



Commentary

An Antiangiogenic Agent Associated with Multi-Targets Exhibits Synergistic Antitumor Effects in Combination with Immunotherapeutics

Liting Xue^{1#}, Kun Wang^{1#}, Xinxin Li¹, Janine Y Yang², Wenjie Song¹, Renhong Tang¹, WenQing Yang^{1*}

¹State Key Laboratory of Translational Medicine and Innovative Drug Development, Jiangsu Simcere Pharmaceutical Co., Ltd., Nanjing, China

²Massachusetts Eye and Ear, Harvard Medical School, Boston, USA

*Corresponding Author: WenQing Yang, Jiangsu Simcere Pharmaceutical Co., Ltd., Nanjing, Jiangsu, China

#These authors contributed equally to this work

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Abstract

Recently, an increasing number of studies have focused on the combination of antiangiogenic agents and Immune Checkpoint Inhibitors (ICIs) in preclinical and clinical settings. In this commentary, we discuss the combination of recombinant human endostatin (Endostar, Simcere) with immunotherapy. Similar to the anti-vascular endothelial growth factor (VEGF) antibody BD0801, Endostar is an antiangiogenic agent with VEGF binding affinity. In addition, Endostar binds to basic Fibroblast Growth Factor (bFGF) and blocks the binding of bFGF to FGF receptor 1 (FGFR1). Endostar in combination with anti-PD-L1 antibody showed synergistic antitumor effects in both colorectal cancer and melanoma mouse models. Furthermore, VEGF concentrations in the serum of tumor-bearing mice were significantly decreased upon Endostar and combination treatments. Significantly, increased CD8⁺ T cell infiltration and reduced Microvessel Density (MVD) in tumors were observed in the combination group. In conclusion, Endostar exerted a synergistic antitumor effect with immunotherapy, revealing a mechanism of synergy in the combination of antiangiogenic agents and immunotherapy involving tumor microenvironment modulation.

Keywords: Angiogenesis; VEGF; Immune checkpoint; Synergy; Anti-tumor therapy

Introduction

This short commentary is based on our recent paper entitled “Antiangiogenic antibody BD0801 combined with Immune Checkpoint Inhibitors (ICIs) achieves synergistic antitumor activity and affects the tumor microenvironment”, which aimed to investigate the synergistic anti-tumor effects of combining anti-Vascular Endothelial Growth Factor (VEGF) antibody BD0801 with ICIs and to explore the underlying mechanisms [1].

Anti-angiogenesis therapy has long been investigated and developed for cancer treatment [2, 3]. Angiogenesis is regulated by the key regulators of vascular development and network patterning,

including VEGF, Hepatocyte Growth Factor (HGF), Fibroblast Growth Factor (FGF) and Transforming Growth Factor (TGF)- β [4]. VEGF, the key factor of vasculogenesis and angiogenesis [5], induces the expression of collagenases [6] and plasminogen activators [7] and promotes proliferation and migration of endothelial cells to form blood vessels, providing an environment and structure of circulation for tumor progression [8,9]. On the other hand, VEGF is associated with immunotherapy because of its effect on immune cells in the Tumor Microenvironment (TME). Overexpression of VEGF leads to structural and functional abnormalities of tumor blood vessels, resulting in reduced recruitment and invasion of immune cells into the tumor tissue through the vascular wall [10]. In addition, VEGF exhausts CD8⁺ T cells by inhibiting their proliferation and upregulating the expression of Programmed Death Receptor 1 (PD-1), T cell

immunoglobulin and mucin domain 3 (TIM-3), lymphocyte activation gene 3 (LAG-3) and cytotoxic T lymphocyte antigen 4 (CTLA-4) [11]. VEGF also increases the immune escape ability of tumor cells by enhancing the expression of programmed cell death ligand 1 (PD-L1) in dendritic cells [12,13].

