Development of bispecific anti-c-Met/PD-1 diabodies for the treatment of solid tumors and the effect of c-Met binding affinity on efficacy

Qingyun Yuan a, Qiaoyan Liang a, Zujun Sun a,b, Xingxing Yuan a, Weihua Hou a, Yuxiong Wang a, Huijie Wang a, and Min Yu a

*Key Laboratory of Metabolism and Molecular Medicine, Ministry of Education and Department of Biochemistry and Molecular Biology, School of Basic Medicine Science, Fudan University, Shanghai, China; aDepartment of Laboratory Medicine, Shanghai Tongji Hospital, Tongji University School of Medicine, Shanghai, China; bDepartment of Oncology, Fudan University Shanghai Cancer Center, Shanghai Medical College, Fudan University, Shanghai, China

ABSTRACT
Although the blockade of the programmed cell death protein 1/programmed cell death ligand 1 (PD-1/PD-L1) pathway has become a promising treatment strategy for several types of cancers, the constitutive activation of c-Met in tumors may cause a low overall response rate to PD-1 inhibitors. Increasing evidence indicates that the dual inhibition of c-Met and PD-1 could improve the efficacy of anti-PD-1/PD-L1 monoclonal antibodies for tumor immunotherapy. In this study, we developed two bispecific single-chain diabodies targeting c-Met and PD-1 for the treatment of solid tumors based on protein homology modeling, and we identified that the binding affinity of diabody-mp to c-Met was 50-folds higher than that of diabody-pm. The results of in vitro studies revealed that both diabodies suppressed HGF-induced proliferation, migration, and invasion of tumor cells, inhibiting the activation of c-Met signaling by antagonizing HGF binding to c-Met. Moreover, they promoted T cell activation by blocking the PD-1 pathway, mediating tumor cellular cytotoxicity through T cell engagement. In vivo studies with mice models demonstrated that diabody-mp exhibited higher therapeutic efficacy than other structural antibodies, greatly enhancing the survival of c-Met-positive tumor-bearing mice compared to single or combined c-Met and PD-1 blockade therapy. Furthermore, diabody-mp, which had a higher c-Met binding affinity, showed better anti-tumoral activity than diabody-pm, which had a lower c-Met binding affinity. In conclusion, bispecific anti-PD-1/c-Met diabody-mp, with high c-Met-associated affinity, inhibited tumor growth by activating T cells, suggesting its therapeutic potential for c-Met-positive solid tumors.

Introduction
Immunotherapy is a promising treatment that provides new hope for patients with advanced tumor that are not treatable with conventional treatment. Activated T cells induce immune tolerance by upregulating the expression of programmed cell death protein 1 (PD-1), a negative immune checkpoint receptor. 1,2 Tumor cells overexpress programmed cell death ligand 1 (PD-L1), a ligand of PD-1, to facilitate immune evasion. 3 Although blocking the PD-1/PD-L1 pathway could partially restore exhaustible T cells in preclinical models 4 and PD-1-based monotherapies have shown remarkable clinical efficacy in some patients with multiple cancers, including melanoma, non-small cell lung cancer, and colorectal cancer, most patients experience poorly sustained clinical benefit and relapse. 5 The objective response rate for nivolumab is 20% in patients with advanced hepatocellular carcinoma (HCC), 6 therefore, there is an urgent need to develop strategies that can improve the response rate of PD-1 inhibitors. PD-1 inhibition combined with radiotherapy, chemotherapy, or targeted therapy could achieve enhanced anti-tumor effect; however, the strategy is associated with increased risk of side effects.

The c-Met tyrosine kinase receptor encoded by the MET proto-oncogene, also known as the hepatocyte growth factor (HGF) receptor, is activated in multiple malignancies through MET amplification, mutation, receptor overexpression, and/or a ligand-dependent mechanism. 7 Dimerization and activation of c-Met receptor after binding with HGF would promote the proliferation, migration, and invasion of tumor cells. 7–9 A previous preclinical study reported that abnormal activation of the c-Met signaling pathway is associated with poor clinical outcomes, and hence, c-Met-targeted therapy could be credible for cancer treatment. 10 Although several c-Met tyrosine kinase inhibitors (TKIs) have been used clinically with positive therapeutic effects, 11,12 HGF produced in the tumor microenvironment results in innate and acquired resistance to TKIs. 13 An alternative approach to the blocking of the HGF/c-Met pathway is the development of therapeutic antibodies; however, it is difficult to produce effective antibodies against both HGF-dependent and HGF-independent c-Met signaling. Moreover, bivalent anti-c-Met antibodies may promote receptor dimerization and activation, 14 and monovalent antibodies may lack potency. 15

CONTACT Huijie Wang wanghj98@hotmail.com Department of Oncology, Fudan University Shanghai Cancer Center, Shanghai Medical College, Fudan University, Shanghai, China; Min Yu minyu@shmu.edu.cn Key Laboratory of Metabolism and Molecular Medicine, Ministry of Education and Department of Biochemistry and Molecular Biology, School of Basic Medicine Science, Fudan University, Shanghai, China

