt signaling pathways.

Fig. 4 Effects of DCT015 on apoptosis-related proteins in AGS (a, b) and BGC823 (c, d) cancer cells. After a 48-h treatment at 5, 10, and 20 μM , DCT015 decreased the expression of anti-apoptotic proteins Mcl-1 and Bcl-2 and increased the expression of pro-apoptotic proteins Bax and caspase-3. The results were presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, compared to the control group

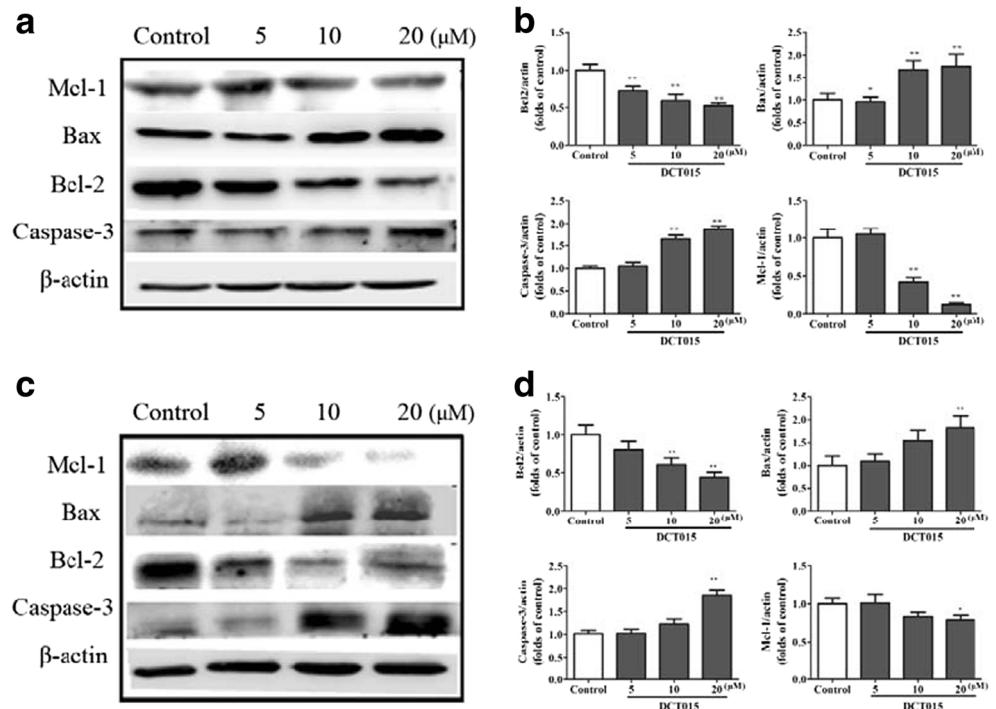
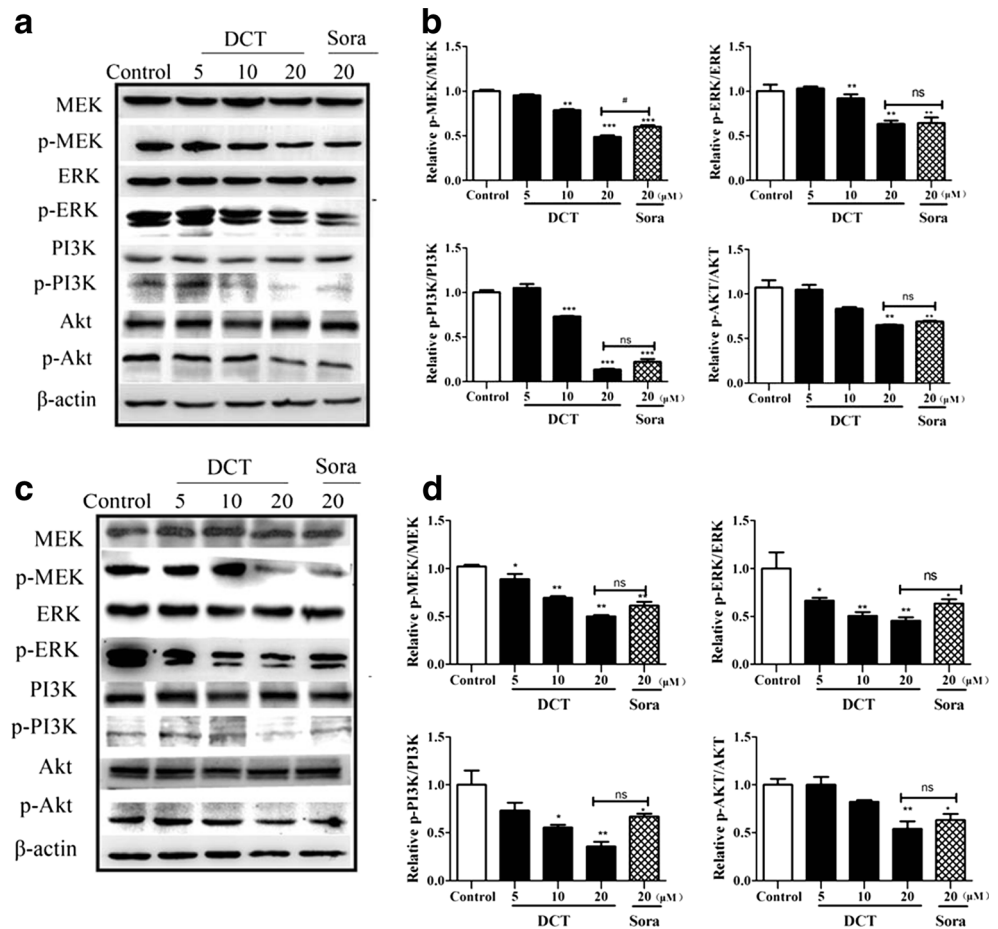


