Low-Dose Apatinib Optimizes Tumor Microenvironment and Potentiates Antitumor Effect of PD-1/PD-L1 Blockade in Lung Cancer

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Abstract

The lack of response to treatment in most lung cancer patients suggests the value of broadening the benefit of anti–PD-1/PD-L1 monotherapy. Judicious dosing of antiangiogenic agents such as apatinib (VEGFR2-TKI) can modulate the tumor immunosuppressive microenvironment, which contributes to resistance to anti–PD-1/PD-L1 treatment. We therefore hypothesized that inhibiting angiogenesis could enhance the therapeutic efficacy of PD-1/PD-L1 blockade. Here, using a syngeneic lung cancer mouse model, we demonstrated that low-dose apatinib alleviated hypoxia, increased infiltration of CD8+ T cells, reduced recruitment of tumor-associated macrophages in tumor and decreased TGFβ amounts in both tumor and serum. Combining low-dose apatinib with anti–PD-1/PD-L1 significantly retarded tumor growth, reduced the number of metastases, and prolonged survival in mouse models. Anticancer activity was evident after coadministration of low-dose apatinib and anti–PD-1 in a small cohort of patients with pretreated advanced non-small cell lung cancer. Overall, our work shows the rationale for the treatment of lung cancer with a combination of PD-1/PD-L1 blockade and low-dose apatinib.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide, with non–small cell lung cancer (NSCLC) accounting for approximately 80% of all cases (1, 2). Treatment for advanced NSCLC has been revolutionized with the availability of targeted therapy against driver oncogenes such as epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (ALK) rearrangements (3, 4), immunotherapy blocking the interaction between programmed cell death-1 (PD-1) and its ligand (PD-L1; ref. 5), as well as antiangiogenic monoclonal antibodies (mAb) and tyrosine kinase inhibitors (TKI; ref. 6). Clinical trials have demonstrated the superior overall survival (OS) resulting from anti–PD-1/PD-L1 monotherapies such as nivolumab, pembrolizumab, and atezolizumab as compared with docetaxel in the second-line setting in patients with advanced NSCLC. Furthermore, pembrolizumab showed superior efficacy and was received with better tolerance compared with platinum doublet chemotherapy as the first-line treatment in patients with PD-L1 expression in at least 50% of tumor cells (7). However, although predictive biomarkers such as PD-L1 expression and tumor mutational load may allow enrichment of the patient population responsive to PD-1/PD-L1 mAbs, only about 20% of NSCLC patients benefit from monotherapy overall (8, 9). New therapeutic approaches, such as combinations of immunotherapies and conventional treatments, are needed to extend the benefits to a larger population of patients with lung cancer.

Evidence suggests that PD-1/PD-L1 mAbs are less efficacious in noninflamed tumors characterized by poor infiltration of lymphocytes, rare PD-L1 expression, increased immunosuppressive components, and more abnormal angiogenesis in the tumor microenvironment (TME; refs. 8, 10). Combining PD-1/PD-L1 mAbs with agents that can modulate the immunosuppressive milieu of TME may overcome primary treatment resistance in patients with advanced NSCLC (8, 11). Antiangiogenic therapy in NSCLC targets the TME through the proangiogenic factor vascular endothelial growth factor (VEGF) and its receptor 2 (VEGFR2; ref. 12). Antiangiogenic therapy enhances the effect of cytotoxic chemotherapy or molecular targeted therapy in patients with advanced NSCLC (6, 12, 13). Studies suggest a bidirectional
relationship between angiogenesis and antitumor immunity in the TME (13–15). Antiangiogenic agents could eliminate immunosuppression by the TME (16–18) and may enhance therapeutic efficacy when given in combination with immunotherapy. Indeed, several preclinical studies have observed promising antitumor effects from antiangiogenic agents combined with immunotherapies in multiple tumor types such as melanoma, renal cell carcinoma, and colon adenocarcinoma (17, 19–21). Trials investigating these combinations in patients with advanced NSCLC are also ongoing; the existing data are preliminary but promising (13, 22). However, challenges remain to be overcome before the full potential of antiangiogenesis and immunotherapy combination can be realized.

