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Anti-VEGFR2-Interferon-α2 regulates the tumor microenvironment and exhibits potent anti-tumor efficacy against colorectal cancer

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Abstract

Interferon-α (IFNα) has multiple anti-tumor effects including direct anti-tumor toxicity and the ability to potently stimulate both innate and adaptive immunity. However, its clinical applications in the treatment of malignancies have been limited because of short half life and serious adverse reactions when attempting to deliver therapeutically effective doses. To address these issues, we fused IFNα2a to the anti-VEGFR2 antibody JZA00 with the goal of targeting it to the tumor microenvironment where it can stimulate the anti-tumor immune response. The fusion protein, JZA01, is effective against colorectal cancer by inhibiting angiogenesis, exhibiting direct cytotoxicity and activating the anti-tumor immune response. Although JZA01 exhibited reduced IFNα2 activity in vitro compared with native IFNα2, VEGFR2 targeting permitted efficient
antiproliferative, proapoptotic, antiangiogenesis and immune-stimulating effects against the colorectal tumors HCT-116 and SW620. JZA01 showed \textit{in vivo} efficacy in NOD-SCID mice bearing established HCT-116 tumors. In conclusion, this study describes an anti-tumor immunotherapy that is highly promising for the treatment of colorectal cancer.

\textbf{Key words}

IFNα2, tumor microenvironment, VEGFR2, DCs, CD8+ T cells, colorectal cancer
Introduction

The tumor microenvironment (TME) includes surrounding blood vessels, immune cells, fibroblasts, bone marrow-derived inflammatory cells, lymphocytes, signaling molecules and the extracellular matrix (ECM).\(^1\) The tumor and the surrounding microenvironment interact constantly. Tumors can influence the microenvironment by releasing extracellular signals, promoting angiogenesis and inducing peripheral immune tolerance, while the immune cells in the microenvironment can affect the growth and evolution of cancerous cells by mechanisms such as immune editing and production of cytokines,\(^2,3\) among which IFN\(\alpha\) is effective in regulating the tumor microenvironment.

IFN\(\alpha\) is a pleiotropic cytokine, which has been used in the treatment of cancer including hematologic tumors and metastatic solid tumors for over 30 years both alone and in combination with chemotherapeutic agents.\(^4,5\) IFN\(\alpha\) has several anti-tumor activities including regulating the immune response through activating dendritic cells, promoting the long-term survival of T cells and enhancing the killing ability of T cells and NK cells.\(^6-8\) Additionally, IFN\(\alpha\) exhibits direct cytotoxicity by inhibiting the proliferation and promoting apoptosis of tumor cells.\(^9,10\)

Although IFN\(\alpha\) can exert efficient anti-tumor effects, because of its short in vivo half-life it requires frequent administration and at therapeutically effective doses produces toxic side effects including severe depression, fever, headache, joint and muscle pain leading many patients to abandon IFN\(\alpha\) treatment.\(^11\) To extend the \(t_{1/2}\), IFN\(\alpha\) has been coupled with PEG, albumin or antibody Fc fragment. These approaches have improved in vivo dynamics but did not eliminate the serious systemic toxicity.\(^12-14\) In contrast, fusion proteins with IFN\(\alpha\) joined to the tumor targeting anti-CD20 antibody showed both improved pharmacokinetics and tumor targeting in the absence of non-specific toxicity.\(^15,16\)
Angiogenesis is critical for tumor growth and metastasis with vascular endothelial growth factor and receptor 2 (VEGF/VEGFR2)-mediated signaling playing an important role.\textsuperscript{17-19} IFNα can inhibit tumor angiogenesis by down regulating the expression of VEGF and HIF-1α, resulting in a synergistic effect with anti-VEGF.\textsuperscript{20,21} The combination therapy of the anti-VEGF antibody Bevacizumab with IFNα was approved by the U.S. FDA in 2009 as first-line treatment for metastatic renal cell carcinoma (mRCC).\textsuperscript{22} However, it had serious side effects and blocking VEGF by Bevacizumab did not completely inhibit tumor angiogenesis.\textsuperscript{23-25} In April 2014, the anti-angiogenesis antibody Ramucirumab targeting VEGFR2, was approved by the FDA for treatment of chemotherapy-resistant gastric cancer.\textsuperscript{26,27}

Thus the combination of IFNα with antibodies that inhibit angiogenesis can both reactivate the immune state of the tumor microenvironment and block the tumor vascular supply.\textsuperscript{22,28} As a consequence, VEGF/VEGFR2 signaling pathway inhibitors and IFNα may play a synergistic role in inhibiting proliferation and promoting apoptosis of cancers.\textsuperscript{10}

Based on these observations, we hypothesized that fusing IFNα2 with anti-VEGFR2 can not only extend the \textit{in vivo} half-life of IFNα2, but the anti-VEGFR2 will also be able to deliver IFNα2 to the tumor site where it will be active in the absence of systemic toxicity. Hence, IFNα2 carried to the tumor site can activate the immune response in the tumor microenvironment, produce direct toxic effect on tumor cells and inhibit tumor angiogenesis. Our earlier studies had shown that anti-VEGFR2 exhibits an anti-tumor effect in breast cancer and leukemia.\textsuperscript{29,30} We now extend these studies and show that anti-VEGFR2-IFNα2 (JZA01) has therapeutic efficacy both \textit{in vivo} and \textit{in vitro} against metastatic colorectal cancer.

\textbf{Results:}

Generation and identification of JZA01
IFNα2 was fused to the carboxy-terminus of the H chain of JZA00 through a G4S linker (Fig. 1A). Western blot analysis, with anti-human kappa chain and anti-human IFNα following non-reducing and reducing SDS-PAGE, demonstrated that the fully-assembled fusion protein JZA01 contained both JZA00 (150 kD) and two IFNα (19 kD) resulting in a molecular weight of around 190 kD with the H chain containing IFNα having a molecular weight of about 75 kD (Fig. 1B). Similar results were seen with proteins purified by protein A affinity chromatography (unreduced Fig. 1C; reduced Fig. 1D).