Fig. 5 DCT015 inhibited the MEK/ERK and PI3K/Akt signaling pathways in AGS cells (a, b) and BGC823 cells (c, d). Gastric cancer cells were treated with DCT015 or sorafenib at different concentrations for 48 h; the levels of phosphorylated MEK, ERK, PI3K, and Akt were analyzed by western blotting with β -actin as a control. The results were presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, compared to the control group



DCT015 inhibited HUVEC viability

The effects of DCT015 on the growth of HUVECs were examined using the MTT assay. DCT015 or sorafenib decreased the viability of HUVECs in a dose-dependent manner with IC_{50} of 4.83 or 5.60 μ M, respectively (Fig. 6).

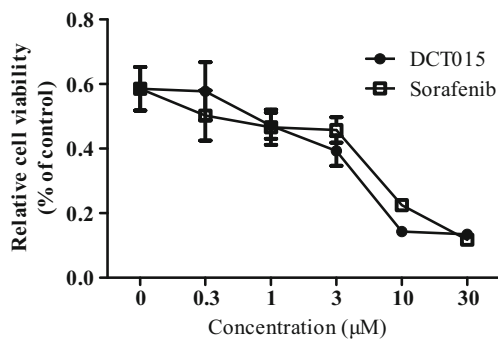


Fig. 6 DCT015 decreased HUVEC viability. HUVECs were seeded in 96-well plates with a density of 6×10^3 cells per well overnight. DCT015 or sorafenib at different concentrations was used to treat the cells. After 24 h, MTT assay was used, and the absorbance was measured at 570 nm. DCT015 and sorafenib markedly decreased the viability of HUVECs at different concentrations after a 24-h treatment. The results were presented as mean \pm SD

DCT015 inhibited VEGF-induced HUVEC migration

We carried out wound healing assays to investigate the effects of DCT015 on HUVEC migration induced by VEGF (Fig. 7a, b). When VEGF was used alone, HUVEC migration was increased by twofold as compared to the control group. However, VEGF-induced migration was significantly inhibited by DCT015 at 1 and 3 μ M or sorafenib at 3 μ M. Notably, DCT015 was more potent than sorafenib at 3 μ M.

DCT015 inhibited VEGF-induced HUVEC tube formation

VEGF-induced HUVEC tube formation is a critical step in the process of angiogenesis [19]. We tested the effects of DCT015 on tube formation induced by VEGF (Fig. 7c, d). Robust and complete tube network formation was observed in VEGF-stimulated HUVECs. When treated with DCT015 at 0.3, 1, and 3 μ M, tube formation was inhibited in a concentration-dependent manner. The inhibition potency of DCT015 was higher than sorafenib at 3 μ M.