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The combinations of immune checkpoint inhibitors and molecular targeted agents have shown great promise in recent years. Previous studies have reported that c-Met signaling plays immunosuppressive roles, such as the impairment of dendritic cell functions and the induction of T cell tolerance, which participate in immune regulation in tumors. The mutation of MET as a driver gene in tumors could cause markedly decreased clinical efficacy of PD-1 inhibition therapy. Data from several clinical trials showed that the deficiency of PD-L1 in tumors might limit the efficacy of anti-PD-1 monoclonal antibodies. However, most patients with MET exon 14-altered lung cancers expressing PD-L1 still have a low overall response rate to PD-1 inhibitors and a short median progression-free survival after treatment. In addition, it has been proposed that c-Met inhibitors promote tumor immune escape in HCC by stabilizing PD-L1. Therefore, dual blocking of c-Met and PD-1 as a feasible strategy could release or improve the efficacy of anti-PD-1/PD-L1 monoclonal antibodies (mAbs) for tumor immunotherapy. Several global clinical trials investigating the combination of PD-1/PD-L1 and c-Met inhibitors are in progress. In 2020, the US FDA accepted priority review applications for nivolumab in combination with cabozantinib in advanced renal cell carcinoma.

Mounting evidence suggests that T cells could play a powerful role in the inhibition of tumor growth in solid tumor patients. Bispecific antibodies (bsAbs) with the specificity of two antibodies could simultaneously target two different antigens or epitopes, and hence, they could overcome MHC restriction to recruit T cells for the elimination of tumor cells. In 2009, the anti-EpCAM/CDC3 catumaxombab, a trifunctional bsAb for the treatment of cancerous ascites, was the first bsAb to receive market approval. In 2014, blinatumomab was approved by the FDA for the treatment of patients with Philadelphia chromosome-negative precursor B cell acute lymphocytic leukemia. At present, popular tumor immunotherapies, such as bsAbs and CAR-T, have been approved for the treatment of hematological malignancies, but most of these therapies do not have the desired effect on solid tumors.

The design of monoclonal antibodies (mAbs) focuses on target selection, and it is also critical to determine the structural design of bsAbs. To achieve the treatment of solid tumors, we designed, expressed, and purified two bispecific anti-c-Met/PD-1 single-chain antibodies, also called diabodies, with different c-Met binding affinities, and we evaluated their anti-tumor activity in vivo and in vitro. Collectively, the results of our studies demonstrated the potential therapeutic benefits of diabodies for various c-Met-positive solid tumors.

Materials and methods

**Cell lines**

A549, MKN45, MHCC-97 H, and HEK293E cells were obtained from American Type Culture Collection, and CHO/hPD-1 EXD (extracellular domain) cells were generated in our laboratory. HEK293E cells were cultured in FreeStyle™293 Expression Medium (Gibco, NY, USA). The hPBMCs and T cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, NY, USA) and IL-2 (10 ng/mL) (Selleckchem, TX, USA). Other tumor cell lines were cultured in RPMI 1640 or Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, MO, USA) supplemented with 10% FBS and 1% penicillin/streptomycin (Hyclone, Shanghai, China) at 37°C and 5% CO₂.

**Expression vector of diabodies construction**

The coding sequences of the anti-c-Met and anti-PD-1 mAbs were created in previous studies in our laboratory. To successfully express the diabodies protein, the Kozak sequence and secretory signal peptide of human IgG were inserted after the CMV promoter of plasmid pCPE4, also called pCPE4-LC-SP. Next, using the previously synthesized anti-c-Met and anti-PD-1 mAbs sequence as templates, we cloned the VL and VH of the anti-c-Met and anti-PD-1 mAbs, respectively. The genes of diabody-mp and diabody-pm were cloned by overlap polymerase chain reaction (PCR). Finally, the genes of the diabodies were linked to the pCPE4-LC-SP plasmid linearized by restriction enzyme (NruI and Not I) digestion.

**Expression and purification**

For the production of diabodies, anti-PD-1 scFv, anti-c-Met scFv, anti-PD-1 mAb, anti-c-Met mAb, and anti-PD-1/c-Met scFv-Fc, a suspension of growing HEK293E cells at a final concentration of 10³ cells/mL were transfected with plasmids by PEI (Polysciences, Shanghai, China). Trypstone N1 was added to the culture at a final concentration of 0.5%. After 7 days of culture in conical flasks gently shaken on the platform of an orbital incubator rotating at 125 rpm, 37°C, and 8% CO₂, the supernatants were harvested and purified using His-tag (Roche, Mannheim, Germany) or Protein A Purification column (GenScript, Nanjing, China) by AKTA Explorer. Eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Flow cytometry**