JZA01 binds specifically to VEGFR2

The affinity of human VEGFR2, also known as tyrosine kinase insert domain receptor (KDR) to immobilized JZA01 or JZA00 was determined by Biacore system with calculations made using the 2:1 binding model. The association increased with increasing concentration of the KDR3 (from bottom to top), ranging from 0.78125 nM to 100 nM. The complex dissociated when buffer flowed through after 120 s. Both JZA01 and JZA00 showed similar binding affinity: The affinity of JZA01 was $K_D = 3.72 \times 10^{-9}$ M ($k_{on} = 3.15 \times 10^5 \text{M}^{-1} \text{s}^{-1}$, $k_{off} = 1.30 \times 10^{-3} \text{s}^{-1}$; Fig. 2A), while that of JZA00 was $K_D = 4.92 \times 10^{-9}$ M ($k_{on} = 2.09 \times 10^5 \text{M}^{-1} \text{s}^{-1}$, $k_{off} = 1.03 \times 10^{-3} \text{s}^{-1}$; Fig. 2B), indicating that the fusion retains the antigen binding ability of the parental antibody. Using flow cytometry, no binding was seen to human hepatic cell L02 (Fig. 2C) while JZA00 and JZA01 bound equivalently to HUVECs (Fig. 2D). To further investigate the expression of VEGFR2 in cancer cells, we detected the expression of VEGFR2 in HCT-116 and SW620 cells using western blot assay and found that there was VEGFR2 expression in HCT-116 and SW620 (Fig. 2E). In conclusion, JZA00 and JZA01 are able to specifically bind with KDR and target to HUVECs.

JZA01 inhibits HUVECs proliferation, migration, invasion and tube formation.
In order to study the anti-angiogenic activity of our fusion antibody, we studied its effects on the proliferation, migration, transwell invasion and tube formation of HUVECs. Following 72 h of treatment, growth inhibition of HUVECs by JZA01 exceeded that of JZA00 and was similar to that of IFNα in a dose dependent manner (Fig. 3A). JZA01 also played a role in inhibiting the migration of HUVECs with the inhibitory effect concentration dependent (Fig. 3B, 3D). All treatments showed inhibition in the transwell invasion assay, with JZA01 being the most effective (Fig. 3C, 3E). In the tube formation assay, JZA01 showed activity similar to IFNα, and both were superior to JZA00 (Fig. 3F, 3G). Thus, JZA01 showed superior anti-angiogenic activity in vitro.

JZA01 inhibits proliferation and promotes apoptosis of colorectal cancer cells

Although SW620 was found to be less sensitive to JZA00 treatment than HCT-116, JZA01 showed greater growth inhibition of both cell lines than JZA00, resembling IFNα (Fig. 4A, 4B). While 50 nM JZA00 barely inhibited colony formation of HCT-116 and SW620, few colonies were observed following treatment with 50 nM JZA01 or 100 nM IFNα (Fig. 4C, 4E). Analysis showed that there was no significant difference between the JZA01 and IFNα treated groups (HCT-116 p = 0.3525, SW620 p = 0.1245) while there was significant difference between the JZA01 and JZA00 treated groups (HCT-116 p = 0.0066, SW620 p = 0.0097) (Fig. 4D, 4F). Detection of apoptotic cells using annexin V/PI staining showed significantly more apoptosis following 48 h treatment with 100 nM JZA01 than with 100 nM JZA00 although less apoptosis was seen in the JZA01 treated cells than in cells treated with 200 nM IFNα. (Fig. 4G, 4I). SW620 cells were more resistant than HCT-116 to JZA00 but both exhibited increased apoptosis following treatment with JZA01 (Fig. 4H, 4J). Although not as effective as free recombinant IFNα, JZA01 was superior to JZA00 in inhibiting proliferation and promoting apoptosis of both cell lines.
JZA01 inhibits migration and invasion of colorectal cancer

HCT-116 (Fig. 5A) or SW620 (Fig. 5C) cells were incubated in 12-well plates until adherent. Supernatant was removed and then a straight line was drawn using a 1 ml tip in the middle of each well before 100 nM JZA00, JZA01 or 200 nM IFNα was added. Photographs were taken at 0 h, 12 h, 24 h, 48 h using an OLYMPUS inverted microscope at 100 times magnification. The migration of SW620 was slower than that of HCT-116. JZA01 was more effective than JZA00 in inhibiting the migration of both HCT-116 and SW620 but was not more effective than IFNα (Fig. 5B, 5D). In the transwell invasion assay (Fig. 5E, 5G), the inhibition by JZA01 of HCT-116 and SW620 was significantly more than that of JZA00 and slightly more than that of IFNα at the corresponding concentration (Fig. 5F, 5H). In conclusion, JZA01 was more effective than JZA00 in inhibiting the invasion and migration of the tumor cells HCT-116 and SW620. JZA01 was similar to IFNα in inhibiting invasion but it was not superior in inhibiting migration.