In this study, we analyzed the impact of apatinib, a small-molecule VEGFR2-TKI, on the TME in murine Lewis lung carcinoma (LLC) models at different doses alone and in combination with an anti–PD-L1. Apatinib has been approved as the third line therapy for patients with advanced gastric cancer by the CFDA (23) and is undergoing evaluation for patients with advanced nonsquamous NSCLC in a phase III trial (NCT02515435). We found that low-dose apatinib alleviated hypoxia and remodeled the immunosuppressive TME to one more permissive for antitumor immunity. In addition, using optimized dosing of apatinib, we observed increased antitumor efficacy when it was combined with anti–PD-L1 in the in vivo experiment. Moreover, in preliminary results from a phase I/B clinical trial, the combination of SHR-1210 (a clinically available PD-1 mAb (24) with low-dose apatinib achieved an objective response rate of 55.6% (5/9) in patients with pretreated NSCLC (ClinicalTrials.gov: NCT03 083041).

Materials and Methods

Cell line, tumor models, and agents

LLC cells were obtained from ATCC at 2014 and cultured at 37°C in a humidified incubator with 5% CO2 in Dulbecco’s modified Eagle medium (DMEM; HyClone) containing 10% fetal bovine serum (Life Technologies) with 100 units/mL penicillin and 100 μg/mL streptomycin. This cell line was not reauthenticated using specific assays in the past years, and only identified by morphology and growth characteristic. Cells were routinely tested to confirm the absence of Mycoplasma contamination and were cultured for less than 20 generations.

Male C57BL/6 mice (6–8 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. Animals were housed and maintained under optimal conditions of light, temperature, and humidity with free access to food and water. For a subcutaneous tumor model, a total of 1 × 10⁶ LLC cells were resuspended in 200 μL PBS and inoculated subcutaneously into the right flank of mice. Tumor dimensions were measured by caliper every other day, and tumor volume (mm³) was estimated using this formula: tumor volume = (long axis) × (short axis)² × π/6. For the metastasis tumor model, 5 × 10⁵ LLC cells in 100 μL PBS were intravenously injected into the tail vein of C57BL/6 mice, and microcomputerized tomography (micro-CT) using the eXplore Locus MicroCT scanner, GE Healthcare) was used to monitor tumor burden in the lung. Apatinib (kindly provided by Hengrui Medicine Co. Ltd) treatment at various doses was initiated 3 days after tumor cell inoculation and administered by oral gavage every day. Anti–PD-L1 (anti-mouse CD274, clone: MIH5, eBioscience) was administered at 200 μg/mouse by intraperitoneal injection every 3 days. At indicated days, tumor-bearing mice were anesthetized and tissues were harvested for analysis and measurement. For comparing the survival time of the mice in each group, additional independent experiments were conducted for observation.

All the animal studies were approved by the Institutional Committee for Animal Care and Use, Tongji University School of Medicine, and were performed in accordance with the institutional guidelines.

