

RESEARCH COMMUNICATION

Tas13D Inhibits Growth of SMMC-7721 Cell via Suppression VEGF and EGF Expression

Huai-Zhen He, Nan Wang, Jie Zhang, Lei Zheng, Yan-Min Zhang*

Abstract

Objective: Taspine, isolated from Radix et Rhizoma Leonticis has demonstrated potential pro-angiogenic effects against cancer. Tas13D, a novel taspine derivative synthesized by structure-based drug design, have been shown to possess interesting biological and pharmacological activities. The current study was designed to evaluate its antiproliferative activity and underlying mechanisms. **Methods:** Antiproliferative activity of tas13D was evaluated by xenograft in athymic mice in vivo, and by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and cell migration assays with human liver cancer (SMMC-7721) cell lines in vitro. Docking between tas13D and VEGFR and EGFR was studied by with a Sybyl/Surflex module. VEGF and EGF and their receptor expression was determined by ELISA and real-time PCR methods, respectively. **Results:** Our present study showed that tas13D inhibited SMMC-7721 xenograft tumor growth, bound tightly with the active site of kinase domains of EGFR and VEGFR, and reduced SMMC-7721 cell proliferation ($IC_{50}=34.7 \mu\text{mol/L}$) and migration compared to negative controls. VEGF and EGF mRNAs were significantly reduced by tas13D treatment in a dose-dependent manner, along with VEGF and EGF production. **Conclusion:** The obtained results suggest that tas13D inhibits tumor growth and cell proliferation by inhibiting cell migration, downregulating mRNA expression of VEGF and EGF, and decreasing angiogenic factor production. Tas13D deserves further consideration as a chemotherapeutic agent.

Keywords: Tas13D - SMMC-7721 cell - EGF - EGFR - VEGF - VEGFR - chemotherapy

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Introduction

Radix et Rhizoma Leonticis (Hong Mao Qi in Chinese, HMQ) is distributed mainly in Shaanxi and Sichuan provinces of China. Taspine (Figure 1a), isolated for the first time from HMQ Radix et Rhizoma Leonticis has many pleiotropic effects such as anti-inflammatory, cell toxin, antiviral, topoisomerase I and II inhibition, (Perdue et al., 1979; Kelly and Xie, 1998; Fayad et al., 2009). In previous studies, we found that taspine had an inhibitory effect on tumor and angiogenesis, and could interact with VEGFR and EGFR. However, silico ADME prediction showed that taspine wasn't a compound with drug-like properties. Biological and cellular assays showed that it had very poor solubility in any solvent. We designed and synthesized tas13D, a taspine derivative by structure-based drug design. In order to identify its binding mode with enzyme of vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptor (EGFR), we performed a molecular docking study using SYBYL and cell inhibition of target compounds. We found tas13D (Figure 1B) had a better effect on SSMC-7721 cell line than taspine.

Tumor cells promote vessel formation through the expression of angiogenic molecules or their induction in the microenvironment (Kerbel, 2000). Among the

pro-angiogenic molecules, vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) have been identified to drive tumor-related angiogenesis (Yancopoulos et al., 2000; Han et al., 2005; Klein et al., 2009; Uchida et al., 2009; Yu et al., 2012) and mediate cell proliferation and migration (Hirsch et al., 2006). The expression of VEGFR and EGFR is common in a number of normal tissues and many human cancers. Overexpression of VEGF and EGF and dysregulation of VEGFR and EGFR-mediated signaling pathways play important roles in tumorigenesis, leading to poor prognosis

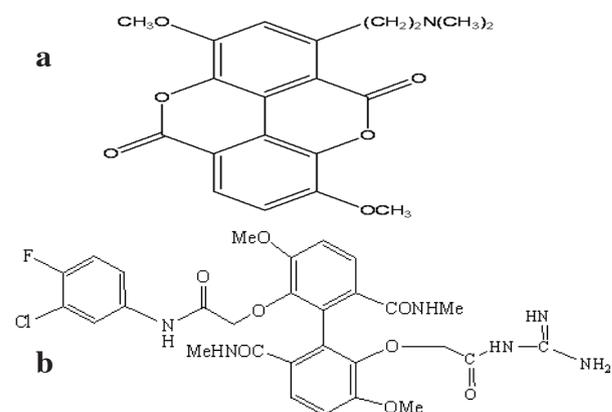


Figure 1. Chemical Structures of Taspine and Tas13D

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(Kim et al., 2001; Marti'nez 2006; Harichand-Herd et al., 2008).