JZA01 regulates VEGFR2, STAT and apoptosis signaling pathway activation and causes cell cycle arrest of colorectal cancer cells

We investigated signaling pathway modulation following JZA01 treatment. With increasing concentrations of JZA01, the amount of p-VEGFR2, p-P38, p-ERK and p-Akt decreased following VEGF treatment (Fig. 6A, 6B). JZA01 treatment for 48 h also decreased the expression of Bcl-2 and Bcl-xL, and increased the expression of Bak and Bax (Fig. 6C, 6D) consistent with the fact that Bcl-xL and Bcl-2 inhibit both apoptosis and proliferation while Bak and Bax promote apoptosis. Cell cycle analysis of HCT-116 and SW620 showed a greater proportion of HCT-116 and SW620 was arrested at the G0/1 checkpoint following JZA01 treatment than following treatment with JZA00 for 48 h (Fig. 6E, 6G). Nevertheless, both JZA01 and IFNα showed similar effect at the same concentration (Fig. 6F, 6H). The activation of p-STAT1 and p-STAT3 was observed.
in HCT-116 and SW620 cells following 30 min treatment with JZA01 (100 nM) and IFNα (100 nM) but not JZA00 (100 nM) (Fig. 6I).

JZA01 treatment enhances DCs maturation and activity

In exploring JZA01 activated immune function, we found that after 48 h treatment with JZA01, the expression of MHC class I on the surface of HCT-116 increased by 17.2% (Fig. 7A) in comparison with the control group while that of SW620 increased by 30.6% (Fig. 7B). There was a small increase of MHC class II expression after JZA01 or IFNα treatment (Fig. S1A, S1B). JZA01 retained ADCC (Fig. S1D, S1E) and CDC activity but with no enhancement compared with JZA00. Type I IFNs have been reported to potently activate dendritic cells. To determine if JZA01 has similar activating activity, mononuclear cells and immature dendritic cells isolated from human peripheral blood mononuclear cells (huPBMCs) were cultured in AIM-V media containing IL-4 (800 U/ml) and GM-CSF (150 ng/ml) for 4 days. Cells were then incubated with JZA00 (50 nM), JZA01 (50 nM) or IFNα (100 nM) for 48 h. Flow cytometry showed that the expression of CD80 (Fig. 7C), CD86 (Fig. 7D), MHC class I (Fig. 7E) and MHC class II molecules (Fig. S1C) on the surface of DCs increased following JZA01 or IFNα treatment, while no significant increase was observed in the JZA00 treated group. In addition, JZA01 was similar to IFNα alone in promoting DCs maturation. When CD8⁺ T cells were labeled with CFSE before co-culturing with pretreated DCs at a ratio 10:1, flow cytometry showed greater proliferation of CD8⁺ T cells following JZA01 and IFNα treatment than treatment with JZA00 (Fig. 7F). The CD8⁺ T cells treated with JZA01 and IFNα showed greater cytotoxicity (Fig. 7G, 7H) and IFNγ production (Fig. 7I, 7J) than that of JZA00 treated groups. Interestingly, we found that when treated with JZA01, CD8⁺ T cells exhibited superior cytotoxicity and IFNγ production towards SW620 than HCT-116 cells, possibly reflecting the increased MHC class I expression. In conclusion, following treatment with JZA01, colorectal
tumor cells and DCs are more likely to present antigens to CD8\(^+\) T cells mainly through increased expression of MHC class I which may induce enhanced production of tumor specific cytotoxic T lymphocytes.

JZA01 suppresses tumor growth and prolongs survival of HCT-116 bearing NOD-SCID mice

Immunodeficient NOD-SCID mice were reconstituted with \(1\times10^7\) huPBMCs isolated from healthy donors together with \(5\times10^6\) HCT-116 cells implanted into the left armpit. The mice with established tumors were treated twice a week with JZA01, JZA00 or IFN\(\alpha\) and tumor growth and survival was monitored for 100 days. All the treatments showed a dose-dependent protection against tumor growth with JZA01 demonstrating superior efficacy compared to both JZA00 and IFN\(\alpha\) (Fig. 8A, 8B). To determine whether CD8\(^+\) T cells and DCs play key roles in IFN\(\alpha\)-mediated immune stimulation, CD8\(^+\) T cells or DCs depleted huPBMCs were used (Fig. 8C, 8D). Tumor growth in CD8\(^+\) T cells or DCs depleted groups was more rapid than in mice reconstituted with non-depleted huPBMCs and treated with the same dose JZA01 (1 mg/kg) suggesting that DCs and CD8\(^+\) T cells were involved in mediating JZA01 stimulated immune protection (Fig. 8E). The low dose of JZA01 (0.5 mg/kg) was more effective in prolonging the survival of HCT-116 bearing NOD-SCID mice than either JZA00 (1 mg/kg) or IFN\(\alpha\) (200 \(\mu\)g/kg) at a higher dose (Fig. 8F). Inconsistent with the \textit{in vitro} data, less protection was seen in the IFN\(\alpha\) treated group, which might reflect the altered pharmacokinetics or off-target systemic toxicity. Mice treated with IFN\(\alpha\) showed obvious signs of toxicity such as hair loss and anorexia while no significant toxicity was seen in the JZA00 or JZA01 treated groups. In conclusion, JZA01 which specifically targeted delivery of IFN\(\alpha\) to activate the DC and CD8\(^+\) T cell immune response, showed better therapeutic efficacy than JZA00 or IFN\(\alpha\) in HCT-116 bearing NOD-SCID mice.

JZA01 influences the tumor microenvironment in HCT-116-bearing NOD-SCID mice
The NOD-SCID mice bearing established HCT-116-tumors were treated with JZA00, JZA01 or IFNα twice a week and sacrificed on day 30 and the alteration of tumor microenvironment was analyzed using immunohistochemistry (IHC) and immunofluorescence (IF). In the tumor sections stained with anti-CD31, tumor neovascularization was found to decrease more in the JZA01 treated group (1 mg/kg), than in the JZA00 (1 mg/kg) or IFNα treated groups (200 μg/kg) (Fig. 9A, 9B). There also was a significant decrease in cell proliferation as indicated by Ki-67 expression in JZA01 treated tumors compared with JZA00 and IFNα treated tumors (Fig. 9C, 9D). Stress-inducible factors HIF-1α and VEGF are indicators of growing tumor tissue. Tumor sections stained with anti-VEGF (Fig. 9E) and anti-HIF-1α (Fig. 9G) showed a greater reduction staining in JZA01 treated group, implying combining anti-VEGFR2 and IFNα2 in JZA01 led to enhanced anti-angiogenic activity (Fig. 9F, 9H). There was more infiltration of CD8+ T cells in JZA01 treated mice than mice treated with IFNα or JZA00 (Fig. 9I, 9J). Furthermore, significantly more secretion and expression of IFNγ was observed in the JZA01 treated group than in JZA00 or IFNα treated groups (Fig. 9K, 9L), indicating that IFNα targeting by anti-VEGFR2 enhanced IFNα stimulated immune responses and potentiated its influence on the tumor microenvironment.