Flow cytometry analysis

Tumors and spleens from the subcutaneous LLC model were harvested on the days indicated. Single-cell suspensions were generated through enzymatic digestion at 37°C for 1 hour in DMEM medium containing collagenase type IV (1 mg/mL Sigma), hyaluronidase (1 mg/mL Sigma) and DNase I (20 U/mL Sigma), or via mechanical dissociation from the collected tissues. Erythrocytes were lysed in the Red Blood Cell lysis Buffer (Sigma). Then single cells were blocked with CD16/32 Ab (clone 93, eBioscience: #14-0161-85, 1:1,000) and were stained with the following Abs against mouse for 30 minutes at 4°C. CD8 (FITC, clone: 4S15, eBioscience: #14-0808-82, 1:300), CD3 (APC, clone: 145-2C11, eBioscience: #17-0031-82, 1:300), PD-L1 (PE, clone: MIH5, eBioscience: #AF1019, 1:300), CD11b (FITC, clone: M1/70, eBioscience: #11-0112-82, 1:300), Ly6G (PE, clone: RB6-8C5, eBioscience: #12-5931-82, 1:300), Ly6C (APC, clone: HK1.4, eBioscience: #17-5932-82, 1:300), F4/80 (APC, clone: BM8, eBioscience: #17-4801-82, 1:300), CD206 (PE, clone: MR6F3, eBioscience: #12-2061-82, 1:300), CD86 (PE-Cyanine7, clone: GL1, eBioscience: #25-0862-82, 1:300), CD45 (FITC, clone: MR6F3, eBioscience: #11-0451-82, 1:300), CD11c (APC, clone: N418, eBioscience: #17-0114-82, 1:300), or isotype controls, which were purchased from eBioscience. Intraacellular Foxp3+ Tregs staining was done by the mouse regulatory T-cell staining kit #3 (eBioscience, clone: FK-165, #88-8115-40). Data were acquired by multiparameter flow cytometry on Accuri C6 flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star Inc.).

Histologic analysis

For IHC analysis, formalin-fixed paraffin-embedded tissue sections were deparaffinized, and after antigen retrieval, the slides were stained with hematoxylin and eosin or with the mouse mAbs against PD-L1 or CD163 (anti–PD-L1, #64988S, 1:100, Cell Signaling Technology; anti-CD163, #ab182422, 1:500, Abcam). Details of the staining method are described in previous studies (25). The CD8/Ki67 ratio after immunofluorescence was used to assess the proliferation of CD8+ T cells, and CD31/collagen IV or CD31/α-SMA was used for vascular analysis (anti–CD8, 1:100, #ab140808-82; anti–Ki67, 1:200, #ab16667; anti–CD31, 1:50, #ab28364; anti–Col IV, 1:100, #ab19080; anti–α-SMA, 1:200, #ab7817; all from eBioscience). Sections were first incubated with primary antibodies and then stained with appropriate fluorophore-conjugated secondary Abs according to the manufacturer’s recommendations. The tissues were also counterstained for cell nuclei using DAPI. To detect the hypoxic area in tumor, Hypoxyprobe-1 (60 mg/kg, Hypoxyprobe-1 Plus Kit, Hypoxyprobe, Inc.) solution was administrated via tail-vein injection 60 minutes before tumors were harvested and fixed with 4% paraformaldehyde. Then tumor tissues were sectioned and...
stained with the primary antibody (FITC-Mab1) and secondary antibody (HRP linked to rabbit anti-FITC IgG) according to the manufacturer’s instructions. Fluorescent images were photographed using a confocal laser-scanning microscope (Nikon eclipse Ti-SR, DS-U1).

Cytokine assay

Peripheral blood and tumor tissues were collected from experimental mice, and indicated cytokines were evaluated. In brief, blood samples and tumor tissues were obtained on days 7, 14, and 21 after apatinib-treatment initiation in apatinib monotherapy experiments or on day 32 in the combination experiments. Serum was separated by centrifugation and then stored at −80°C. Tumor tissues were also stored at −80°C until they were used for multiplex assay or single analyze ELISA analysis. The circulating and intratumoral IL10, IFNγ, TNFα, GM-CSF were measured using the U-plex biomarker group 1 (nvs) Assays from Meso Scale Discovery. TGFB was analyzed using the ELISA kit from eBioscience. All samples were tested in duplicate.