BD0801, a humanized rabbit anti-VEGF monoclonal antibody undergoing phase 3 clinical studies, was developed by Simcere Pharma (Nanjing, China). We have reported the anti-tumor effects and mechanisms of BD0801 combined with PD-1/PD-L1 blockade in tumor mouse models to provide a guidance for BD0801 application in further clinical practice [1]. The combination of BD0801 and ICIs exert better anti-tumor effects compared with single agents in both lung and colorectal cancer mouse models by enhancing T-cell mediated immunity and improving vasculature normalization in the TME.

Due to biological complexity and drug resistance considerations associated with antiangiogenic therapies, multi-target anti-angiogenic therapeutics are increasingly considered efficient regimens to be combined with immunotherapy for difficult-to-treat malignancies. Anlotinib, an anti-angiogenic agent targeting VEGF receptors (VEGFR), Platelet-Derived Growth Factor Receptor (PDGFR) and epidermal growth factor receptor (EGFR), has sufficient activity and acceptable toxicity when used concomitantly with anti-PD-1 treatment in patients with advanced NSCLC [14,15]. Likewise, Nintedanib is a triple vascular kinase inhibitor targeting VEGFR, FGFR and PDGFR which has been shown to be a durable and synergistic antitumor activity in combination with PD-1 blockade [16,17]. Endostar is a commercial anti-angiogenic drug associated with multiple targets. The current study is designed to investigate the potential therapeutic benefits of Endostar in combination with immune checkpoint blockade approaches in preclinical models, with a hope to expand Endostar's clinical application.

Endostar is an Anti-Angiogenetic Agent Binding to Multiple Targets

Endostar, a novel recombinant human endostatin, was approved by Center For Drug Evaluation (CDE) China, for the treatment of non-small cell lung cancer in 2005 [18]. It exerts strong antiangiogenic and tumor growth inhibitory effects by targeting multiple targets in angiogenesis. Endostar inhibits angiogenesis by binding the VEGF receptor KDR/Flk-1 and inhibits the signal transduction of VEGF [19]. Although Endostar and BD0801 have similar effects, Endostar modulates other components in angiogenesis as well. It directly regulates multiple signaling pathways such as Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2) phosphorylation, nucleolin phosphorylation and FAK/C-Raf/MEK1/2/ P38 /ERK1 signaling [20-23]. In addition, Endostar adjusts the TME by increasing the PH value inside, improving the inter-tissue pressure [24]. Most notably, Endostar attenuates melanoma tumor growth by suppressing FGF mediated angiogenesis [25]. FGF receptors (FGFRs) are a family of Receptor Tyrosine Kinases (RTKs) involved in cell proliferation, differentiation and angiogenesis [26].

In this report, we examined the binding affinity and blocking ability of Endostar to VEGF, bFGF, and bFGF/FGFR1. To quantitate the blocking effect on the binding of Endostar to VEGF and bFGF, human VEGF and/or bFGF were incubated with different concentrations of Endostar before addition to ELISA plates. As shown in Figure 1A and Figure 1B, Endostar was able to bind to both VEGF and bFGF at doses of 30 mM and 100 mM, respectively, as well as to the mixture of VEGF and bFGF. Endostar inhibited the binding of bFGF to FGF receptor 1 (FGFR1) by occupying bFGF (Figure1C). These results showed that Endostar can efficiently block the binding of bFGF and FGFR1, which is consistent with Ling's work [27].