The present study was to evaluate the effects of tas13D, a novel taspine derivative, on the proliferation and migration of SMMC-7721 cell line plus the activity of anti-tumour, and to examine its inhibition by modulating VEGF and EGF expressions as well. Human hepatoma cell line SMMC-7721 established in 1977 expressed EGFR and VEGFR in previous reports (Li, et al., 2005). SMMC-7721 cell line was recognized the most sensitive to Tas13D among 5 cancer cells compared in previous studies. Herein, docking study was used to investigate the action between tas13D and VEGFR and EGFR, SMMC-7721 cell to investigate the action on VEGF and EGF by tas13D, and human tumor models xenografted in athymic mice to study the anti-tumor activity in vivo.

Experimental data presented here showed that tas13D displayed the anti-tumor activity in vivo. The anti-tumor activity lied in the ability to inhibit SMMC-7721 cell proliferation and migration, and downregulate the mRNA expressions as well as production of VEGF and EGF. All these results indicate that tas13D with potent anti-tumor activity might be helpful for tumor treatment.

Materials and Methods

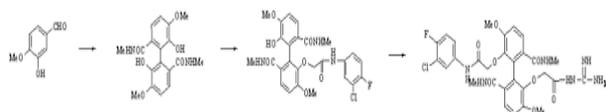
Tas13D was synthesized by School of Medicine, Xi'an Jiaotong University. Dimethylsulfoxide (DMSO), MTT, RPMI-1640, trypsin, and RNase were from Sigma (St. Louis, MO, USA). Total RNA extracted kit was from Fastagen (Fastagen, China). Revert AidTM first strand cDNA synthesis kit was from Fermentas (Hanover, Lithuania). ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). Other reagents used were analytical grade. The SMMC-7721 cell was purchased from Shanghai Institute of Cell Biology in the Chinese Academy of Sciences. SMMC-7721 cells were grown in RPMI-1640 medium containing 10% bovine serum, antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin), at 37 °C in a 5% CO₂ atmosphere.

Animals

Male (18-22 g) BALB/c mice and male BALB/C nude mice (18-22 g) were supplied by the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). Mice were housed and cared for under standard conditions, with a 12 h:12 h day/night cycle. All experimental procedures utilizing mice were in accordance with National Institute of Health guidelines.

Tas13D synthesis

We used commercially available isovanillin as the starting material. Tas13D was obtained via bromination, benzylation, oxidation, ulmman reaction, catalytic hydrogenation and substitution reaction. The basic preparation of tas13D was as follows:



Anti-tumor effect of tas13D on SMMC-7721 cell lines xenografted in athymic mice

Solid tumor models were developed from SMMC-7721 cell lines. The male immunodeficient BALB/c nude mice (18-22 g) were randomized and then implanted with 0.2 ml SMMC-7721 cell of 2×10⁷ cell/ml s.c. into the right axilla. Tumors were measured once every three days and tumor volumes (V_t) [(L×W²)/2] were calculated from caliper measurements. The relative tumor volume (RTV) was expressed as the V_t/V₀ index, where V_t is the tumor volume on the day of measurement and V₀ is the volume of the same tumor at the start of the treatment. The results were expressed as median T/C where T/C (%) equals median RTV of treated animals/median RTV of control animals ×100. Mice were injected with gefitinib (100 mg/kg in normal saline; n=8) or tas13D (100 mg/kg and 200 mg/kg in normal saline; n=8) or vehicle alone (normal saline; n=8). Drugs were given once a day for 14 days. Mice weight and tumor volume were recorded when animals were killed. The inhibition ration was expressed by the formula:

Inhibition rate (%) = (T_{Control} - T_{treatment})/T_{Control} × 100%. T_{Control}, tumor weight of control group, T_{treatment}, tumor weight of administration group. All experimental procedures utilizing mice were in accordance with the National Institute of Health guidelines and Animal Research Committee of Xi'an jiaotong university.