Patient cohort

The phase IB trial (NCT03083041), begun at our institute in March 2017, investigates the efficacy and toxicity of SHR-1210 combined with apatinib at two doses in PD-L1 expression unselected patients with advanced NSCLC. Results from a phase III clinical trial of patients with advanced nonsquamous NSCLC treated with apatinib recommended a dose of 750 mg, oral, daily. Our goal was to determine the safety, tolerability, and pharmacokinetics of SHR-1210 in combination with lower doses of apatinib (250 or 375 mg/day, orally). SHR-1210 was administrated at a dose of 200 mg every 2 weeks.

All the enrolled patients lacked EGFR-activating mutations or EML4–ALK translocations, had failed at least two previous systemic treatments, and had received no prior immune-checkpoint inhibitors. Additional eligibility requirements included age ≥ 18, with at least one measurable lesion per Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1), Eastern Cooperative Oncology Group (ECOG) performance score of 0 or 1, and adequate end-organ and hematologic function. Tumor measurements were performed based on CT scan imaging by investigator per RECIST v1.1 every 2 cycles (4 weeks/cycle) for the first 6 months, and then reexamined every 3 cycles. The cutoff date for data to be analyzed for this paper was December 31, 2017. This study was approved by the Ethics Committee of Shanghai Pulmonary Hospital, Tongji University School of Medicine. Before the initiation of any study-related procedures, written informed consent was obtained from each participant.

Statistical analysis

Results were presented as mean ± SEM. All data analysis was performed using GraphPad Prism software (version 5.0, GraphPad Software, Inc.). For the comparison among treatment groups in the in vitro study, one-way ANOVA was performed. Survival curves were plotted via Kaplan–Meier method, and differences were analyzed with log-rank (Mantel–Cox) test. Survival time was defined from the day of tumor cell inoculation until the mice expired naturally or euthanized. No additional power analysis was conducted to calculate the sample size, and the numbers of mice enrolled in experiments were determined empirically based on our experience with similar assays and from the numbers generally used by other researchers. P < 0.05 was considered statistically significant. In the figures, symbols were used as *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Results

Low-dose apatinib alleviates hypoxia and suppresses tumor growth in vivo

Previous studies suggested that anti-VEGF/R2 treatment could alleviate intratumoral hypoxia and immunosuppression by modulating abnormal tumor vasculature in a dose- and time-dependent pattern (26). Therefore, we began our research by scheduling apatinib administration and investigated the changes in intratumoral vessels based on the subcutaneous lung cancer model. Previous reports indicated that 50 to 200 mg/kg/day apatinib was necessary to achieve anticancer effects in different mouse models (27–29), and low-dose antiangiogenic agents could promote vascular normalization (30). We thus treated tumor-bearing mice with low-dose apatinib (60 mg/kg, hereafter referred to as "Apa-60") or CMC (0.5% (w/v), carboxymethyl cellulose—Na solution) as a control via oral gavage 3 days after tumor inoculation, and then assessed tumor vascular structure and hypoxia at different time points. We focused our analysis on day 7 (referring to previously defined time window of normalization in other studies; refs. 25, 31), days 14 and 21 after apatinib administration. On day 7, the dual staining of CD31 and alpha-smooth muscle actin (α-SMA) showed that perivascular cell coverage was significantly higher in the Apa-60 group compared with the control group (Fig. 1A and B), though the tumor microvascular density (MVD, defined as CD31+ vessel numbers per field) and basement membrane (BM) showed no difference (Fig. 1C; Supplementary Fig. S1A). Afterward, we observed increased perivascular cell coverage and BM as well in the Apa-60 group on days 14 and 21 (Fig. 1D and E; Supplementary Fig. S1A). In addition, we found that Apa-60 tended to reduce hypoxia (measured by Hypoxyprobe-1) and HIF1α expression in tumor tissue on day 7, though did not reach statistical significance (Supplementary Fig. S1B). Then on days 14 and 21, hypoxia in the Apa-60 group significantly decreased compared with the control group (Fig. 1F–H; Supplementary Fig. S1B). Furthermore, because normalizing abnormal tumor vessels might increase nutrient and oxygen supply to cancer cells and could theoretically accelerate tumor growth, we monitored tumor volume in each group every other day. We saw a sustained but modest decrease in tumor growth after Apa-60 treatment compared with the untreated group (Supplementary Fig. S2A and S2B).