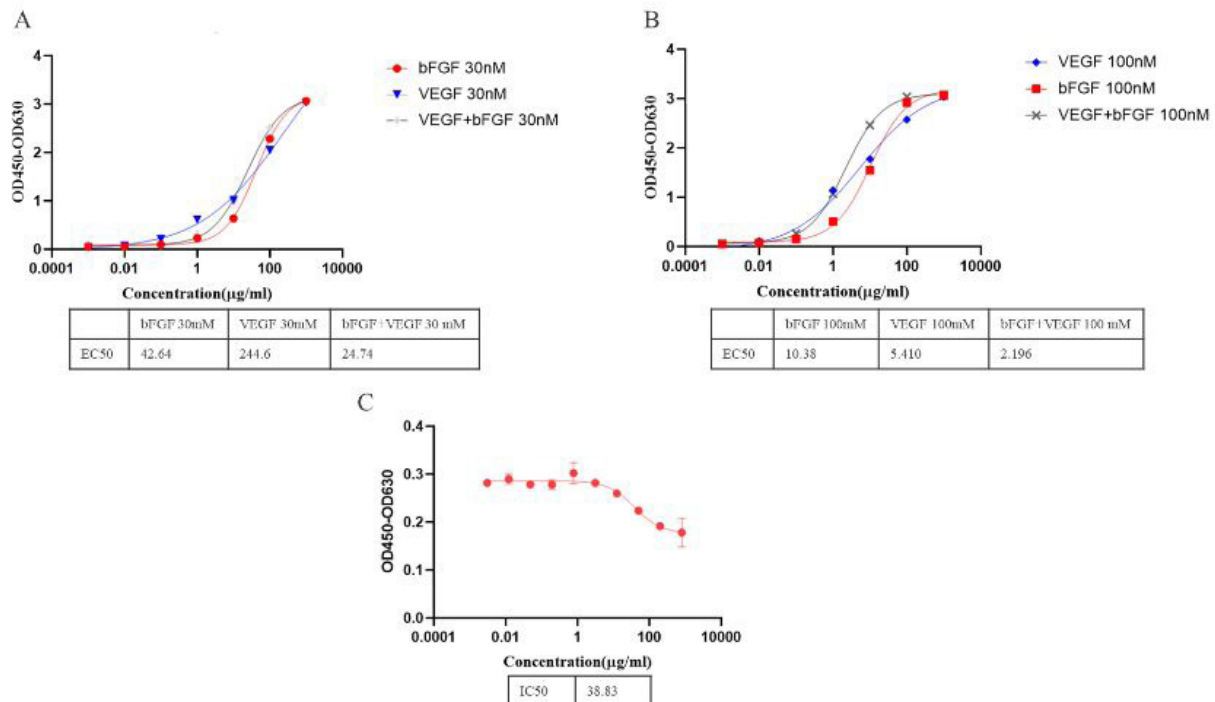


Figure 1: Binding of Endostar to VEGF and bFGF.

(A) Binding affinity of Endostar to 30 nM VEGF, 30 nM bFGF and a mixture of VEGF and bFGF. EC50: concentration for 50% of maximal effect; (B) Binding affinity of Endostar to 100 nM VEGF, 100 nM bFGF and a mixture of VEGF and bFGF. (C) Blocking curve of Endostar to bFGF/FGFR1 binding, 800 µg/ml Endostar was diluted as 4 folds.

Endostar in Combination with ICI Demonstrates Synergistic Antitumor Effects in Multiple Animal Models

As attractive therapies for malignant tumors, numeral anti-PD-1 and PD-L1 antibodies have been developed to stimulate the immune system by blocking their interaction [28]. However, long-term response is still limited, and approximately 87% of cases are not responsive to PD-1/PD-L1 blockade [29]. Some tumor types, including pancreatic, colorectal and prostate cancers, exhibit intrinsic resistance to PD-1/PD-L1 axis blockade [30]. Primary or acquired drug resistance is caused by tumor immune escape via alternative immunosuppressive mechanisms [31]. Overall, the TME, tumor immunogenicity, antigen presentation, and tumor signal transduction pathways all play important roles in response and resistance to immune checkpoint blockade, resulting in the failure of PD-1/PD-L1 blockade used alone. In addition, abnormal blood vessels in the tumor tissue block the extravasation of tumor-responsive T cells and create an immunosuppressive microenvironment that allows tumor cells to escape immune surveillance in the host [32]. It has been generally accepted that,

at least some of the anti-VEGF/VEGFR-based therapeutics can alleviate immune suppression, in addition to their main roles of anti-angiogenesis. Combining immunotherapy with different mechanism-based treatments and modalities, including anti-angiogenic agents or multi-target inhibitors, may be able to reverse the immunosuppressive tumor microenvironment and facilitate better clinical outcome.