Cell viability Assay

The effect of tas13D on SMMC-7721 cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium broide (MTT) assay. Briefly, exponentially growing cells were harvested and plated in 96-well plates at a concentration of 2 × 10⁴ cells/well. After 24 h incubation at 37 °C, cells were treated with tas13D at various concentrations for 48 h. Then, 20 µl of MTT (5 mg/ml) was added to each well and incubated at 37 °C for 4 h. After the supernatant was discarded, 150 µl of DMSO was added to each well, and the optical density of cells was determined with a microplate reader (Bio-RAD instruments, USA) at 490 nm and expressed as absorbance values (Sikander et al., 2011).

SMMC-7721 migration assay

The SMMC-7721 migration activity was tested as described (Shyu et al., 2004; Liang et al., 2005). SMMC-7721 cells were plated at a density of 1 × 10⁵ cells/well of 6-well plates and incubated at 37 °C for 24 h. Once cells were attached completely, they were scraped to form a line in the middle of the plates. The width of the line was about 500 µm. After scraping the cells, the medium was changed into serum-free medium containing 0.1 ng/ml EGF and 1 mol/ml thymidine. At the same time, the cells were treated without or with tas13D (3.47, 6.94 and 13.89 µmol/L). The widths of the line were measured after incubation at various intervals (0, 12, 24 and 48 h).

Docking study

In an effort to elucidate the binding modes of tas13D with VEGFR and EGFR, it was constructed with Sybyl/Sketch module, optimized using Powell's method with the

Table 1. Sequence Details of Individual Pairs of Primers

| Gene | Forward primer | Reverse primer |
|----------------|---------------------------------|--------------------------------|
| VEGF | 5'- CGGCGAAGAGAAGAGACACATTG -3' | 5'- CGGGAAGGGAAGGGAAGGAC -3' |
| EGF | 5'- TGCTGCGTGGTGGTGGTCTTG -3' | 5'-CAGCCAGGAGAAATCAAACAGAGG-3' |
| β -actin | 5'-ATCGTGCCTGACATTAAGGAGAAG-3' | 5'- CTGCGACTCCTCATCTCTCTGC -3' |

Table 2. Effects of Tas13D on SMMC-7721 Cell Xenografted in Athymic Mice

| Groups | No. of animals | | Tumor size (mm ³) | | T/C (%) | | Inhibition rate(%) | |
|----------------------|----------------|-----|-------------------------------|------------------|--------------------|-------|--------------------|-------|
| | Start | End | Start | End | | | | |
| Control | 8 | 8 | 25.74 \pm 4.14 | 98.2 \pm 25.67 | 555.40 \pm 162.2 | / | / | / |
| Tas13D (100.0 mg/kg) | 8 | 8 | 24.47 \pm 3.95 | 98.4 \pm 31.2 | 503.13 \pm 118.6 | 90.4 | | 22.64 |
| Tas13D (200.0 mg/kg) | 8 | 8 | 25.47 \pm 4.28 | 119.4 \pm 21.5 | 372.07 \pm 96.57 | 54.99 | | 47.17 |

Athymic mice with SMMC-7721 transplant tumor were treated with (injection), Tas13D 100 or 200 mg/kg every day for 14 days. T/C (%) = TRTV/CRTV \times 100; RTV, relative tumor volume

tripos force field with convergence criterion set at 0.05 kcal/(\AA mol), and assigned with Gasteiger-Hückel method (Mou et al., 2009). The docking study was performed using Sybyl/Surflex module, the residues in a radius of 6.5 \AA around VEGFR (PDB ID: 2X1W) and EGFR (PDB ID: 1IVO) selected as the active site. Other docking parameters implied in the program were kept default.