Discussion

Current treatment of colorectal cancer is surgery combined with chemotherapy or radiotherapy. In recent years, drugs targeting cell receptors have significantly prolonged patients overall survival (OS) and improved the quality of life. For example, Bevacizumab with 5-fluorine (5-FU) or capecitabine based combination chemotherapy has become the first-line and second-line treatment for metastatic colorectal cancer. In clinical trials, Bevacizumab combined with IFNα can effectively prolong the survival of patients with mRCC.
However, serious adverse reactions including hypertension, proteinuria, fatigue, and anorexia nervosa have limited its clinical application.\textsuperscript{34,35} Therefore, it is urgent to reduce the toxic side effects and enhance therapeutic efficacy for the treatment of metastatic colorectal cancer.

In our study, the anti-VEGFR2 antibody JZA00 was fused with IFNα2. We found that our fusion antibody, JZA01, was more effective in inhibiting angiogenesis than JZA00 both \textit{in vivo} and \textit{in vitro}. It showed anti-angiogenic activity similar to IFNα \textit{in vitro} but was more effective \textit{in vivo}. JZA01 can regulate the VEGFR2 signaling pathway, down regulate the expression of VEGFR2 related downstream signaling proteins and reduce the expression of HIF-1α and VEGF to further inhibit tumor angiogenesis.\textsuperscript{36} In addition, JZA01 was able to inhibit the invasion and migration of HUVECs and colorectal cancer cells demonstrating its potential for inhibiting tumor metastasis. Our data revealed that both SW620 and HCT-116 were more sensitive to direct killing, inhibition of proliferation and induction of apoptosis by JZA01 than by JZA00. Although data showed that fusing IFNα to anti-VEGFR2 decreased the direct cytotoxicity of IFNα, anti-VEGFR2 and IFNα can play a synergistic role in suppressing tumor angiogenesis and metastasis. In HCT-116 tumor bearing NOD-SCID mice, JZA01 was more effective in inhibiting the tumor growth and prolonging the survival than IFNα alone and was able to do so without toxic side-effects.\textsuperscript{37,38} Subsequent IHC and IF analysis showed that JZA01 was more effective than IFNα and JZA00 in stimulating the release of cytokines suggesting it has improved efficacy on regulating the tumor microenvironment \textit{in vivo}.

Most current strategies to improve the therapeutic effect of antibodies focus on increasing the direct tumor cell cytotoxicity of antibodies and cytotoxic drug conjugates.\textsuperscript{39,40} In contrast, JZA01 can not only inhibit proliferation, arrest cell cycle and produce apoptosis of tumor cells but can also inhibit tumor angiogenesis. More importantly, it can target to the tumor microenvironment and activate the immune response. In our
study, we found that JZA01 can more effectively upregulate the expression of MHC class I on the colorectal cancer cell lines HCT-116 and SW620 than JZA00, thereby facilitating the direct presentation of tumor cell antigens. Analysis of the expression of CD80, CD86, MHC class I and MHC class II on the surface of DCs showed that JZA01 can promote the maturation of DCs, so that DCs will be better able to present antigens to CD8⁺ T cells and promote proliferation and IFNγ release of CD8⁺ T cells. DCs are the most important APCs for CTL induction. Thus, activating DCs with JZA01 confers better immune recognition and killing of tumor cells. In vivo experiments showed that the anti-tumor effect of JZA01 decreased in mice depleted of DCs or CD8⁺ T cells suggesting that DCs and CD8⁺ T cells played a key role in the anti-tumor effect of JZA01. The CD8⁺ T cells infiltration and IFNγ release by mice treated with JZA01 was much greater than in mice treated with JZA00 or IFNα. Taken together, the IFNα in JZA01 retains the ability to activate the immune response. Furthermore, JZA01 is more effective than JZA00 or IFNα alone in activating the immune response which can probably be attributed to the tumor microenvironment targeting ability of anti-VEGFR2.

Overall, this study has some important implications for the field of antitumor immune therapy. We fused hIFNα2 with the anti-VEGFR2, to deliver IFNα2 to the tumor microenvironment, thereby inhibiting tumor angiogenesis, directly killing tumor cells and activating the immune response. We found that anti-VEGFR2-IFNα2 was also effective against tumor cells such as SW620 which had not been previously shown to be sensitive to anti-VEGFR2 treatment. Antibody-cytokine fusions, such as Ab-IFNα fusion proteins provide a new strategy for promoting the immunotherapy of cancer. Collectively, this study provides a new approach for the treatment of colorectal cancer and opens up several novel avenues for optimizing targeted immune therapy that may impact clinical cancer therapy.