For cellular c-Met and PD-1 binding studies, MKN45 and CHO/hPD-1 EXD cells were harvested and resuspended in culture medium, respectively. The cells were added to a round bottomed 96-well plate (Coring Falcon, NY, USA) at 100 µL/well and incubated with the indicated concentrations of diabody-mp or diabody-pm in triplicate wells at 4°C for 1 hr. Next, the cells were incubated with anti-His-tag antibody-FITC (GenScript, Nanjing, China), followed by the analysis of the cells on Intellicyt iQueScreener. Data analysis was done by software FlowJo. The mean fluorescence intensity (MFI) of the cells was used to evaluate antibody affinity. When the MFI reaches half of its maximum value, the corresponding concentration value (EC50) of diabodies is the KD value of diabodies relative to c-Met or PD-1. For the T cell activation assay, the tumors were enzymatically digested using collage-nase I, collagenase IV, and hyaluronidase (YEASEN, Shanghai, China) for 1 hr at 37°C. The TILs were separated by Percoll separation (Gibico, NY, USA) and incubated with antibodies (BioLegend, CA, USA) against FITC-anti-human CD4, FITC-anti-human CD8, and PE-anti-human CD69. For apoptosis
detection, the tumor cells were plated in 12-well dishes and incubated overnight at 37°C and 5% CO₂. The cells were then pre-treated with IFN-γ (10 ng/mL) (PeproTech, NJ, USA) for 24 hr, followed by co-culturing with activated T cells for 48 hr with or without 100 nM of diabody-mp or diabody-pm. The immunostaining of single cell suspensions was performed using an Annexin V-FITC/PI Apoptosis Detection Kit (Meilun, Dalian, China) according to the manufacturer’s instructions. Sample analysis was carried out using an Accuri C6 flow cytometer (BD Biosciences, CA, USA) and data analysis was performed using the Accuri software.

qRT-PCR

Cells were lysed and total RNA was extracted following standard laboratory protocols. The RNA was reverse-transcribed to complementary DNA using a 5x All-In-One RT MasterMix (Abm, Zhenjiang, China). Quantitative real-time PCR was performed using the ChamQ™ Universal SYBR® qPCR Master Mix (Vazyme, Nanjing, China).

Enzyme linked immunosorbent assay

For the cell supernatants, MKN45 cells were seeded in a 96-well plate and cultured overnight at 37°C and 5% CO₂. Next, the cells were pre-treated with IFN-γ (10 ng/mL) for 24 hr, after which the medium was removed. Upon the addition of diabody-mp or diabody-pm at the indicated concentrations, the cells were co-cultured with stimulated human T cells at 37°C for 48 hr. The cell supernatants were collected and analyzed using human IFN-γ and human IL-2 ELISA kits (MULTISCIENTES, Hangzhou, China). For tumor plasma, the tumors were dissociated in RPMI 1640 medium and then centrifuged to collect the supernatant. The supernatant was concentrated with Ultra-4 Centrifugal Filters-3 K MWCO (MerckMillipore, MA, USA). For blood plasma, blood was collected by cardiac puncture and centrifuged at 1500 g for 10 min. The supernatant was collected and analyzed using a human IFN-γ ELISA kit.

Luciferase assay

The Jurkat-PD-1⁺ cell line stably expressing hPD-1 was engineered by transfecting the cells with PCDH-CMV-MCS-EF1-GFP+Puro vector expressing PD-1, followed by selection with puromycin. The Jurkat-PD-1⁺ cells, from which PD-1 has been knocked out, were engineered by transfecting the cells with the PX459 vector cloned into gRNA targeting PD-1, as described in Zheng’s protocol27 (Oligo1: 5’-CACCAGACAGCTCCTCCGATGTT-3’; Oligo2: 5’-AAACACACATCGGAGAAGTGC-3’). The expression and knock-out of PD-1 were validated by western blotting and FACS. Jurkat-PD-1⁺ or Jurkat-PD-1⁻ cells were co-transfected with pGL3-basic vector expressing luciferase regulated by the binding of NFAT with the IL-2 promoter and phRL-TK vector as a control plasmid. MKN45 cells were seeded in a 96-well plate and cultured overnight at 37°C and 5% CO₂. After removing the medium, MKN45 cells were pre-treated with IFN-γ (10 ng/mL) for 24 hr. Jurkat-PD-1⁺/NFAT cells were activated with PHA-L (2 μg/mL) and IL-2 (10 ng/mL) for 48 hr and then co-cultured with MKN45 cells upon the addition of 100 nM of diabody-mp, diabody-pm, or anti-PD-1 mAb at 37°C for 6 hr. Luciferase activity was determined using the Dual Luciferase Reporter Assay Kit (Vazyme, Nanjing, China).

Western blotting

A549 or MHCC-97 H cells were plated in 6-well plates and incubated overnight at 37°C and 5% CO₂. The cells were incubated with 100 nM of diabody-mp, diabody-pm, anti-c-Met mAb, and JNJ-38877605 (Selleckchem, TX, USA) at 37°C for 2 hr. They were stimulated with or without 1 nM of HGF (GenScript, Nanjing, China) before collection and lyised with RIPA cell lysis buffer (Meilun, Dalian, China). Equal amounts of cell lysate were subjected to 10% SDS-PAGE. Immunoblotting was performed with primary antibodies against c-Met (ProteinTech, #25869-1-AP), Phospho-Met (Tyr1234/1235) (CST, #3077), Akt (CST, #4691), phospho-Akt (Ser473) (CST, #4060), p44/42 MAPK (CST, #4695), Phospho-p44/42 MAPK (Thr202/Tyr204) (CST, #4370), and GAPDH (CST, #5174) overnight at 4°C. HRP-conjugated secondary antibodies (CST, MA, USA) were incubated at room temperature for 1 hr.

Proliferation assay

A549 and MHCC-97 H cells were dispensed in 100 μL aliquots, seeded in 96-well plates, and allowed to adhere to the plates overnight. The cells were treated with HGF, followed by incubation with diabody-mp, diabody-pm, anti-c-Met mAb, and JNJ-38877605. They were further incubated in 10% Cell Counting Kit-8 solution (Meilun, Dalian, China) and diluted in culture medium for an additional 2 hr at 37°C. The absorbance at 450 nm was determined in each well.