RNA isolation and real-time PCR

Quantification of VEGF, EGF and an internal reference gene (β -actin) was done using a fluorescence based real-time detection method. Total RNA of the SMMC-7721 cells treated by tas13D was isolated using total RNA extracted kit. The total RNA was reversely transcribed in a reaction solution of 20 μ l using the Revert Aid First Strand cDNA Synthesis kit. The sequence details of individual pairs of primers of VEGF, EGF and β -actin are shown in Table 1. RT-PCRs were carried out with the Thermal Cycler Dice™ Real Time System (TaKaRa, Japan) in 96-well reaction plates. Reaction volumes were 25 μ l containing 2 μ l cDNA. Thermal cycling conditions included pre-incubation at 95 $^{\circ}$ C for 2 min followed by 40 PCR cycles at 95 $^{\circ}$ C for 20 s and 60 $^{\circ}$ C for 1 min. All reactions were run in triplicate. iCycler software was used to analyze the calibration curve by plotting the threshold cycle (Ct) vs. the logarithm of the number of copies for each calibrator. The relative amount of mRNA for each gene was normalized based on that of the housekeeping gene β -actin [$E^{-\Delta\Delta C_t}$]. Measurements yield Ct values that are inversely proportional to the amount of cDNA in the tube. For example, a higher Ct value means that more PCR cycles are required to reach a certain level of cDNA detection. Gene expression values (relative mRNA levels) are expressed as ratios (differences between the Ct values) between the gene of interest and an internal reference gene (β -actin). This reference gene provides a baseline measurement for the amount of RNA isolated from a specimen (Desbois-Mouthon et al., 2006; Lin et al., 2006; Mu et al., 2006).

VEGF and EGF secretion in vitro

SMMC-7721 cells (5×10^4 cells per well) were cultured in 24-well culture plates for 24 h. Then, the cells were incubated for another 24 h after the medium was changed to serum-free medium. When tas13D was added to the well, the final concentrations for SMMC-7721 cells were

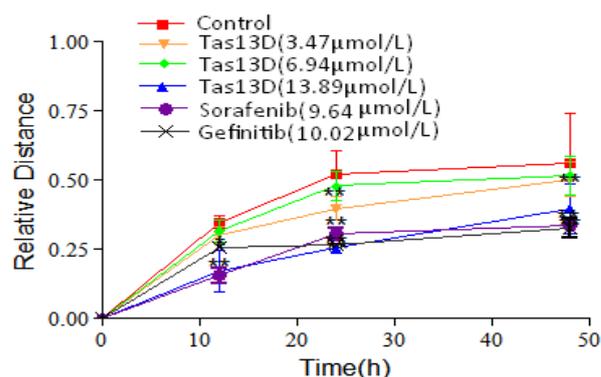


Figure 2. Effects of Tas13D on the SMMC-7721 Cell Migration. Dose-dependent inhibition of tas13D on migration, positive control (gefitinib, \times ; sorafenib, \bullet), negative control (\blacksquare), 3.47 μ mol/L (\blacktriangledown), 6.94 μ mol/L (\square), 13.89 μ mol/L (\blacktriangle). Values were expressed as means \pm SD (n=5). *p < 0.05, **p < 0.01 vs. the control group

3.47, 6.94 and 13.89 μ mol/L. The 48-h cultured medium was collected. VEGF and EGF protein concentrations were quantified by a commercially available VEGF and EGF ELISA kit (Shimamura et al., 2001). Ods were measured at 450 nm.

Statistic analysis

Data were expressed as mean \pm S.D. Statistical analysis was undertaken using the SPSS10.0 statistical software (SPSS, Chicago, IL, USA). ANOVA was used to analyze statistical differences between groups under different conditions. p < 0.05 was considered significant.

Results

Therapeutic effect of tas13D in vivo

The anti-tumor properties of tas13D were evaluated using human tumor models xenografted in athymic mice, with gefitinib employed here as a positive control group. Tas13D significantly inhibited tumor growth in SMMC-7721 xenografted athymic mice in a dose-dependent manner (Table 2). Compared with the control group, the group treated with tas13D had significantly inhibited tumor growth at concentrations of 22.64% and 47.17%, respectively. Furthermore, there was no substantial change in athymic mice body weight during the experiment, which implied that the anti-tumor activity of tas13D took precedence over the toxicity on athymic mice.