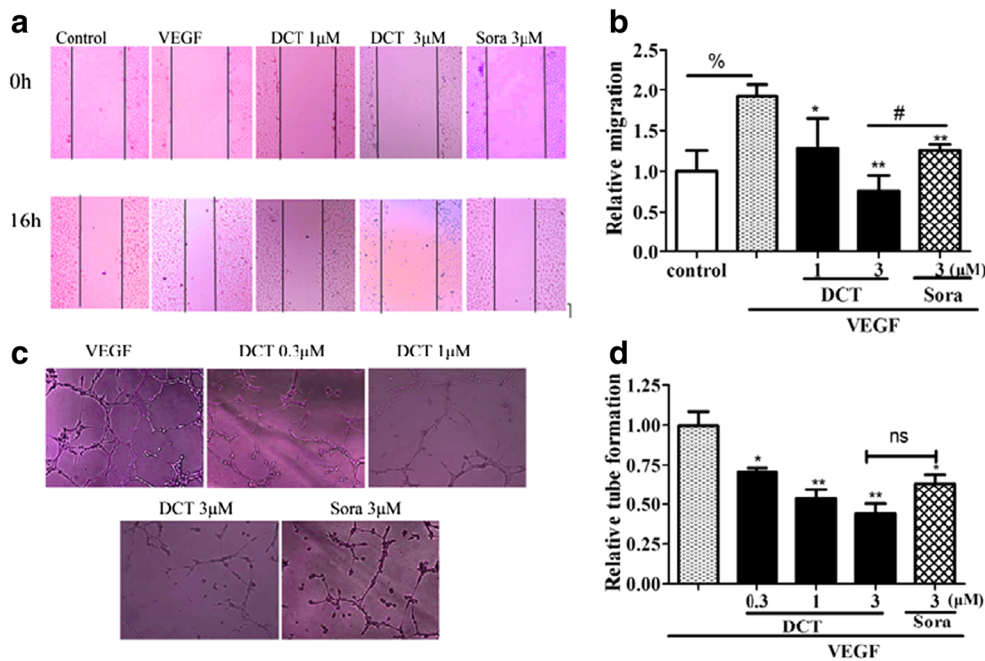


Fig. 7 DCT015 inhibited VEGF-induced HUVEC migration and tube formation. **a, b** DCT015 inhibited HUVEC migration by wound healing assay. Incubated HUVECs were wounded by pipette tips and then treated with DCT015 or sorafenib in the presence of VEGF (30 ng/mL) for an additional 24 h. The wound width was determined with ImagePro Plus software. DCT015 significantly inhibited the VEGF-induced migration with a superior potency to sorafenib. **c, d** DCT015 inhibited the tube formation of HUVECs. The incubated HUVECs in the 96-well plate

containing Matrigel were treated with various concentrations of DCT015 or sorafenib with or without VEGF (30 ng/mL) for 24 h. Tubular structures were quantified by calculating the ratio of confluent area of network with ImageJ software. The results showed that DCT015 significantly inhibited the VEGF-induced tube formation. The results were presented as mean ± SD. %*p* < 0.01, compared to the control group. **p* < 0.05, ***p* < 0.01, compared to the VEGF group. #*p* < 0.01, compared to the sorafenib group

Effects of DCT015 on VEGF-induced VEGFR2 signaling pathways in HUVECs

VEGFR2 phosphorylation leads to the activation of various downstream signaling substrates, which are responsible for angiogenesis processes such as endothelial cell

migration and tube formation. To investigate the potential mechanism of anti-angiogenesis induced by DCT015, several downstream key kinases in MEK/ERK and PI3K/Akt signaling pathways were examined. As shown in Fig. 8a, b, VEGF (30 ng/mL) significantly induced phosphorylation of VEGFR2, which was blocked by