Next, we titrated the dose of apatinib to assess whether higher doses of apatinib treatment could induce more extensive vascular normalization and tumor growth inhibition. High-dose (180 mg/kg, the maximum tolerance dose based on preliminary data, hereafter referred to as "Apa-180") or intermediate-dose (120 mg/kg, hereafter referred to as "Apa-120") apatinib treatment showed significantly greater retardation in tumor growth than in the Apa-60 treatment group (Supplementary Fig. S2A and B). However, the MVD, perivascular cell coverage, and BM in both Apa-120 and Apa-180 groups were less than in the Apa-60 group on days 14 and 21 (Fig. 1E; Supplementary Fig. S1A), and hypoxia was aggravated in higher-dose groups (Fig. 1F–H; Supplementary Fig. S1B).

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Low-dose apatinib treatment increases the infiltration of lymphocytes in tumor

Studies have demonstrated a mechanistic link between vasculature and immune-cell infiltration in tumor (17, 18, 21). Thus, we assessed the intratumoral immunologic changes post apatinib treatment by flow cytometry in the subcutaneous model. Numbers of lymphocytes (measured as CD3+ cells) in the Apa-60 group significantly increased compared with those in the higher-dose and control groups on days 7 and 21 after treatment initiation (Fig. 2A). We observed a simultaneous change in the number of tumor-infiltrating CD8+ T cells. On day 14, CD8+ T cells in the Apa-60 group increased, compared with the higher-dose treatment groups. On day 21, a statistically significant increase of tumor-infiltrating CD8+ T cells was observed in Apa-60 when compared with the other groups (Fig. 2B). To further evaluate the proliferation of intratumoral CD8+ cells, dual staining of CD8 and Ki67 was conducted in tumor sections. The proportion of Ki67+/CD8+ double-positive cells in CD8+ cells was highest in the Apa-60 group on day 21 (Fig. 2C and D), suggesting enhanced proliferation of CD8+ T cells in tumor post low-dose apatinib treatment.

Figure 1.
Low-dose apatinib improved tumor vascular normalization. Subcutaneous tumor-bearing mice were treated with different doses (60, 120, 180 mg/kg) of apatinib or CMC as control by oral gavage daily. Tumor vasculatures were analyzed on days 7, 14, and 21 after apatinib treatment initiation (n = 6–7 per group). A, Representative immunofluorescent staining for perivascular cell coverage (the ratio of α-SMA/CD31 double-positive area over CD31+ area) on day 7. Red, CD31 staining; green, α-SMA staining; blue, DAPI staining. Scale bar, 50 μm. B, Quantification of perivascular cell coverage on day 7. C, Quantification of MVD on day 7. D, Representative immunofluorescent staining for BM (collagen IV positive vessel) on day 14. Red, CD31 staining; green, collagen IV staining; blue, DAPI staining. Scale bar, 50 μm. E, Quantification of BM on day 14. F, G, H, Representative images and comparisons of Hypoxyprobe+ and HIFα+ areas in core tumor regions on day 14. Hypoxyprobe+ and HIFα+ areas are presented as percentage per total sectional area. Scale bar, 100 μm. All of the data are from one experiment representative of two independent experiments.
Low-dose apatinib hinders immunosuppressive myeloid recruitment better than high-dose apatinib

We next analyzed the effects on immunosuppressive cells that could attenuate or inhibit T-cell–mediated antitumor immune responses in the TME by cells such as myeloid-derived suppressor cells (MDSC), tumor-associated macrophages (TAM), and regulatory T cells (Treg). We found that on days 14 and 21 after apatinib treatment, numbers of intratumoral immune cells were analyzed by flow cytometry on day indicated (n = 6–7 per group).