Two different animal models were developed and employed to examine the potential synergy of Endostar with immune checkpoint blockade *in vivo*. To verify the effect of anti-PD-L1 combined with Endostar, S91 melanoma-bearing mice were treated with anti-PD-L1 antibody, PEGylated Endostar (PEG-Endostar) or a combination of the two agents. As shown in Figure 2A, 5 mg/kg of anti-PD-L1 antibody or 30 mg/kg PEG-Endostar as a single agent reduced tumor growth compared with the vehicle. Meanwhile, the combination of 5 mg/kg anti-PD-L1 antibody and 30 mg/kg PEG-Endostar exhibited a significantly stronger suppression of tumor growth compared with single agent treatment, indicating synergistic antitumor effects for Endostar and immunotherapy. In

addition, CT26 colorectal tumor bearing mice were treated with anti-PD-L1 antibody, Endostar or the combination of the two. As shown in Figure 2B, 10 mg/kg anti-PD-L1 antibody or 45 mg/kg Endostar as a single agent reduced tumor growth compared with vehicle. Meanwhile, the combination of 10 mg/kg anti-PD-L1 antibody and 45 mg/kg Endostar exerted a significantly stronger suppression of tumor growth compared with single agent treatments, suggesting synergistic antitumor effects for Endostar and immunotherapy.

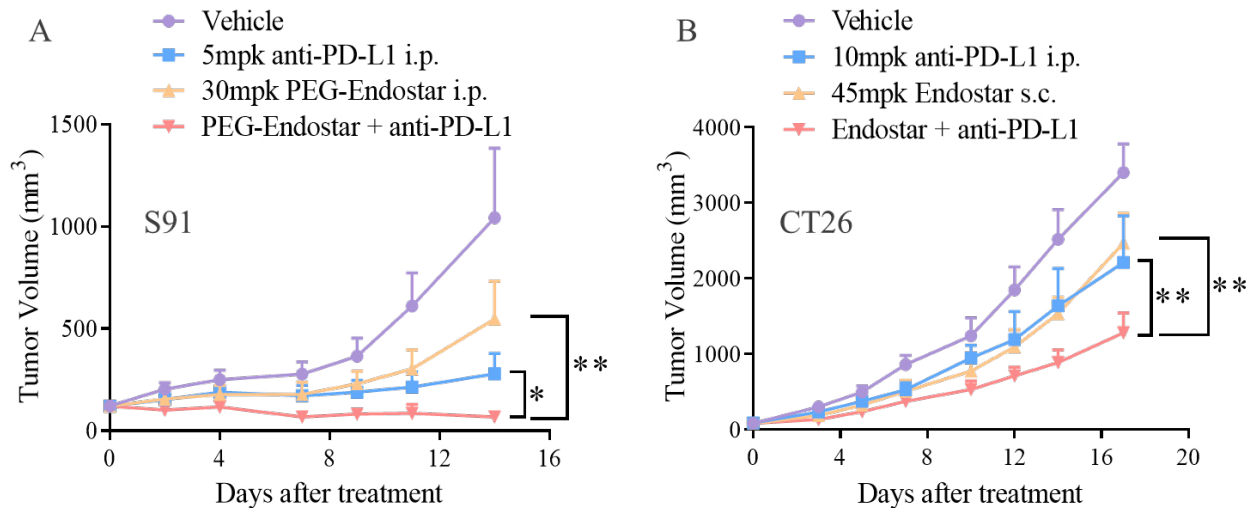


Figure 2: Antitumor activity of Endostar combined with anti-PD-L1 antibody.