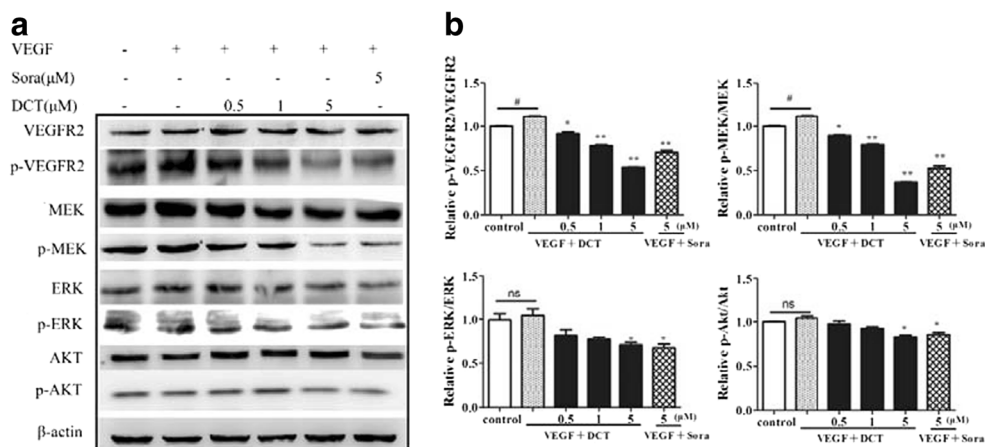


Fig. 8 DCT015 inhibited the activation of VEGFR2-mediated signaling pathways in HUVECs. HUVECs were starved with 2 % serum-complete DMEM medium for 24 h and then treated with different concentrations of DCT015 or sorafenib for 24 h. After being induced with VEGF (30 ng/mL) for 2 h, cells were treated for Western blotting analysis. VEGF stimulated VEGFR-2 autophosphorylation in HUVECs. DCT015 and

sorafenib suppressed the activation of VEGFR-2 and its downstream key kinases MEK, ERK, and Akt in HUVECs. The densities of the bands were quantified using ImageJ software. The results were presented as mean ± SD. **p* < 0.05, ***p* < 0.01, compared to the VEGF group. #*p* < 0.05, compared to the control group

DCT015 and sorafenib. The phosphorylation levels of MEK, ERK, and Akt were also inhibited by DCT015 and sorafenib, while total levels of VEGFR2, MEK, ERK, and Akt were not affected. These data suggested that the anti-angiogenic activity of DCT015 in endothelial cells was through downregulation of the activation of VEGFR2-mediated signal pathways, which was similar to the effects of sorafenib.

Discussion

Gastric cancer has severely impacted the health and resulted in high mortality rate all over the world [1]. The first- and second-line chemotherapies cannot achieve a satisfying clinical trial. In order to improve the OS and life quality of the patients with GC, there is an urgent need for more specific and effective treatment options. Currently, some targeted drugs, such as ramucirumab, apatinib, and trastuzumab, have been used for treating AGC [3, 5, 6]. Moreover, some small molecular inhibitors targeting the key kinases of Ras/Raf/MEK/ERK and PI3K/Akt signaling pathways are being investigated in clinical trials [8, 12].

Preclinical studies suggest that sorafenib, a known oral multi-kinase anti-cancer drug, can inhibit cancer cell proliferation and angiogenesis, which acts on tumor growth and tumor vasculature [15, 16]. Sorafenib has shown encouraging anti-tumor efficacy in a phase II study of gastric cancer [17]. It is worth further investigating sorafenib as an anti-gastric cancer drug. However, sorafenib can induce some side effects [16, 20]. Therefore, it is necessary to develop some novel sorafenib derivatives in order to improve the efficacy and adverse effects for treating gastric cancer.

We are trying our best to develop a novel sorafenib derivative as an anti-gastric cancer drug with fewer side effects. After the chemical modification, in a series of derivatives with promising anti-tumor activities, DCT015 was selected to evaluate the pharmacological effects and the effects on the targeted signaling pathways in tumor growth and angiogenesis. Among 12 cancer cell lines, AGS, BGC-823, MNK45, and SGC7901 gastric cancer cells were found to be more sensitive to DCT015 than sorafenib. AGS gastric cancer xenograft model was used to evaluate the general *in vivo* anti-tumor activity of DCT015 and sorafenib. DCT015 not only inhibited tumor volume and tumor weight in a dose-dependent manner but also demonstrated more significant efficacy than sorafenib at the same dose (60 mg/kg). In addition, no obvious adverse effects, such as weight loss and mortality, were observed in all treated groups during the course of treatment. Furthermore, IHC assay showed that DCT015 strongly

decreased Ki67-positive cells and inhibited the expression of CD31 in tumor tissues, suggesting that DCT015 could significantly suppress gastric tumor growth through inhibiting cell proliferation and angiogenesis *in vivo*.

Based on *in vitro* and *in vivo* anti-tumor activity of DCT015 in our experiments, we further conducted experiments to understand the possible action mechanisms of DCT015 for the treatment of gastric cancer. We determined the apoptotic effect of DCT015 or sorafenib on AGS gastric cancer cells by Hoechst staining and PI staining. Furthermore, we found that DCT015-induced apoptosis may be dependent on the downregulation of anti-apoptotic proteins Bcl-2 and Mcl-1 and the upregulation of pro-apoptotic proteins Bax and caspase-3. Our data suggested that there was an imbalance between the expression of pro-apoptotic and anti-apoptotic proteins after the treatment with DCT015.

