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Short communication

Inhibitory effect of taspine derivative TAD1822-7 on tumor cell growth and angiogenesis via suppressing EphrinB2 and related signaling pathways

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The aim of this study was to investigate the inhibitory effect of TAD1822-7, a synthesized taspine derivative, on cancer, through effects on tumor cell growth and angiogenesis via the suppression of EphrinB2. The obtained data showed that TAD1822-7 decreased Bel-7402 cell viability and colony

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formation ability, and suppressed cell migration. TAD1822-7 effectively inhibited blood vessel formation in an aortic ring assay to examine angiogenesis. Moreover, it also downregulated the expression of VEGFR2, VEGFR3, CD34, PLCγ, Akt, MMP2, MMP9, and CXCR4, and suppressed the expression of EphrinB2 and its PDZ protein, PICK1, in Bel-7402 cells. These results indicated that TAD1822-7 is a potential anti-angiogenic agent that can inhibit the viability and migration of Bel-7402 cells via the suppression of EphrinB2 and the related signaling pathways.

Keywords: TAD1822-7, EphrinB2, Bel-7402 cells, proliferation, migration, anti-angiogenesis

Hepatocellular carcinoma (HCC) is one of the common malignant cancers with high morbidity and mortality (1). Ephrins are the ligands for erythropoietin-producing hepatoma amplified sequence (Eph) family receptor tyrosine kinases (RTKs), which are membrane-bound proteins (2). EphrinB2 is a subtype of EphrinB, which is a ligand for the EphB2, EphB3, and EphB4 kinases, the only known ligand of EphB4 is EphrinB2 (3). The ephrin-mediated interactions between EphrinB2 and EphB4 are essential for tumor angiogenesis (4).

Angiogenesis plays a pivotal role in tumor progression, including primary capillary germination, branching, and remodeling, or form a mature vascular network (5). The stimulation of EphrinB2 which phosphorylates its cognate receptor EphB4 provides signals for cell survival and migration in cancers (6). Meanwhile, the upregulation of EphrinB2 can activate VEGF/VEGFR signaling during the tumor formation, which leads to the activation of the PI3K/Akt and PLC-γ/PKC pathways, and other related downstream protein expressions (7).

Taspine has been reported to have antitumor activity (8), and TAD1822-7 (Fig. 1a) is one of its derivatives, designed and synthesized as a lead compound containing biphenyl scaffold for better tumor suppression effects (8). However, the activity of TAD1822-7 against HCC has never been reported, and the related mechanism is still unknown. The current study focused on the inhibitory effects of TAD1822-7 on HCC and the involved mechanisms.

EXPERIMENTAL

Materials

The taspine derivative TAD1822-7 was synthesized in School of Pharmacy, Xi'an Jiaotong University (8). EphB4 Fluorescence-chimera (B4-Fc)

was purchased from Beijing Biotechnology Co., Ltd. (MDL No. MFCD01867528, Beijing, China). DAPI, MTT, RPMI-1640 medium and Dulbecco's Modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thrombin was obtained from Guoao Pharmaceutical (Changchun, China). Fetal bovine serum (FBS) was purchased from HyClone (Logan, Utah, USA). G418 was obtained from Gibco (Grand Island, NY, USA). The RIPA lysis buffer was purchased from Beijing BLKWB Biotechnology Co., Ltd (Beijing, China). The enhanced Chemiluminescence (ECL) Plus Reagent kit was obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA). EphrinB2 (Cat. #6957-1), MMP9 (Cat. #2551-1), and MMP2 (Cat. #1948-1) antibodies were purchased from Epitomics (Burlingame, CA, USA). VEGFR2 (Cat. #26415-1-AP), VEGFR3 (Cat. #20712-1-AP), PICK1 (Cat. #10983-2-AP), Syntenin-1 (Cat. #22399-1-AP), CD34 (Cat. #14486-1-AP), CXCR4 (Cat. #11073-2-AP), and GAPDH (Cat. #60042-1-IG) antibodies were purchased from Proteintech Group (Chicago, IL, USA). Akt (Cat. #4685), PLCγ1 (Cat. #5690), and anti-mouse IgG (H+L; Cat. #14709), and anti-rabbit IgG (Cat. #14708) secondary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cells and animals

The Bel-7402, HepG2, A549, PANC-1, and SGC-7901 cell lines were purchased from Shanghai Institute of Cell Biology in the Chinese Academy of Science (Shanghai, China) and cultured in RPMI-1640 medium supplemented with 10 % FBS. EphrinB2/HEK293 cells were constructed at Food and Drug Research & Test Center, Xi'an Jiaotong University and cultured in DMEM supplemented with 300 mg·mL^{-1} G418, penicillin/streptomycin and 10 % FBS.