Tumor cells migration and invasion

For cell migration detection, A549 and MHCC-97 H cells were plated in 6-well plates and incubated at 37°C and 5% CO₂ for 24 hr. At > 90% cell confluence, the medium was removed and the cells were wounded with a tip. The detached cells were removed by washing the cells with a serum-free medium. Diabody-mp, diabody-pm, or JNJ-37788605 was added to serum-free medium with or without 1 nM HGF. The wounds were photographed at 0 hr, 24 hr, and 48 hr. For cell migration detection, 8-μm transwell inserts in 24-well plates (Corning Falcon, NY, USA) were coated with Matrigel basement membrane matrix (50 μL/well) (BD Biosciences, Shanghai, China). The cells were re-suspended in 200 μL serum-free RPMI 1640 medium and cultured in the upper chamber. RPMI 1640 medium (with 20% FBS) was added to the bottom wells. Diabody-mp, diabody-pm, or JNJ-37788605 was added to the medium in both inserts. The cells in the upper chamber were removed after 24 hr of incubation at 37°C. The migrated cells were fixed with 4% paraformaldehyde (PFA) and stained with 0.1% crystal violet solution for 30 min at room temperature. The wound
area and the number of migrated cells were quantified using ImageJ software.

**T cell-mediated tumor cell killing assay**

A549, MHCC-97 H, or MKN45 cells were pre-treated with IFN-γ (10 ng/mL) for 24 hr and co-cultured in 12-well plates with either of the diabodies and with or without activated T cells for 48 hr. The wells were washed twice with PBS to remove the T cells. The surviving tumor cells were fixed with 4% PFA and stained with 0.1% crystal violet solution. They were quantified by a spectrometer at OD (570 nm), followed by elution with 33% acetic acid.

**Mouse xenograft studies**

All the animal experiments were in accordance with the ethical standards of the Department of Laboratory Animal Science of Fudan University, and this study was approved on March 6, 2019. BALB/c and NOD-SCID mice (6 weeks old) (Jihui, Shanghai, China) were raised and treated under specific-pathogen-free conditions. The mice were injected subcutaneously in the right flank with single-cell suspensions of MKN45 or MHCC-97 H cells (5 × 10^6). The immune system of mice was reconstructed by administering intraperitoneal injection of 10^7 hPBMCs every 2 weeks. Diabody-mp, diabody-pm, anti-PD-1 scFv and anti-c-Met scFv, anti-PD-1/c-Met scFv-Fc, anti-PD-1 mAb, capmatinib, and toripalimab were administered to the mice as indicated. The vehicle group was administered 0.5% methylcellulose supplemented with 1% polysorbate-80. Tumor volume was measured as indicated time and calculated as follows: volume (cm^3) = (width) × length × 0.5.

**Patient and healthy donor material**

All the experiments involving human samples were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards, based on informed consent. Tumor tissues were obtained from a patient with HCC who underwent surgery at Fudan University Shanghai Cancer Center. The samples were enzymatically digested using collagenase I, collagenase IV, and hyaluronidase (YEASEN, Shanghai, China) for 1 hr at 37°C. Single-cell suspensions were filtered with 70-μm filter units (Corning Falcon, NY, USA), and then incubated with diabody-mp or diabody-pm at 37°C for 48 hr. The hPBMCs were isolated from healthy individuals using Ficoll-Paque (GE Healthcare, Little Chalfont, UK). Autologous T cells were enriched using RosetteSep™ human T cell enrichment cocktail kits (STEMCELL technology, Canada). The hPBMCs were activated with PHA-L (2 μg/mL) (Sigma-Aldrich, MO, USA), and T cells were activated with 100 ng/mL of anti-human CD3 and anti-human CD28 antibodies (PeproTech, NJ, USA) for 48 hr.

**Homology modeling**

Similarity searches were conducted for the sequences of diabody-mp and diabody-pm in the DS database, and high score sequences were selected as templates based on E-values (< 1 × 10^-5). After the selected template structures were loaded and aligned, we aligned the model sequence to the template sequence. Next, homology models were built using the MODELER program and selected according to PDF Total Energy or DOPE Score. Ramachandran plot was used to evaluate the structural rationality of the model after further energy optimization of the protein. The binding modes of diabody-mp or diabody-pm to c-Met were predicted using ZDOCK, a computational protein-protein docking program. Possible binding poses were searched and refined using the CHARMM-based optimization procedure RDOCK. The most reasonable binding pose was selected according to RDOCK scores on the basis of annotated CDR loops of both light and heavy chain of diabody-mp and diabody-pm in the Kabat numbering scheme.