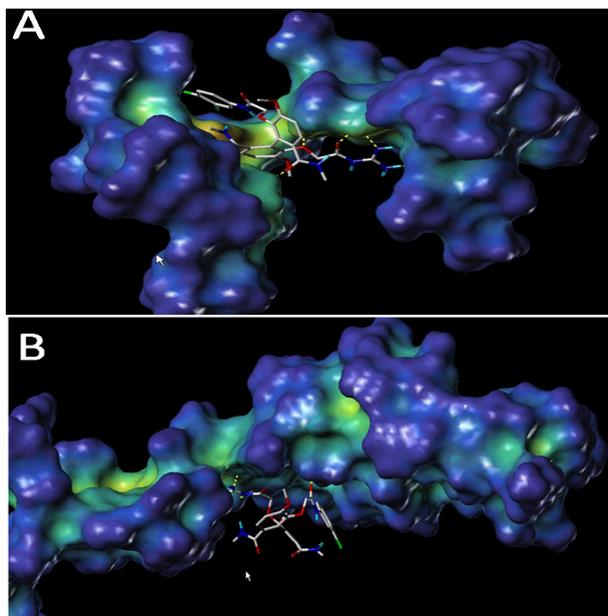


Figure 3. Docking Study by the Binding Modes of Tas13D with VEGFR and EGFR. Sybyl/Surflex docked conformation of tas13D in the active site of VEGFR (PDB ID: 2X1W) and EGFR (PDB ID: 1IVO). (A) Molcad surface cavity depth of EGFR; (B) Molcad surface cavity depth of VEGFR

Effect of Tas13D on SMMC-7721 cell viability

We examined the effect of tas13D and taspine on the viability of SMMC-7721 cell by MTT. Tas13D and taspine showed dose-dependent inhibition on cell growth. The 50% cell growth inhibition (IC₅₀) of tas13D and taspine were 34.74 and 46.02 μmol/L, respectively. It suggests tas13D has a better antiproliferative effect on the SMMC-7721 cells than taspine.

Effects of tas13D on migration

In solid tumors, angiogenesis is characterized as a critical step for growth, migration and metastasis. In vitro SMMC-7721 cells were treated with tas13D to test its effect on migration. As shown in Figure 2, results indicated that tas13D could significantly reduce cell motility in a time- and dose-dependent manner at the concentrations of 3.47, 6.94 and 13.89 μmol/L, respectively. The positive control of sorafenib and gefinitib showed the same inhibition.

Docking study

Docking of tas13D in the active site of VEGFR and EGFR showed many H-bond interactions between inhibitor and amino acid residues of receptor. Virtual docking of VEGFR in complex with tas13D also showed that tas13D bound tightly to the active site of receptor (Figure 3). The results indicate that the compound might act through the signal pathway of EGFR and VEGFR, and could interact with EGF and VEGF.

Effects of tas13D on quantitation of extracted mRNA

Quantitative PCR was carried out to understand whether tas13D could influence synthesis of VEGF and EGF transcript. As shown in Figure 4, the mRNA levels of VEGF and EGF were statistically significant compared

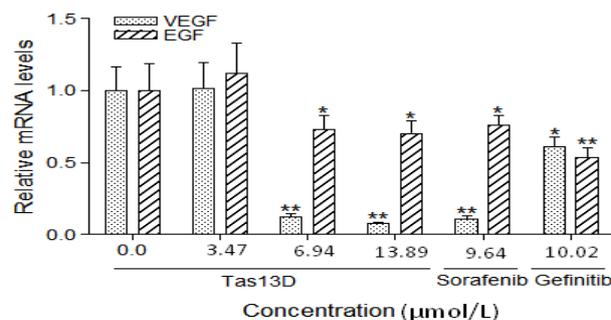


Figure 4. Effects of Taspine on VEGF, EGF and β-actin mRNA Expression in SMMC-7721 Cells. SMMC-7721 cells were treated with taspine, and then the cDNA was synthesized from total mRNA of the cells by reverse transcription. The mRNA levels of VEGF, EGF and β-actin were analyzed by quantitative PCR. The relative amount of mRNA for each gene was normalized based on that of the housekeeping gene β-actin [$E^{-\Delta\Delta Ct}$]. Values were expressed as means ±SD (n=5). *p<0.05, **p <0.01, vs. control. All samples were run in triplicate