Sprague-Dawley rats (40–60 g, 2–4 weeks of age, 3 male and 3 female) were purchased from Animal Research center of Xi'an Jiaotong University, and were sacrificed through CO_2 inhalation. The animal experimental protocol was approved by the Animal Ethics Committee of Xi'an Jiaotong University (license number: XJTULAC 2018-0542).

Cell viability test

The cell viability test was assessed by MTT assay and cell colony survival assay. In the MTT assay, cells were treated for 48 h with 4, 8 and 16 μM TAD1822-7 dissolved in RPMI-1640 medium and the medium was used as control, and then incubated with MTT solution, the formed crystals were dissolved with DMSO, and the absorbance at 490 nm was measured by Molecular Devices FlexStation® 3 microplate reader (San Jose, CA, USA). In the colony survival assay, cells were plated at 200 cells per well in a 12-well

plate and treated with TAD1822-7 when the colonies were visible and countable. The colonies were fixed in methanol, stained with crystal violet, and observed under the microscope.

EphB4 Fluorescence-chimera (B4-Fc) binding to EphrinB2

The binding ability of B4-Fc to EphrinB2 on cytomembrane of Bel-7402 was assessed by EphrinB2 expression analysis. Bel-7402 cells were seeded in 96-well plates, incubated with 1 mg·mL^{-1} B4-Fc dissolved in RPMI-1640 medium for 4 h at 37 ℃, and observed by using a FITC fluorescence microscope. To determine the target role of TAD1822-7 on EphrinB2, HEK293/EphrinB2 cells were seeded in 96-well plates and co-cultured with 1 mg·mL^{-1} B4-Fc and 4, 8 and 16 μM TAD1822-7 dissolved in DMEM for 6 h at 37 ℃，and the medium was used as control. Subsequently, the cells were washed with PBS twice, fixed in 4 % paraformaldehyde (PFA) for 15 min, stained with 1 μg·mL^{-1} DAPI for 15 min, and observed by using a fluorescence microscope.

Cell migration test

The cell migration test was performed using the Transwell system, in which there is an 8 mm pore of Millicell polycarbonate membrane for the cells to

migrate through. Serum-starved Bel-7402 cells were plated at a density of 1 × 10^4 cells per well in the upper chamber of the system and treated with TAD1822-7. After migration, the cells on the lower surface were fixed, stained with 0.2 % crystal violet, and observed under a microscope.

Aortic ring culture

Dissected thoracic aortas from Sprague-Dawley rats, transferred immediately to PBS, the adventitia was removed, and the small vessels around the aorta were carefully transverse truncated with 1 mm cuts and cultured in polymerized fibrinogen with thrombin dissolved in DMEM in 48-well plates. More polymerized fibrinogen with thrombin was put on the aortic ring, and the aortic ring was cultured in the sandwich of the medium. After 7 days, the sprouting vessels were observed under a stereomicroscope and treated for 48 h with 4, 8 and 16 μM TAD1822-7 in DMEM and DMEM was used as control. Neo-microvessels were observed and imaged through the experiment.

Western blotting analysis

After treatment with 0, 4, 8 and 16 μM TAD1822-7 for 48 h, the proteins in the Bel-7402 cells were extracted by using RIPA lysis buffer, denatured,

separated by electrophoresis, and transferred to polyvinylidene fluoride membranes. After incubation with the corresponding primary antibodies and secondary antibodies, the membranes containing the proteins were exposed by using an ECL kit. The Image-Pro Plus 5.1 (Rockville, MD, USA) was used for the quantification of protein expression.

Statistical analysis

The data were expressed as the mean ± S.D. and a two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, with statistical significance was accepted at $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$) All statistical analyses were computed by using GraphPad Prism 6.0 (La Jolla, CA, USA).

RESULTS AND DISCUSSION

TAD1822-7 inhibited cell viability and colony formation

The cell viability of Bel-7402, HepG2, A549, PANC-1, and SGC-7901 cells treated by TAD1822-7 were investigated by using MTT assay (Fig. 1b), which revealed an inhibitory effect, especially in Bel-7402 cells. Meanwhile, when treated with 0, 4, 8 and 16 μM TAD1822-7 until the colonies were visible and

147 countable, the colony formation was also similarly inhibited (Fig. 1c). Hence,
148 TAD1822-7 has a potential antitumor activity in HCC and that may be
149 regulated by EphrinB2.