Material and methods
Mice and cell lines

All animal experiments were conducted under protocols approved by the Ministry of Health of the People’s Republic of China (Document No. 55, 2001) and the Guidelines for the Care and in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation and approved by the Science and Technology Department of Jiangsu Province (SYXK (SU) 2016-0011). All PBMC healthy donors were given informed consent for this study and approved by the ethical committee of China Pharmaceutical University. The NOD/SCID mice were purchased from Model Animal Research Center of Nanjing University (MARC). Animals used in experiments were between 4 and 8 weeks of age. The Chinese hamster ovary cell line CHO-pro (Amprotein Co., Ltd, Hangzhou, China) was cultured in IMDM medium (Gibco, Grand Island, USA), supplemented with 5% (v/v) fetal bovine serum (FBS, Gibco, Auckland, NZ). Human umbilical vascular endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC) and were maintained in endothelial culture medium (ECM) supplemented with 5% (v/v) FBS and 1% (v/v) endothelial cell growth supplement (ECGS, ScienCell, San Diego, CA). The human colorectal cancer cell lines HCT-116 and SW620 cultured in 1640 medium (Gibco, Life, America) were obtained from the cell bank of the Chinese Academy of Science (Shanghai, China).

Construction, expression and purification of JZA00 and JZA01

The construction of anti-VEGFR2-IFNα2 was conducted as we previously reported. In brief, the variable region of Ramucirumab was used to replace the variable region of anti-CD138.42 IFNα2a was fused to the carboxy terminus of the heavy chain with a flexible pentapeptide (Gly-Gly-GlyGly-Ser). Subsequently, the recombinant plasmid was introduced into CHO-pro cell through lipofectin mediated transfection. Stable clones were selected using 1 mg/ml G418, 1 mM Histidinol (Sigma-Aldrich) and screened by ELISA (HRP
conjugated goat-anti-human IgG Fc, Sino Biological Inc., China) and western blot analysis (HRP conjugated goat-anti-human IgG H+L, Sino Biological Inc., China). The fusion protein was purified from culture supernatants using protein A affinity chromatography (GE Healthcare, UPPsala, Sweden) and characterized by reducing and non-reducing SDS-PAGE (10%).

Binding affinity analysis

The binding affinity of JZA01 to tyrosine kinase insert domain receptor (KDR, human VEGFR2) was measured by the Biacore X100 (GE Healthcare, Sweden). Firstly, anti-human IgG (Fc) antibody was fixed on the CM5 chip with Human Antibody Capture Kit (GE Healthcare, Sweden). Then JZA01 was captured, KDR was added into running buffer (HBS-EP, pH 7.4) with different concentrations ranging from 0.78125 nM to 100 nM. One flow cell of the sensor chip was set as a control. The captured antibody-analyte complex was removed by regeneration solution (3 M magnesium chloride). The association ($k_{on}$) and dissociation ($k_{off}$) rate constants were calculated and analyzed using the bivalent analytemodel and the equilibrium dissociation constant ($K_D$) was calculated ($K_D = k_{on}/k_{off}$).

Cell proliferation assay

Cells were plated into 96-well plates at 4×10³ cells/well. After incubation with different concentrations of treatments at 37°C for 72h, cell proliferation was quantified by MTT assay. Inhibition (%) was calculated as $(1- OD_{experiment}/OD_{control}) \times 100\%$.

Colony formation

$1\times10^3$ cells were incubated with different treatments (JZA00 50nM, JZA01 50nM, IFNα 100 nM) in 1ml RPMI 1640 medium supplemented with 10% (v/v) FBS at 37°C for 7 days. Supernatant was removed, cells were
fixed with 4% (v/v) paraformaldehyde for 20 min at room temperature and stained with 1% (w/v) crystalline violet. Images were taken by Canon camera and the number of cell clones was counted with the Image-Pro-Plus program. Inhibition (%) = (1 - \frac{\text{number}_{\text{experiment}}}{\text{number}_{\text{control}}}) \times 100%.

Tube formation

Matrigel (BD, Bioscience, USA) diluted 1:1 with serum-free ECM was plated in 96-well plates, 50 μl/well and allowed to polymerize at 37°C for 1 h. Then 2×10^4 HUVECs incubated with different concentrations of treatments in 100μl of ECM supplemented with 2% (v/v) FBS, 1% (v/v) ECGS was added into each well. After 2–16 hours incubation, endothelial tubes were photographed with an OLYMPUS inverted microscope under 100-time magnification and counted with the Image-Pro-Plus program. Inhibition(%) = (1- \frac{\text{number}_{\text{experiment}}}{\text{number}_{\text{control}}}) \times 100%.

Transwell invasion

1×10^4 HUVECs suspended in ECM supplemented with 1% (v/v) FBS and various concentrations of the treatments were incubated in the upper well of 24-well transwell chambers (Millipore, Billerica, USA), which were pretreated with matrigel (BD, Bioscience, USA). The lower chambers were filled with 600μl of ECM containing 5% (v/v) FBS and 1% (v/v) ECGS. After a 12–24 h incubation, the upper cells were removed while the lower cells were fixed with 4% polyoxyymethylene for 20 min and then stained with 1% (w/v) crystalline violet before being photographed under an OLYMPUS inverted microscope at 100-time magnification. The number of the invasive cells was determined with the Image-Pro-Plus program. Inhibition (%) = \left[\frac{(\text{control}_{\text{number}} - \text{experiment}_{\text{number}})}{\text{control}_{\text{number}}}\right] \times 100%.

Scratch assay
2×10^5/well HUVECs suspended in ECM containing 1% (v/v) ECGS and 5% (v/v) FBS were incubated in a 12-well plate until adherent. Supernatant was removed and then a straight line was drawn in the middle of each well with a 1 ml tip. ECM supplemented with different concentrations of the treatments was then added into the 12-well plate. Images were taken using an OLYMPUS inverted microscope at 100× magnification. The length of migration was analyzed using the Image-Pro-Plus program. Inhibition (%) = (1-length_{experiment}/length_{control}) × 100%.