**Figure 2.**

Effect of apatinib treatment on tumor-infiltrating lymphocytes. Subcutaneous tumor-bearing mice were treated as described in Fig. 1 and intratumoral immune cells were analyzed by flow cytometry on day indicated (n = 6–7 per group). **A,** Left, percentages of CD3+ T cells in tumor on days 7, 14, and 21 after apatinib treatment. Right, flow-cytometric analysis of CD3+ T cells on day 21. **B,** Left, percentages of CD8+ T cells in tumor on days 7, 14, and 21. Right, flow-cytometric analysis of CD8+ T cells on day 21. **C,** Representative immunofluorescent staining of sections from different treatment groups on day 21. Red, CD8 staining; green, Ki67 (proliferation marker) staining; blue, DAPI staining. Scale bar, 100 μm. **D,** Histograms show the quantitation of CD8+ T-cell proliferation on day 21. All of the data are from one experiment representative of two independent experiments.
Figure 3.
Changes in intratumoral MDSCs and TAMs after apatinib treatment. Subcutaneous tumor-bearing mice were treated as described in Fig. 1, and MDSCs and TAMs were analyzed on day indicated (n = 6–7 per group). A, Representative images of the gating strategy to define MDSCs: Mo-MDSCs (CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6Chigh) and PMN-MDSCs (CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>low</sup>). B, C, Percentages of Mo-MDSCs and PMN-MDSCs in each group on days 7, 14, and 21 after apatinib treatment. D, Representative images of gating strategy to define TAMs. TAMs were defined as CD11b<sup>+</sup>F4/80<sup>+</sup>. E, Percentages of TAMs from each group on days 7, 14, and 21. F, IHC images for CD163 staining of tumor sections on day 21. Scale bar, 100 μm. G, Histograms show the proportions of CD163<sup>+</sup> cells in each group on day 21. All of the data are from one experiment representative of two independent experiments.
CD11b⁺Ly6G⁺Ly6Chigh mononuclear-MDSCs (Mo-MDSCs; Fig. 3A and B) and CD11b⁺Ly6G⁺Ly6Chlow polymorphonuclear-MDSCs (PMN-MDSCs; Fig. 3A and C) in the Apa-60 group were comparable with those of the control group. However, when compared with Apa-120 and -180 groups, the percentages of both Mo-MDSCs and PMN-MDSCs were reduced in the Apa-60 group. In addition, low-dose apatinib significantly decreased the proportion of CD11b⁺F4/80⁺ TAMs among total viable cells as compared with the other groups on days 14 and 21 (Fig. 3D and E). Next, we examined CD163⁺ (marker for M2-like macrophage) cells in tumors on day 21 by IHC staining and found that the number of CD163⁺ cells in the Apa-60 group dropped (Fig. 3F and G). No differences were observed between the Apa-60 group and other treatment groups in the amounts of intratumoral transcription factor forkhead box P3 (Foxp3)-positive Tregs (Supplementary Fig. S3).

Changes of intratumoral PD-L1 expression following apatinib treatment
To analyze the effect of apatinib treatment on PD-L1 expression, we investigated changes of intratumoral PD-L1 as well. We found that on day 7, in comparison with the control group, PD-L1 expression was reduced in groups treated with apatinib monotherapy (Fig. 4A and B). At days 14 and 21, no difference in expression was evident between treated and control groups (Fig. 4B–D).