**Immunohistochemical and immunofluorescence staining**

Tumors were removed from sacrificed mice and fixed in 4% PFA for 24 hr. The samples were embedded in paraffin and sectioned into slides. Individual slides were then incubated overnight at 4°C with primary antibodies against Ki67 (ProteinTech, #27309–1-AP), VEGFA (ProteinTech, #19003–1-AP), MMP9 (CST, #13667), and CD8 (ProteinTech, #66868–1-lg). Tumors were removed from sacrificed mice, embedded into OCT blocks, and frozen for cryostat sections (8 μm thick). Cryostat sections were fixed with 4% PFA for 15 min at room temperature and blocked with a blocking solution for 30 min at room temperature. The samples were stained with primary antibodies against CD8 (ProteinTech, #66868–1-lg), Granzyme B (ProteinTech, #13588–1-AP), and CoraLite488-conjugated goat anti-mouse (ProteinTech, #SA00013–1) and CoraLite594-conjugated Goat anti-rabbit (ProteinTech, #SA00013–4) secondary antibodies at room temperature for 1 hr. 4’,6-Diamidino-2-phenylindole (Dalian Meilun Biotechnology, Dalian, China) was used for nuclear staining, and the EVOS M5000 microscope was used for image analysis. Data were analyzed using ImageJ software.

**Statistical analysis**

Statistical analyses were conducted using GraphPad Prism 7. The data are presented as means ± SD; pairwise multiple comparisons were carried out using two-way ANOVA tests, unpaired t-tests, and log-rank (Mantel-Cox) test. A p-value less than 0.05 indicated statistical significance (**p < .001; *p < .01; *p < .05; ns: not significant p > .05).

**Results**

**Design of single-chain PD-1 specific diabodies with high and low affinities to c-Met**

We developed two bispecific single-chain diabodies targeting c-Met and PD-1 for solid tumor treatment (Figure 1a-b). On one hand, only light and heavy chain variable regions of
anti-c-Met and anti-PD-1 mAbs, diabodies (55 kDa) may show improved pharmacokinetics and tissue penetration. On the other hand, the diabodies have different affinities with c-Met (diabody-pm [low, KD = 32.48 nM] and diabody-mp [high, KD = 0.63 nM]) (Figure 1c). Therefore, diabody-mp with superior target retention by optimization of c-Met-associated
affinity may theoretically have higher drug exposure level in tumors. This improves the anti-tumor efficacy without the damage of normal tissues caused by long-term drug retention in the periphery.

Previous studies on the prediction of protein structure by homologous modeling found that diabodies could be developed into extremely flexible structures (Figure 1d) by exchanging the light and heavy chain variable regions of anti-c-Met and anti-PD-1 mAbs (Figure 1a). The Ramachandran plot showed high rationality of homology modeling of diabody-mp and diabody-pm (Figure 1e). Furthermore, we annotated the antibody sequence of the diabodies by labeling the complementary-determining region (CDR) using Discovery Studio, a computer protein simulation software (Figure 1f). Next, using the ZDOCK database to predict the binding mode of diabody-mp or diabody-pm with c-Met (PDB ID: 2RFS), we found that the binding details were quite different despite some common interaction interface, as diabody-mp has a relatively longer CDR loop (Figure 1g, white arrow). This result may shed light on the possible mechanism associated with the relatively high binding affinity of diabody-mp to c-Met.

**Diabodies suppress HGF-induced proliferation, migration, and invasion of tumor cells**

The c-Met tyrosine kinase receptor is activated through a ligand-dependent and/or ligand-independent mechanism. To detect the anti-c-Met effect of diabodies, we screened HGF-dependent A549 cells, HGF-independent MHCC-97 H cells with high c-Met expression, and MKN45 cells with low c-Met expression as c-Met-positive tumor cell lines for subsequent experiments (Figure 2a). Unlike anti-c-Met mAb and c-Met inhibitor NJN-38877605, which played certain inhibitory roles in either HGF-dependent or HGF-independent settings, the diabodies significantly inhibited HGF-induced proliferation of both A549 and MHCC-97 H cells (Figure 2b). In addition, both diabodies inhibited HGF-induced migration and invasion of A549 and MHCC-97 H cells (Figure 2c-d). Together, our data illustrated that both diabody-mp and diabody-pm suppressed HGF-induced proliferation, migration, and invasion of tumor cells in vitro.

**Diabodies inhibit the activation of c-Met signaling by antagonizing HGF binding to c-Met**

To further investigate the effect of the diabodies on cellular phospho-c-Met, western blotting was used to quantify exogenous HGF (1 nM)-induced phospho-c-Met, and we analyzed the PI3K/Akt and MAPK signaling cascades triggered by the activation of c-Met tyrosine kinase in the lysates of A549 and MHCC-97 H cells. Both diabodies and NJN-38877605 reduced HGF-induced c-Met phosphorylation in A549 cells, but anti-c-Met mAb was less effective in blocking HGF-induced c-Met phosphorylation (Figure 2e, left). Moreover, both diabodies showed dose-dependent inhibition of the c-Met signaling pathway in A549 cells (Figure 2f). In contrast to the HGF-dependent A549 cells, MHCC-97 H cells expressed high levels of constitutively phosphorylated c-Met. Although the inhibitory effect of the diabodies on phospho-c-Met was not significant, the diabodies similarly inhibited HGF-induced elevation of phospho-Akt and phospho-Erk (Figure 2e, right). Furthermore, treatment with the bivalent anti-c-Met mAb mimicked the activity of native HGF, which induced c-Met phosphorylation in both ligand-dependent A549 cells and ligand-independent MHCC-97 H cells (Figure 2g). Remarkably, the diabodies did not induce c-Met phosphorylation in the absence of HGF, which was consistent with our design ideas (Figure 2g-h). These results suggested that both diabodies inhibited the activation of c-Met signaling by antagonizing HGF binding to c-Met.