Apoptosis assay

HCT-116 or SW620 cells were incubated in 6-well plates with various treatments (JZA00 100 nM, JZA01 100 nM, IFNα 200 nM) at 37°C for 48h and the supernatant was removed and cells were washed three times with PBS followed by staining with annexinV-FITC and propidium iodide (PI) for 30 min. After being washed with PBS for three times, the samples were analyzed by flow cytometry to distinguish populations of early apoptotic (annexin V+/PI−), late apoptotic (annexin V+/PI+), and necrotic (annexin V−/PI+) using annexin V/PI Apoptosis Assay Kit (Sangon Biotech, Shanghai, China). The percentage of apoptotic cells (annexin V+) was calculated as the sum of both early apoptotic (annexin V+/PI−) and late apoptotic (annexin V+/PI+).

Cell cycle assay

3×10^5 HCT-116 cells and SW620 cells were incubated in 6-well plates with various treatments (50 nM) at 37°C for 48 h. After that, the supernatant was removed and cells were washed three times with PBS and then fixed in 75% ethanol overnight. Subsequently samples were stained with PI (Beyotime Biotech, Shanghai, China) for 30 min and washed with PBS for three times before flow cytometry assay. All the assays
were performed using a BD FACS flow cytometer and PI fluorescence intensity represents the different cell cycles. The proportion of cells from each cell cycle was calculated with the MFLT32 software.

MHC class I and MHC class II expression on the tumor surface

3×10^5 HCT-116 or SW620 cells were cultured in 6-well plates at 37°C with different treatments (JZA00 50 nM, JZA01 50 nM, IFNα 100 nM) for 48h. After that, cells were respectively incubated with the anti-MHC class I (Bioscience, 560965) and anti-MHC class II (Bioscience, 560943) for 1 h before being analyzed by flow cytometry.

Maturation of dendritic cells

Mononuclear cells and immature dendritic cells were isolated from human peripheral blood mononuclear cells (huPBMCs) and incubated in AIM-V medium (Gibco, #087-0112DK) containing IL-4 (800 U/ml) and GM-CSF (150 ng/ml) for 4 days. Half of the medium was changed every two days. Different treatments (JZA00 50 nM, JZA01 50 nM, IFNα 100 nM) were given on the fourth day for 48 h. After that, cells were respectively incubated with different antibodies (anti-CD80, Biosciences 560925; anti-CD86, Bioscience 560956; anti-MHC class I, Bioscience 560965; anti-MHC class II, Bioscience 560943) for 1 h in PBS containing 2% FBS and detected by flow cytometry.

CD8⁺ T cells activation and cytotoxicity assay

CD8⁺ T cells isolated from the huPBMCs (from 5 healthy donors) using a human CD8⁺ T isolation kit (BD Bioscience 557766) were labeled with CFSE (BD Bioscience 565082) and then co-cultured for 48 h with tumor cells and DCs (T:DC = 10:1) which had been pretreated as we previously described in the maturation of dendritic cells. Flow cytometry analysis was then performed and expression of IFNγ was detected by the ELISA kit (Arigo, AGR80117). Data were analyzed with the FlowJo 7.6 software and GraphPad Prism 5. The ADCC and CDC assays were performed as previously reported.29
Cytotoxicity was detected by the CytoTox 96 Nonradioactive Cytotoxicity kit (Promega, Madison, USA). LDH analysis was performed according to the manufacture’s protocol. \[ \% \text{Cytotoxicity} = \left( \frac{\text{experimental} - \text{effector spontaneous} - \text{target spontaneous}}{\text{target maximum} - \text{target spontaneous}} \right) \times 100\% . \]

HCT-116 transplanted NOD-SCID mice model and in vivo efficacy

5×10^6 HCT-116 cells mixed with 1×10^7 non-activated human PBMCs from 5 healthy donors were implanted into the left armpit of 6-week-old NOD/SCID mice (Model Animal Research center of Nanjing University). CD8^+ T cells were removed with human CD8^+ T isolation kit (BD Bioscience, 557766) and DCs were removed with DC immunomagnetic beads (BD Bioscience, 558420). There were 8 groups (6/group): control (without huPBMCs), control (with huPBMCs), JZA00 (1 mg/kg), JZA01 (1mg/kg), JZA01 (0.5 mg/kg), JZA01 (1 mg/kg, without DCs), JZA01 (1 mg/kg, without CD8^+ T cells), IFNα2 (200 μg/kg). All treatments were administered twice a week by tail vein injection from day 0 to a month. Growth of the tumor was measured every day and the tumor volume was determined using the formula \[ V = \frac{\text{length} \times \text{width}^2}{2} . \] The survival of the NOD-SCID mice with xenografts was monitored until 100 days.

Immunohistochemistry and immunofluorescence analysis

The mice were sacrificed and the tumors were made into paraffin tissue sections. Paraffin sections were cut into 5 μm sections and fixed in 4% paraformaldehyde. For immunohistochemical (IHC) staining, the sections were incubated with CD31, Ki67, VEGF, HIF-1α or CD8 specific antibody (Cell Signaling Technology, Boston, USA), and horseradish peroxidase-labeled secondary antibody and analyzed with the Vectastain ABC Kit (Dako, Copenhagen, Denmark). For immunofluorescence (IF) staining, the sections were incubated with anti-IFN-γ (Abcam, Cambridge, UK) and slides were
mounted with Vectashield mounting media containing DAPI before analyzed under fluorescence microscope.

**Statistical analysis**

All data are indicated as mean ± SD. Data were compared using the student’s t test and P < 0.05 was considered statistically significant. Analyses were performed with the GraphPad Prism software (San Diego, CA).