150 *TAD1822-7 and B4-Fc competitively bind to EphrinB2*

151 The B4-Fc staining of Bel-7402 cells revealed a high expression of EphrinB2
152 (Fig. 2a). The protein expression of EphrinB2 was effectively downregulated
153 in both Bel-7402 cells (Fig. 2b,c) and EphrinB2/HEK293 cells (Fig. 2d,e) after
154 the treatment of 0, 4, 8 and 16 μM TAD1822-7, which indicated that
155 TAD1822-7 could suppress EphrinB2 expression. In addition,
156 EphrinB2/HEK293 cells were treated with 0.5 $mg \cdot mL^{-1}$ B4-Fc and 0, 4, 8 and 16
157 μM TAD1822-7 simultaneously for 4 h, and then stained with DAPI. The
158 results showed that TAD1822-7 inhibited the binding of B4-Fc to EphrinB2 in
159 EphrinB2/HEK293 cells (Fig. 2f), indicating that TAD1822-7 and B4-Fc could
160 competitively bind to EphrinB2 and TAD1822-7 to inhibit the activation of
161 EphrinB2.

162 *TAD1822-7 inhibited Bel-7402 cell migration*

163 TAD1822-7 dose-dependently inhibited the migration ability of Bel-7402
164 cells in the transwell migration assay (Fig. 3a,b). MMP2 and MMP9 are critical

components in cancer cell invasion and migration (9), and CXCR4 is associated with cancer cell migration (10). In this study, TAD1822-7 inhibited the expression of MMP2, MMP9, and CXCR4 in Bel-7402 cells in a dose-dependent manner (Fig. 3c,d), which was thought to confer the anti-migration effect.

TAD1822-7 inhibited angiogenesis in the aortic ring model

In the aortic ring assay, the entire angiogenesis process can be reproduced *in vitro* through the culture of rat aortic explants in a three-dimensional cell growth matrix under chemically defined growth factors conditions. After the sprouting vessels were observed, 0, 4, 8 and 16 μM TAD1822-7 were added and cultured for 48 h. The result showed that TAD1822-7 inhibited the number of sprouting vessels (Fig. 4a), which indicated an inhibitory effect on angiogenesis. Pitulescu, M.E. *et al*. have reported EphrinB2 was crucial in tumor angiogenesis, which can be regarded as a promising prognostic indicator and a target to modulate angiogenesis in cancer therapies (11).

CD34 protein expression is found on early hematopoietic and vascular-associated tissue and also facilitates in cell migration (12). VEGFR2 is the main mediator for the mitogenic, angiogenic, and enhanced permeability properties of VEGF-A, and VEGFR3 is also reported to be involved in

angiogenesis in cancer; the blocking of those proteins are thought to suppress angiogenic sprouting and vascular network formation (8). TAD1822-7 suppressed the expression of VEGFR2 and VEGFR3 in Bel-7402 cells in a dose-dependent manner after treatment for 48 h, and CD34 expression was also downregulated by TAD1822-7 (Fig. 4b,c).

TAD1822-7 regulated EphrinB2 PDZ-binding motif protein of Bel-7402 cells

EphrinB2 plays a crucial role in tumor angiogenesis, for which the presence of the C-terminal PDZ motif is required to allow the recruitment of the related functional proteins around the EphrinB2 intracellular region. Syntenin-1 and PICK1 are the PDZ-binding motif protein of EphrinB2, and may affect the activation of EphrinB2 (13). The expression of PICK1 was suppressed drastically by TAD1822-7, but there was a minimal effect of TAD1822-7 on Syntenin-1 (Fig. 4d,e), which indicated that TAD1822-7 inhibited the activation of EphrinB2 through its PICK1 intracellular tyrosine kinase domain.

TAD1822-7 inhibited the related downstream signal pathways

VEGFR2 is essential for many angiogenic processes in tumor vascularization that regulate the downstream signaling pathway to affect cell

function. TAD1822-7 has the ability to inhibit the protein expression of PLC-γ and Akt (Fig. 4f,g). The PLCγ/PKC and PI3K/Akt signaling pathways are two of the diverse molecular signaling pathways related to tumor cell growth, and the activation of these two pathways may lead to aberrant cell proliferation (14). TAD1822-7 inhibited the expression of PLC-γ and Akt in Bel-7402 cells. The activation of VEGFR3 is known to cause PI3K-dependent activation of Akt and PKC (15). We suspected that TAD1822-7 inhibited the expression of EphrinB2, thereby suppressing the related PI3K/Akt and PLCγ/PKC signaling pathways. The inhibition of EphrinB2 by TAD1822-7 will negatively regulate VEGFR2, VEGFR3, and CD34 to inhibit angiogenesis.

CONCLUSIONS

EphrinB2 is an angiogenesis-related transmembrane protein that is upregulated and requires the presence of its C-terminal PDZ motif for activity in tumor cells. TAD1822-7 is a synthesized taspine derivative for tumor growth inhibition, which inhibits cell viability and migration in Bel-7402 cells via competitively binding to EphrinB2 and regulating EphrinB2 related signaling molecules expression. Meanwhile, TAD1822-7 was demonstrated that it has the potential function of anti-angiogenesis. Furthermore, the results

suggested TAD1822-7 is a potential anti-tumor agent for hepatocellular carcinoma.