Then, we further demonstrated that the anti-tumor activities of DCT015 were associated with blocking the MEK/ERK and PI3K/Akt signal pathways. The MEK/ERK signaling pathway has pivotal roles in cell proliferation, survival, motility, transcription, metabolism, and differentiation [21]. Furthermore, the MEK/ERK pathway produces profound regulatory effects on the apoptosis-related molecules including Bcl-2 and caspase families [22]. The activation of the MEK/ERK pathway is associated with progression and poor prognosis of gastric cancer [23]. As an important pathway in cancer, some small molecular inhibitors targeting Raf and/or MEK have been developed [24]. In the present study, our results suggested that the inhibition effect of DCT015 on the MEK/ERK pathway had been consistent with the inhibitory effect of sorafenib.

The PI3K/Akt signal pathway plays key roles in cell proliferation, growth, and angiogenesis, which has been one major target pathway for cancer therapy [25]. Moreover, the PI3K/Akt pathway could enhance the mitochondrial death pathway in AGS gastric cancer cell line [26]. However, a recent report showed that the inhibition of PI3K/Akt could induce MEK/ERK activation [25, 27]. The simultaneous inhibition of both MEK/ERK and PI3K/Akt signaling pathways could be a more optimal strategy to maximize cancer therapy [25]. Preclinical studies have demonstrated that co-targeting of the MEK/ERK and PI3K/Akt pathways resulted in synergistic cancer inhibition [28]. Also, the inhibition of two signaling pathways has been a more effective means to induce apoptosis than the action on a single signal pathway. This might provide a further rationale for inhibiting both signaling pathways [29]. In our study, DCT015 simultaneously blocked both MEK/ERK and PI3K/Akt signaling pathways, with similar potency to sorafenib.

Angiogenesis has been strongly linked with tumor growth and metastasis in most human tumors, and VEGF

is the most potent and specific angiogenic factor [30]. Anti-angiogenesis targeting the VEGF/VEGFR2 pathway has been attractive against tumor [3, 5]. Furthermore, as the downstream key proteins in the VEGF/VEGFR2 pathway, activation of the MEK/ERK or/and PI3K/Akt has been shown to promote endothelial cell proliferation, migration, and tube formation [14, 31, 32]. Some drugs targeting VEGF or VEGFR2 have been evaluated, such as monoclonal antibody bevacizumab, and multi-target tyrosine kinase inhibitors such as sunitinib and sorafenib. In the present study, DCT015 significantly inhibited VEGF-induced migration and tube formation of HUVECs, with higher potency than sorafenib. The action mechanism studies demonstrated that DCT015 significantly inhibited the kinase activity of VEGFR2 and the phosphorylation levels of the downstream key proteins including MEK, ERK, PI3K, and Akt.

In our study, not only that the apoptosis induction potency of DCT015 was higher than that of sorafenib in gastric cancer cell lines but also that the inhibition potency of DCT015 in migration and tube formation was higher than that of sorafenib in HUVECs. Furthermore, DCT015 had more significant anti-cancer effects than sorafenib by measuring tumor volumes and tumor weights in AGS gastric cancer model. In our primary pharmacokinetics test, nude mice bearing AGS cells were orally given with DCT015 or sorafenib at 60 mg/kg. Plasma peak concentration and drug exposure level (AUC_{0-72h}) in the DCT015 group were over fivefold higher than those in the sorafenib group, respectively (unpublished data). The above factors could contribute to superior *in vivo* anti-tumor effects of DCT015 compared to sorafenib.

Conclusion

As a novel sorafenib derivative, DCT015 displayed strong *in vivo* anti-tumor effects in gastric cancer, which may be associated with not only anti-proliferative activities against gastric cancer cells by inhibiting both MEK/ERK and PI3K/Akt signaling pathways but also anti-angiogenesis by inhibiting the VEGF/VEGFR2 signaling pathway. Our findings demonstrated that DCT015 is a promising orally anti-cancer drug for treating gastric cancer, though further investigation in preclinical and/or clinical experiments is required.

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Compliance with ethical standard

Conflict of interest None

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