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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Figure 1. Construction and characterization of JZA01. (A) Diagram of JZA01. IFNα2 was linked to the C terminal of H' chain of JZA00 with a G4S linker using overlap PCR yielding JZA01. (B) Western blot analysis of purified JZA01 with anti-kappa chain and anti-IFNα shows it was properly assembled and of the correct molecular weight. Lane 1: JZA00 non-reducing; lane 2: JZA01 non-reducing; lane 3: JZA00 reducing; lane 4: JZA01 reducing. JZA01 and JZA00 were purified using protein A affinity chromatography and the purity was identified with (C) non-reducing SDS-PAGE and (D) reducing SDS-PAGE; lane 1: JZA01; lane 2: JZA00.
Figure 2. JZA01 binds to KDR and to cells expressing VEGFR2. (A) The binding affinity of JZA01 with KDR was determined with the Biacore system. To prepare chips, JZA01 was captured on a CM5 chips with bound anti-human IgG. Increasing concentrations of the KDR (from bottom to top), ranging from 0.78125 nM to 100 nM then flowed over the chip for 120 s. Binding affinity was calculated using the 2:1 model. (B) Binding affinity of JZA00 with KDR was determined as described in A with concentrations of KDR ranging from 0.78125 nM to 100 nM. L02 cells (C) or HUVEC (D) were incubated with 100 nM JZA00 or JZA01 for 1 h, washed three times with PBS, then incubated with FITC labeled anti-human kappa chain and analyzed by flow cytometry assay. (E) Western blot analysis of VEGFR2 expression using either JZA01 or JZA00 as the primary antibody and HRP conjugated goat-anti-human IgG H+L (Sino Biological Inc., China) as the secondary antibody. Lane 1: JZA01 HUVEC; Lane 2: JZA01 HCT-116; Lane 3: JZA00 HCT-116; Lane 4: JZA01 SW620; Lane 5: JZA00 SW620.
Figure 3. JZA01 inhibits the proliferation, migration, transwell invasion and tube formation of HUVECs. (A) HUVECs were incubated with different concentrations of JZA01, JZA00 or IFNα and proliferation analyzed by MTT assay. Inhibition (%) was calculated as (1 - OD_{experiment}/OD_{control}) × 100%. (B, D) The inhibition of the migration of HUVECs by JZA01 added at different concentrations was analyzed by the scratch assay. The distance of migration at 0 h, 12 h, 16 h, 20 h, 24 h was measured using Image-Pro Plus 6.0 after being photographed by an inverted OLYMPUS microscope at 100× magnification. Inhibition(%) = (1 - length_{experiment}/length_{control}) × 100%. (C, E) Photomicrographs and quantitative analysis of transwell invasion assay indicated that JZA01 suppressed the invasion of HUVECs better than IFNα or JZA00 in a dose-dependent manner. Inhibition(%) = [(control – experiment)/ control] ×100%. (F, G) HUVECs were incubated for 4 h with different concentrations of the JZA00, JZA01 or IFNα in a 96-well plate pretreated with matrigel. The endothelial tubes were photographed by an inverted OLYMPUS microscope at 100× magnification with the number of endothelial tubes counted using Image-Pro Plus 6.0. Inhibition(%) = (1 - number_{experiment}/number_{control}) × 100%. Quantitative analysis were presented as the mean ± SD, n = 5, *p<0.05, **p<0.01, ***p<0.001.
Figure 4. JZA01 inhibits the proliferation, colony formation and promotes apoptosis of HCT-116 and SW620. HCT-116 (A) or SW620 (B) cells were incubated with different concentrations of JZA00, JZA01 or IFNα for 72 h and proliferation were quantified by MTT assay. Inhibition(%) = (1- OD_{experiment}/OD_{control}) × 100%. HCT-116 (C, D) or SW620 (E, F) cells were diluted to 1000 /ml and incubated in 6-well plates with JZA00, JZA01 or IFNα at 50 nM for 7 days. The numbers of colonies formation were measured by Image-Pro Plus 6.0. Inhibition(%) = (1- number_{experiment}/number_{control}) × 100%.