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REFERENCES

1. D. S. Dizon, L. Krilov, E. Cohen, T. Gangadhar, P. A. Ganz, T. A. Hensing, S. Hunger, S. S. Krishnamurthi, A. B. Lassman, M. J. Markham, E. Mayer, M. Neuss, S. K. Pal, L. C. Richardson, R. Schilsky, G. K. Schwartz, D. R. M. A. Spriggs, Villalona-Calero, G. Villani and G. Masters, Clinical Cancer Advances 2016: Annual Report on Progress Against Cancer From the American Society of Clinical Oncology, *J. Clin. Oncol*. **34 (**2016) 987–1011; https://doi.org/10.1200/JCO.2015.65.8427
2. N. Holder and R. Klein, Eph receptors and ephrins: effectors of morphogenesis, *Development* **10** (1999) 2033–2044.
3. J. E. Chrencik, A. Brooun, M. L. Kraus, M. I. Recht, A. R. Kolatkar, G. W. Han, J. M. Seifert, H. Widmer, M. Auer and P. Kuhn, Structural and biophysical characterization of the EphB4*ephrinB2 protein-protein interaction and receptor specificity, *J. Biol. Chem*. **38** (2006) 28185–28192; https://doi.org/10.1074/jbc.M605766200
4. S. S. Gerety, H. U. Wang, Z. F. Chen and D. J. Anderson, Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development, *Mol. Cell*. **3** (1999) 403–414.
5. J. Folkman, Angiogenesis in cancer, vascular, rheumatoid and other diseases, *Nat. Med*. **1** (1995) 27–31; https://doi.org/10.1038/nm0195-27
6. S. R. Kumar, J. Singh, G. Xia, V. Krasnoperov, L. Hassanieh, E. J. Ley, J. Scehnet, N. G. Kumar, D. Hawes, M. F. Press, F. A. Weaver and P. S. Gill, Receptor tyrosine kinase EphB4 is a survival factor in breast cancer, *Am. J. Pathol*. **169** (2006) 279–293; https://doi.org/10.2353/ajpath.2006.050889
7. D. Yang, C. Jin, H. Ma, M. Huang, G. Shi, J. Wang and M. Xiang, EphrinB2/EphB4 pathway in postnatal angiogenesis: a potential therapeutic target for ischemic cardiovascular disease, *Angiogenesis* **19** (2016) 297–309; https://doi.org/10.1007/s10456-016-9514-9
8. C. Wang, J. Dong, Y. Zhang, F. Wang, H. Gao, P. Li, S. Wang and J. Zhang, Design, synthesis and biological evaluation of biphenyl urea derivatives as novel VEGFR-2 inhibitors, *Medchemcomm* **11** (2013) 1434–1438; https://doi.org/10.1039/C3MD00192J
9. Q. Y. Chen, Y. Zheng, D. M. Jiao, F. Y. Chen, H. Z. Hu, Y. Q. Wu, J. Song, J. Yan, L. J. Wu and G. Y. Lv, Curcumin inhibits lung cancer cell migration and invasion through Rac1-dependent signaling pathway, *J. Nutr. Biochem*. **25** (2014) 177–185; https://doi.org/10.1016/j.jnutbio.2013.10.004

10. Y. Chen, G. Stamatoyannopoulos and C. Z. Song, Down-regulation of CXCR4 by inducible small interfering RNA inhibits breast cancer cell invasion in vitro, *Cancer Res*. **63** (2003) 4801–4804.
11. M. E. Pitulescu and R. H. Adams, Eph/ephrin molecules--a hub for signaling and endocytosis, *Genes Dev*. **24** (2010) 2480–2492; https://doi.org/10.1101/gad.1973910
12. J. S. Nielsen and K. M. McNagny, Novel functions of the CD34 family, *J. Cell Sci*. **121** (2008) 3683–3692; https://doi.org/10.1242/jcs.037507
13. T. Makinen, R. H. Adams, J. Bailey, Q. Lu, A. Ziemiecki, K. Alitalo, R. Klein and G. A. Wilkinson, PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature, *Genes Dev*. **19** (2005) 397–410; https://doi.org/10.1101/gad.330105
14. T. V. Byzova, C. K. Goldman, N. Pampori, K. A. Thomas, A. Bett, S. J. Shattil and E. F. Plow, A mechanism for modulation of cellular responses to VEGF: activation of the integrins, *Mol Cell*. **6** (2000) 851–860.
15. T. Makinen, T. Veikkola, S. Mustjoki, T. Karpanen, B. Catimel, E. C. Nice, L. Wise, A. Mercer, H. Kowalski, D. Kerjaschki, S. A. Stacker, M. G. Achen and K. Alitalo, Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3, *EMBO J*. **20** (2001) 4762–4773; https://doi.org/10.1093/emboj/20.17.4762.