(A).Tumor volumes of S91 tumor-bearing DBA/2 mice treated with vehicle, anti-PD-L1 antibody, PEG-Endostar or PEG-Endostar and anti-PD-L1 antibody combination (n=6/group). (B) Tumor volumes of CT26 tumor-bearing BALB/c mice treated with vehicle, anti-PD-L1 antibody, Endostar or Endostar and anti-PD-L1 antibody combination (n=6/group). P-values were calculated in two-way ANOVA; *P < 0.05; **P < 0.01.

To investigate the possible mechanisms of actions behind the phenotypic changes, we analyzed *ex vivo* samples collected at end of these experiments. In the CT26 model, serum VEGF concentrations in tumor-bearing mice were significantly decreased upon Endostar and combo treatments, respectively (data not shown). In addition, increased tumor infiltrated CD8⁺ T cells and reduced Microvessel Density (MVD) in tumors were observed in the combination group (data not shown). VEGF interacts with immune cells to decrease the recruitment and invasion of these cells and inhibits CD8⁺ cell proliferation, creating an immunosuppressive microenvironment [10,11]. In addition, VEGF enhances microvascular density (MVD), which increases common vascular endothelial cell markers including CD31 and CD34 [33,34]. These results suggest that the synergistical anti-tumor efficacy of the Endostar/aPD-1 combination may be associated with normalized vasculature and improved TME.

Discussion

This study has limitations. Endostar is an anti-cancer agent with very broad biological effects; numeral targets have been proposed to be associate with Endostar's MOA. Although we demonstrated that Endostar could improve therapeutic activities

of anti-PD-1 antibodies in multiple animal models, the exact mechanism of action remains to be elucidated. Endostar also has a broad anti-angiogenic effect on endothelial cells. Besides targeting VEGF and bFGF, Endostar decreases migration and invasion of endothelial cells by targeting Matrix Metalloproteinases (MMPs), especially MMP-2, MMP-9 and MMP-13 [35,36]. Endostar can inhibit the FAK/Ras/p38-MAPK/ERK signaling cascade through another target, $\alpha 5 \beta 1$ -integrin [37,38]. Furthermore, Endostar blocks Wnt signaling via downregulation of β -catenin in a GSK-3 β -independent manner, which suppresses the transcription of important genes involved in the cell cycle including Myc and cyclin D1 [39]. In addition, Endostar plays a role in several other targets such as integrins, Activating Protein 1 (AP-1) and thrombospondin-1 [40]. Moreover, Endostar blocks the metastasis, invasion, and angiogenesis of ovarian cancer cells by reducing the activation of PD-L1 and signal transducer and activator of transcription 3 (STAT3) [41]. Therefore, we speculate that, in comparison with BD0801 or other anti-angiogenic agents with a single target, Endostar in combination with immunotherapy might exert a better or durable anti-cancer effects in tumor cells addicted to various abnormal signaling pathways. More studies are needed to support this hypothesis; some experiments are currently in

progress in our laboratory.

To our knowledge, this paper may be the first report to fully demonstrate synergistic anti-tumor activities of Endostar in combination with immune-therapy *in vivo*. Preclinical investigation of Endostar in combination with anti-PD-1 antibody has been attempted and data has been reported by Wu, et al. [42]. However, in the animal experiments described by Wu, et al. [42], an important single agent treatment control was missing in the study design. Therefore, more experiments are warranted in order to fully support the data interpretation.

Conclusions

Endostar is an anti-angiogenic agent associated with multiple targets. Endostar in combination with anti-PD-1 therapeutics demonstrates synergistic anti-tumor activities in preclinical settings. Combinatorial treatment using Endostar and immunotherapy is currently being tested in clinical trials in solid tumor patients in China. More experiments are warranted in order to fully understand Endostar's MOA including its anti-angiogenic and immune-regulatory activities in cancer.

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