**Diabodies promote T cell activation by blocking PD-1/PD-L1 axis**

The binding of the TCR-CD3 complex on the effector T cells with the MHC-I complex on the tumor cells leads to the activation of effector cells. However, the interaction of PD-1 on the effector T cells with PD-L1 on the tumor cells causes T cell depletion and tumor immune escape. Herein, we engineered the Jurkat cell line that stably express or knockout PD-1 with luciferase expression regulated by the binding of the transcription factor NFAT with the promoter of IL-2. Furthermore, we measured the bioactivity of the diabodies by conducting a reporter gene assay with PHA-L-activated Jurkat cells expressing PD-1 (effector cells) and IFN-γ-induced tumor cells expressing PD-L1 (target cells). Both diabodies increased luciferase expression in Jurkat-PD-1+ cells, but not in Jurkat-PD-1− cells (Figure 3a), suggesting that they could promote target-specific activation of Jurkat cells by blocking PD-1/PD-L1 interaction.

To determine the ability of the diabodies to activate T cells, a single-cell suspension was prepared from the fresh tumor tissue of a patient with HCC using the enzyme digestion method, and the suspension was incubated with the diabodies. An increase in the expression level of CD69 (the lymphocyte activating marker) was observed in tumor-infiltrating CD8+ and CD4+ T effector cells in the diabody-treated groups compared with that in the control group (Figure 3b), indicating that diabodies could promote the activation of CD8+ and CD4+ T cells in the tumor microenvironment. Together, these results demonstrated that diabody-targeted PD-1 could promote the activation of T cells and redirect the human immune system.

**Diabodies mediate cellular cytotoxicity of HGF-dependent and HGF-independent tumor cells through T cell engagement**

To evaluate the broad therapeutic potency of the diabodies in HGF-dependent and HGF-independent settings, we examined the non-small cell lung carcinoma cell line A549, HCC cell line MHCC-97 H, and gastric carcinoma cell line MKN45 as c-Met-expressing targets in an in vitro apoptosis assay. Human T cells as effectors were isolated by negative selection from the peripheral blood of healthy donors. PI and Annexin V FACS analyses revealed that both diabodies promoted the apoptosis of tumor cells with diverse c-Met aberration through T cell engagement (Figure 3c). Moreover, the diabodies induced
Figure 2. Diabodies inhibit the proliferation and movement of tumor cells and the activation of c-Met signaling by antagonizing HGF binding to c-Met. a The mRNA levels of c-Met detected by quantitative reverse transcription polymerase chain reaction in A549, MHCC-97 H, and MNK45 cells. Expression level of p-c-Met (Y1234/5) and total c-Met protein levels in A549, MHCC-97 H, and MNK45 cells treated with or without HGF (1 nM). b A549 or MHCC-97 H cells treated with 100 nM of diabody-mp, diabody-pm, anti-c-Met mAb, or JNJ-38877605 and stimulated with 1 nM HGF were subjected to a Cell Counting Kit-8 assay, and viable cells were measured on days 1–5. c Representative images of A549 cells and MHCC-97 H cells incubated with 100 nM of diabody-mp, diabody-pm, and JNJ-38877605 (positive control) from HGF-induced wound healing assay; scale bar, 400 µm. d Representative images of A549 and MHCC-97 H cells treated with 100 nM of diabody-mp, diabody-pm, and JNJ-38877605 (positive control) from HGF-induced transwell assay; scale bar, 400 µm. e Western blot analysis of the activation of c-Met downstream molecules in A549 or MHCC-97 H cell lysates. Cells were treated with 100 nM of diabody-mp, diabody-pm, anti-c-Met mAb, and JNJ-38877605 for 2 hr and analyzed by immunoblotting with the indicated antibodies. HGF (1 nM) was added to stimulate cells at 37 °C for 15 min before sample collection. f Diabody-mp or diabody-pm inhibits HGF-induced c-Met signaling activation in a dose-dependent manner. A549 cells were incubated with the indicated concentration of diabody-mp or diabody-pm at 37 °C for 2 hr. g Western blot analysis of c-Met and other phosphorylated-targets in A549 and MHCC-97 H cell lysates. The cells were treated with HGF, anti-c-Met mAb, diabody-mp, and diabody-pm at indicated time point. h Design ideas of diabody-mp.
human T cell-mediated killing (Figure 3d), which was likely due to the increased expression of cytokines IFN-γ and IL-2 (Figure 3e). Moreover, the diabodies induced dose-dependent secretion of IFN-γ and IL-2 after co-culturing with MKN45 cells (Figure 3f), suggesting that the diabody-induced activation of T cells can only occur in tumor cells. This also indicates the safety of the diabodies in normal cells. Accordingly, our results revealed that both diabodies induced T cell-mediated
cellular cytotoxicity against c-Met-positive tumor by improving cytokine secretion.