Quantitative analysis is presented as the mean ± SD, n = 5, *p<0.05, **p<0.01, ***p<0.001. HCT-116 (G) and SW620 (I) cells were incubated with JZA00 100 nM, JZA01 100 nM or IFNα 200 nM for 48h and analyzed by flow cytometry following staining with PI and Annexin V. (H, J) Annexin V positive cells in Q2 and Q3 were considered apoptotic and are shown quantitatively as the mean ± SD, n = 3, *p<0.05, **p<0.01, ***p<0.001.
Figure 5. JZA01 inhibits the migration and invasion of HCT-116 and SW620. For the scratch test, HCT-116 (A) or SW620 (C) cells were incubated in 24-well plates with JZA00, JZA01 or IFNα and photographed at 0 h, 12 h, 24 h, and 48 h (B, D).
D) The migration lengths were measured by Image-Pro Plus 6.0 and data were presented as the mean ± SD, n = 3, *p<0.05, **p<0.01, ***p<0.001. Photomicrographs and quantitative analysis of transwell invasion assay indicated that JZA01 suppressed the invasion of HCT-116 (E, F) and SW620 (G, H) cells in a dose-dependent manner. %inhibition = [(control – experiment)/ control] ×100%. Data were presented as the mean ± SD, n = 5, *p<0.05, **p<0.01, ***p<0.001.
Figure 6. JZA01 down regulates VEGFR2, STAT and apoptosis signaling pathways and causes cell cycle arrest of colorectal cancer cells. (A) Western blot analysis for p-VEGFR2/VEGFR2, p-P38/P38, p-ERK/ERK, p-AKT/AKT in HUVECs treated with different concentrations of JZA01 for 48 h. (B) Gray scanning and data statistics of A based on the integrated density of β-actin. (C) Western blot analysis of Bcl-2 family proteins in HUVECs treated with different concentrations of JZA01 for 48 h. (D) Gray scanning and data statistics of C based on the integrated density of 0 nM. (E, F) Cell cycle analysis by flow cytometry of HCT-116 (E, F) or SW620 (G, H) cells that incubated with JZA00, JZA01 or IFNα for 48h. Data were presented as the mean ± SD, n = 3, *p<0.05, **p<0.01, ***p<0.001. (I) Western blot analysis of STAT activation in HCT-116 and SW620 following treatment with JZA01 (100 nM) for 30 min.
Figure 7. JZA01 treatment enhances DCs maturation and activity. (A) Flow cytometry assay of the expression of MHC class I of HCT-116 under different treatments (JZA00 50 nM, JZA01 50 nM, IFNα 100 nM) for 48 h. (B) Flow cytometry assay of the expression of MHC class I of SW620 under different treatments (JZA00 50 nM, JZA01 50 nM, IFNα 100 nM) for 48 h. (C, D, E) Immature dendritic cells were isolated from non-activated huPBMCs and incubated under different treatments for 72 h. The expression of CD80, CD86 and MHC class I was detected by the flow cytometry assay. (F) CD8⁺ T cells labeled with CFSE were co-cultured for 48 h with DCs (10:1) pretreated with different treatments in Fig. 7C and isolated using human CD8⁺ T cells isolation kit (BD Bioscience;557766) before detected by flow cytometry assay. (G, H) CD8⁺ T cells together with DCs (10:1, in Fig. 7F) as effectors, E:T ratio 1:1, 5:1, 10:1, time point of 24 h, Abs 50 nM, IFNα 100 nM, cytotoxicity% was analyzed by LDH release assay. Data were presented as mean of three repeats. %Cytotoxicity = [(experimental - effector spontaneous - target spontaneous)/(target maximum - target spontaneous)]×100. Fig. 7G represents the cytotoxicity% of HCT-116, Fig. 7H represented the cytotoxicity% of SW620. (I, J) CD8⁺ T cells together with DCs (10:1, in Fig. 7F) as effectors, E:T ratio 10:1, time point of 24 h, Abs 50 nM, IFNα 100 nM, the production of IFNγ was measured by ELISA kit. I, HCT-116 as target cells; J, SW620 as target cells.
Figure 8. JZA01 has superior anti-tumor efficacy in HCT-116-bearing NOD-SCID mice. (A, B) Female NOD-SCID mice 4–6 weeks of age (n = 6 per group) were injected in the left armpit with 5 × 10^6 HCT-116 cells and 1×10^7 unstimulated huPBMC. (A) Mice was treated with 1 mg/kg (high dose) or 0.5 mg/kg (low dose) JZA01 (i.v.) twice a week with tumor
growth followed for a month. (B) Mice were treated with 1 mg/kg of JZA01 or JZA00, or IFNα (200 μg/kg) twice a week and tumor volumes were monitored for a month. (C, D) Female NOD-SCID mice 4–6 weeks of age were injected in the left armpit each with $5 \times 10^6$ HCT-116 cells and $1 \times 10^7$ unstimulated huPBMC from which dendritic cells (C) or CD8$^+$ T cells (D) had been removed. Mice ($n = 6$ per group) were treated with 1 mg/kg JZA01 twice a week (i.v.) beginning from day 0 for a month with tumor volumes monitored. (E) Tumor inhibition of different dosage groups. JZA01 high dose significantly improved tumor inhibition compared to other groups. Data represent the mean ± SD ($n = 6$ per group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (F) The survival of tumor-bearing NOD-SCID mice for 100 days. Data are from the experiments shown in panels A-D. The JZA01 treated group had better survival compared with IFNα treated group. Log-rank tests: JZA01 high dose vs. JZA00, $P < 0.001$; JZA01 high dose vs. JZA01 low dose, $P = 0.0012$; JZA01 high dose vs. IFNα2, $P < 0.001$; JZA01 high dose vs. JZA01 without DCs, $P = 0.0409$; JZA01 high dose vs. JZA01 without CD8$^+$ T, $P = 0.0080$. 
Figure 9. JZA01 inhibits the HCT-116 tumor growth at least in part through influencing the tumor microenvironment in vivo. (A) CD31+ blood vessels were identified with an anti-CD31 antibody (brown staining). Photomicrographs show...
representative pictures from 3 independent tumor samples. (B) Semiquantitative and statistical analysis of CD31⁺ blood vessels. The CD31 density was calculated with Image Pro Plus. Relative expression of CD31 (%) = 
\[(\text{Experiment/Control}) \times 100\%\]. (C) Expression of Ki-67 in paraffin sections of xenografted tumor identified with an anti-Ki-67 antibody (brown staining). (D) The tumor cells expressing Ki-67 were counted with Image-Pro-Plus. Ki-67 Positive(%) = (cell expressing Ki-67/total cells) × 100%. (E) Staining of VEGF using anti-VEGF (brown staining). (F) Semiquantitative and statistical analysis of VEGF. (G) Expression of HIF-1α identified by anti-HIF-1α (brown staining). JZA01 was more effective in down-regulating the expression of HIF-1α and VEGF than IFNα or JZA00. (H) Semiquantitative and statistical analysis of HIF-1α. VEGF and HIF-1α density were quantified with Image-Pro-Plus. Relative expression(%) = (Experiment/Control) × 100%. (I) The infiltration of CD8⁺ T cells was identified with an anti-CD8 antibody (brown staining). (J) Semiquantitative and statistical analysis of the infiltration of CD8⁺ T cells. The relative number of CD8⁺ T cells was calculated with Image-Pro-Plus. (K) Expression of IFNγ in the tumor microenvironment was identified by staining with anti-IFNγ (red fluorescence) following different treatments. Expression of IFNγ in JZA01 group is the highest. All pictures were photographed by an inverted OLYMPUS microscope at 400× magnification. (L) Semiquantitative and statistical analysis of IFNγ expresison made with Image-Pro-Plus. Relative expression (%) = (Experiment/Control) × 100%. 