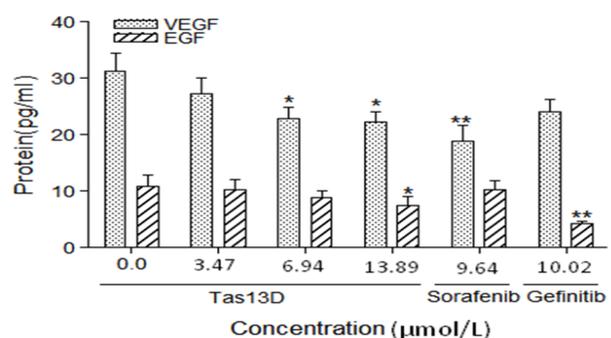


Figure 5. Effect of Tas13D on VEGF and EGF Expressions of SMMC-7721 Cells. VEGF expressions were inhibited in a dose-dependent manner compared with the control group. EGF expressions decreased obviously at the concentration of 13.89 μmol/L. Values were expressed as means ±SD (n=5). *p <0.05, **p <0.01, vs. control

to the negative control (p<0.05). The VEGF mRNA expressions in the tas13D-treated group were significantly downregulated, so were the VEGF mRNA expressions in the positive control of sorafenib. The inhibitory effect on EGF mRNA, consistent with that of the gefinitib, was increased with the increased concentration of tas13D.

Effect on VEGF and EGF secretion

ELISA for VEGF and EGF showed that tas13D could inhibit VEGF and EGF production in a dose-dependent manner at the concentrations (0, 3.47, 6.94 and 13.89 μmol/L) compared with the control group in SMMC-7721 cells (p<0.05) (Figure 5). The VEGF expressions decreased obviously at different concentrations. There were significant differences between the tas13D group and the control group. However, The EGF expressions only decreased at the concentrations of 13.89 μmol/L.

Discussion

Taspine was isolated for the first time from HMQ Radix et Rhizoma Leonticis (Hong Mao Qi in Chinese, HMQ). Tas13D was a novel derivative from taspine, a component that has pharmacologic actions. The efficacy of tas13D firstly was examined by xenografts in nude mice in

vivo, and the cell viability and migration were evaluated. The data obtained here showed tas13D had a better inhibitory effect on SMMC-7721 cell, the MTT and cell migration results showed tas13D could inhibit SMMC-7721 cell proliferation and migration (Figure 3). At the same time, the efficacy treated with tas13D was consistent with the suppression of growth of established human SMMC-7721 xenografts in nude mice (Table 2). In order to investigate the mechanisms of inhibition by tas13D on the proliferation and migration of SMMC-7721 cell line as well as the activity of anti-tumour, docking study was applied to analyze the interaction between tas13D and VEGFR and EGFR. The docking study performed by Sybyl/Surflex module indicates tas13D might act through the signal pathway of EGFR and VEGFR.

Solid tumors induce neovascularization, and the resultant angiogenesis stimulates tumor growth and metastasis (Folkman, 1992; Myoung et al., 2003; Carmeliet, 2005). In the process of tumor growth, the two-way paracrine action between tumor cells leads to obvious increase of tumor blood vessels and promote the tumor growth (Ishiwata et al., 1999; Yancopoulos et al., 2000). VEGF is a multifunctional cytokine that acts as one of the most potent angiogenic factors. EGF has also been identified to drive tumor-related angiogenesis (Uchida C et al., 2009; Tao et al., 2012). VEGF in coordination with EGF has profound effects on tumor cells, and could robustly induce cells growth and migration. Recent insights continue to support the notion that blockade of even a single growth factor might limit disease-induced vascular growth, with the most compelling evidence supporting approaches based on blockade of VEGF and EGF (Ishiwata et al., 1999; Yancopoulos et al., 2000). Compared to the negative control, VEGF and EGF mRNA expressions in SMMC-7721 cells (Figure 4) were significantly inhibited by tas13D treatment in a dose-dependent manner. Protein was translated by RNA. The measurement of VEGF and EGF using ELISA revealed that tas13D could inhibit production of VEGF and EGF (Figure 5). These data show tas13D inhibits tumor growth, cell proliferation and cell migration by downregulating mRNA expressions of VEGF and EGF, decreasing angiogenic factor production of VEGF and EGF in the VEGFR and EGFR signaling pathways.

In summary, according to our research in vivo and in vitro, tas13D displayed the anti-tumor activity, which lied in its ability to inhibit SMMC-7721 cell proliferation and migration through a two-way paracrine system as downregulated mRNA expressions along with the production of VEGF and EGF. All these results indicate that tas13D could act as a potential inhibitor for tumor therapy.

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