**Diabody-mp inhibits the growth of tumors with high and low expression of c-Met in vivo**

No significant difference in anti-tumor activity was observed between both diabodies. Subsequently, to examine the therapeutic efficacy of two diabodies in NOD-SCID mice engrafted with human peripheral blood mononuclear cells (hPBMCs), we established transplant solid tumor models with low c-Met expression in MKN45 cells and high c-Met expression in MHCC-97 H cells. All the mice were injected once daily for 30 consecutive days with 1 mg/kg of diabody-mp or diabody-pm. Although the two distinct bispecific diabodies inhibited tumor growth in vivo, diabody-pm provided limited therapeutic benefits for low c-Met expression in the MKN45 tumor model, whereas diabody-mp therapy substantially decreased tumor burden in both high and low expression of c-Met tumors model. Moreover, all tumors progressed in the control mice treated with the vehicle (Figure 4a-c). In addition, diabody-mp suppressed spontaneous liver metastasis in the MKN45 tumor model, compared with the control group (Figure 4d). To explore the potential mechanisms associated with the anti-tumor activity of the diabodies, we assessed the activation of lymphocytes in an MKN45 tumor model and observed the upregulation of CD69 in CD8+ and CD4+ T cells. It is worth noting that diabody-mp induced higher CD69 expression in CD8+ T cells than diabody-pm (Figure 4e). Moreover, we identified that diabody-mp inhibited tumor proliferation, metastasis, and angiogenesis by inhibiting the expression of Ki67, MMP9, and VEGFA in tumor tissues (Figure 4f). However, diabody-pm did not significantly inhibit the expression of MMP9 and VEGFA (Figure 4f). In general, our observations in tumors models with both high and low expression of c-Met suggested that dual c-Met and PD-1 blockade by diabodies, especially diabody-mp, showed marked anti-tumor activity in vivo.

**Diabody-mp exhibits stronger tumor inhibition than other structural antibodies**

Furthermore, we evaluated if diabody-mp showed preferable therapeutic efficacy in vivo, compared with its IgG-like bispecific antibody counterparts (we focused on the scFv-Fc format, which is one of the most commonly used formats of bsAbs), the combination of its two antibodies functional region (anti-PD-1 scFv combined with anti-c-Met scFv), and anti-PD-1 mAb. The result showed diabody-mp exhibited stronger tumor growth inhibition in mice with subcutaneous MKN45 tumor transplants (Figure 5a). The diabody-mp-treated tumor had greater infiltration of CD8+ cytotoxic T lymphocytes than the other treated tumors (Figure 5b). In addition, diabody-mp increased the secretion of cytokine IFN-γ in the tumor microenvironment, but the level of IFN-γ in the peripheral blood was almost undetectable (Figure 5c), suggesting that diabody-mp activates the lymphocytes mainly in the tumor microenvironment. This indicates the safety and efficacy of diabody-mp and that it could effectively avoid cytokine storm generated by the activation of the systemic immune cells.

**Diabody-mp shows superior anti-tumor efficacy than single or combined c-Met and PD-1 blockade therapy**

We further compared the anti-tumor effects of single or combined c-Met and PD-1 blockade with diabody-mp in mouse MKN45 tumor models. The results showed that diabody-mp, but not single or combined capmatinib and toripalimab therapy, had the strongest tumor-inhibition effect (Figure 5d). We analyzed the tumor microenvironment by immunofluorescent detection, providing evidence that combined c-Met and PD-1 inhibition by diabody-mp exhibited higher therapeutic benefits than single or combined agents, possibly due to increased activation of CD8+ T cells (Figure 5e). Although the effect of diabody-mp on CD8+ T cell infiltration was slightly higher than that of combination therapy, there is no significant difference between two groups (Figure 5e). Moreover, diabody-mp prominently extended the survival of MKN45-bearing mice (Figure 5f), whereas combination with capmatinib and toripalimab had reduced effects, and the single agent did not show a considerable effect on the survival of the mice. Generally, these data demonstrated the superior anti-tumor activity of diabody-mp over that of single or combined c-Met and PD-1 blockade therapy in subcutaneous MKN45 gastric carcinoma mice models.

**Discussion**

Approximately 20–60% of solid tumors are poorly recognized by CD8+ T cells due to the deficiency of MHC-I, which is one of the principal reasons for tumor immune escape. Bispecific diabodies could overcome MHC restriction and recruit T cells to eliminate tumors. Moreover, unlike monovalent and bivalent anti-c-Met mAbs, diabodies do not activate c-Met receptor tyrosine kinase but induce the apoptosis of different c-Met-positive tumor cells through T cell engagement. Diabody-mp improves the anti-c-Met affinity by optimizing the c-Met variable region. The high c-Met binding affinity of diabody-mp may play a part in increasing the level of drug exposure in solid tumors by improving tissue permeability and tumor targeting.

Multiple factors determine the affinity and efficacy of antibodies, including the conformational flexibility of binding modules as well as the accessibility of epitopes. Early research proved that single mouse variable (V) domains could potentially target cryptic epitopes. Therefore, it is desirable to engineer anti-c-Met variable (V) domains and anti-PD-1 scFv fragment into diabody-mp with improved binding affinity to c-Met. The diabody provides a new c-Met-binding specificity, which is hardly formed by intact antibodies because their limited diversity of CDR loop lengths restricts antibody-antigen interaction.

Inappropriate activation of cells expressing the Fc receptor could produce a massive level of cytokines and the associated toxic effects, which are undesirable for some antibodies, particularly bispecific antibodies that engage effector cells. Smaller recombinant antibody fragments without the Fc region as
reliable alternatives retain the targeting specificity but possess other superior properties, such as improved avidity and tissue penetration. Bispecific diabodies, as intermediate-sized multivalent molecules (55 kDa) without the Fc region, are ideal tumor-targeting reagents, which could provide rapid tissue penetration and high target retention. Nevertheless, diabodies...
Figure 5. Diabody-mp exhibits improved tumor inhibition compared with other structural antibodies and single or combined c-Met and PD-1 blockade therapy. 

a Individual tumor growth curve. BALB/c mice were inoculated with MKN45 cells and treated as indicated for a month from day 10. The tumor growth was monitored at the indicated times point. n = 5 mice per group.

b Representative images of immunohistochemical staining of CD8⁺ performed on MKN45 tumors treated as indicated; scale bar, 100 µm.

c Quantification of IFN-γ secreted in the blood and tumor microenvironment of mice bearing the MKN45 tumors and treated as indicated. 

d Tumor growth curves of MKN45 gastric carcinoma in BALB/c mice treated with diabody-mp (1 mg/kg, once-daily, i.p.), capmatinib (10 mg/kg, once-daily, i.g.), toripalimab (10 mg/kg, once-weekly, i.v.) or capmatinib combined with toripalimab for a month. n = 8 mice per group.

e Immunostaining of CD8 (CTL marker) and granzyme B (activity of T cell) in the MKN45 tumor sections; scale bar, 100 µm.

f Mice survival curves were generated from MKN45-bearing mice. n = 8 mice per group.
have short half-lives as a result of the defect in the Fc region, which imparts extended circulation. To maintain the effective concentration in vivo and ensure anti-tumor efficacy, diabodies need to be administered more frequently than traditional antibody drugs with long half-lives.

However, increasing clinical data have shown that the efficacy of immune checkpoint inhibitors with a half-life of only a few days is consistent with that of similar drugs with a half-life of several weeks. Target occupancy rate has been used as a pharmacokinetic biomarker for many therapeutic antibodies, one of which is the PD-1 inhibitor. There is no significant relationship between the half-life of anti-PD-1 antibody in the plasma and PD-1 occupancy on the T cell surface. More efficient and persistent procedures that can block PD-1/PD-L1 in vivo may provide improved therapeutic effects. In other words, as long as anti-PD-1 antibody occupies enough PD-1 on the T cell surface at the beginning of treatment, the anti-tumor effect is guaranteed regardless of the half-life of the PD-1 inhibitor in the plasma. Research has shown that most anti-PD-1 antibodies maintain a PD-1 occupancy rate of more than 70% for several weeks after a single injection, hence, their anti-tumor efficacy would not be affected by the half-life and frequency of medication. As shown in Figure 5a-b, there was almost no significant difference in the inhibition of tumor growth in mice administered diabody-mp once daily or twice weekly. Further related comparison and analysis could be carried out in the follow-up study.

Furthermore, immune checkpoint inhibitors with a long half-life may produce immune-related adverse reactions. It was recently reported that immune checkpoint inhibitors with a half-life of more than 15–20 days and a target binding rate of more than 70% after several months of treatment caused a series of severe side effects in clinical trials. Although the mechanism has not been fully elucidated, some researchers speculated that the severe side effects may be associated with the long half-life of such drugs, suggesting that diabodies with a short half-life might decrease the risk of adverse reactions. In mouse models, no obvious difference was observed between the diabody-treated groups and the control group in terms of activity, weight, and eating behavior. Moreover, hematoxylin and eosin staining of the liver and lung sections from the mice in the diabody-treated groups showed normal histological morphology without cell denaturation and inflammatory cell infiltration, suggesting no obvious organ toxicity. In addition, as shown in Figure 5c, diabody-mp increased the secretion of IFN-γ in the tumor microenvironment, but the level of IFN-γ in the peripheral blood was almost undetectable. This demonstrated that by activating lymphocytes mainly in the tumor microenvironment, diabody-mp could effectively avoid cytokine storm generated by the activation of the systemic immune cells.

Taken together, our results indicate that bispecific diabody-mp exhibited improved tumor growth inhibition, which greatly enhanced the survival of tumor-bearing mice compared with other structural antibodies and single or combined c-Met and PD-1 blockade therapy. The results of the in vitro studies indicate that there was no significant difference in the anti-tumor activities of the two diabodies. However, in vivo studies in mice models revealed that diabody-mp with higher c-Met binding affinity exhibited stronger anti-tumor activity than diabody-mp with lower c-Met binding affinity, suggesting the potential of diabody-mp in the treatment of c-Met-positive solid tumors.

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ORCID
Qingyun Yuan http://orcid.org/0000-0002-5313-9239

Author contributions
Min Yu, Huijie Wang, and Qingyun Yuan designed the study. Qingyun Yuan prepared all the materials and designed all the experiments. Qingyun Yuan, Weihua Hou, and Yuxiong Wang performed expression and purification of the antibodies. Qingyun Yuan and Xingxing Yuan assisted with all in vitro experiments. Qingyun Yuan and Qiaoyan Liang contributed to the in vivo experiments. Qingyun Yuan performed data collection, analysis, and interpretation. Qingyun Yuan wrote the first draft of the manuscript. All authors revised and approved the manuscript. Min Yu supervised the entire project.

Ethics approval
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All procedures performed in studies involving animals were in accordance with the ethical standards of the department of laboratory animal science of Fudan university and permitted on March 6, 2019.

Consent to participate
Informed consent was obtained from all individual participants included in the study.

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