Low-dose apatinib reduces transforming growth factor-β (TGFβ) amounts
Tumor cells can communicate with other components of the tumor milieu via soluble cytokines. We found on day 14 after apatinib monotherapy less intratumoral TGFβ, which could inhibit T-cell infiltration and be immunosuppressive, in the Apa-60 group. On day 21, we found less TGFβ in tumor in the Apa-60 group compared with the other groups, which was consistent with the changes of intratumoral CD8⁺ T cells (Fig. 5A). Furthermore, we observed that TGFβ in serum was reduced as well in the Apa-60 group compared with the Apa-120 and -180 groups on day 21 (Supplementary Fig. S4A). As for other cytokines, including interleukin-10 (IL-10), interferon-γ (IFNγ), tumor necrosis factor-α (TNFα), and granulocyte-macrophage colony stimulating factor (GM-CSF), no differences were observed in any group (Fig. 5B–E; Supplementary Fig. S4B–S4E).

Combining anti-PD-L1 with low-dose apatinib optimizes antitumor effects
Abnormal angiogenesis and immunosuppression in the TME are barriers to effective cancer immunotherapy (9, 32). Because we have observed that low-dose apatinib, in contrast to higher-dose
treatment, induced greater tumor vascular normalization and converted TME from immunosuppressive to immunosupportive, we hypothesized that low-dose apatinib plus anti–PD-L1 combination treatment could enhance antitumor effects. Prior to the initiation of animal experiments, we confirmed that LLC cells express PD-L1, which could be upregulated by IFNγ in vitro (Supplementary Fig. S5A and S5B). We also observed that apatinib did not show potent effects on the apoptosis of LLC cells

**Figure 5.** Expression of inflammatory cytokines in tumor from different treatment groups. Tumor tissues were collected from subcutaneous tumor-bearing mice on the days indicated in Fig. 1. Expression of cytokines were detected by multiplex assay or ELISA (n = 5 per group). Graphs show the concentration alterations of TGFβ (A), IL10 (B), IFNγ (C), TNFα (D), GM-CSF (E) in tumor from mice in each treatment group on day 7, 14, 21 after apatinib treatments of various doses. Each sample was tested in duplicate. The data are from one experiment representative of two independent experiments.
in vitro (Supplementary Fig. S6A-C). Tumor-bearing mice were treated on the following schedules: varying doses (60/120/180 mg/kg) of apatinib or CMC treatment once a day, followed by the administration of anti–PD-L1 or IgG control started on day 14 (Fig. 6A). As expected, in the subcutaneous LLC model, we observed that apatinib plus anti–PD-L1 treatment could attenuate tumor growth compared with either monotherapy group or control. Moreover, tumor growth was more prominently retarded in low-dose apatinib plus anti–PD-L1 treatment as compared with higher-dose combination treatment during the therapeutic period (Fig. 6B and C). No statistical differences in body weight were found among all groups (Fig. 6D), suggesting no overt toxicity. Moreover, we observed that although high-dose apatinib could inhibit tumor growth effectively in the early experimental stage (consistent with previous data; Supplementary Fig. S2B), tumor growth subsequently accelerated and eventually reached no difference compared with control and groups treated solely with anti–PD-L1 (Fig. 6B). Thus, apatinib monotherapy, even used at high dose, was not sufficient to achieve long-term tumor suppression. To verify our hypothesis that low-dose apatinib plus anti–PD-L1 could induce enhanced antitumor effect by modulating the TME, mice were sacrificed, and intratumoral immune components post combination treatment were examined. Consequent with the observations in apatinib monotherapy experiments, we found that compared with other combination treatments, low-dose apatinib plus anti–PD-L1 resulted in a better immunosupportive milieu, accompanied by more CD8+ T cells, but fewer PMN-MDSCs, M0-MDSCs, and TAMs, as well as intratumoral M2-like macrophages infiltrated in tumors (Supplementary Fig. S7), TGFB in both the serum and tumor was the lowest in the Apa-60 plus anti–PD-L1 group compared with other groups (Supplementary Fig. S8A and S8B). We observed no differences in the percentages of splenic CD8+ T cells, MDSCs, or macrophages in each group (Supplementary Fig. S9), suggesting that the distinct therapeutic effects should be attributed to the changes of immune profiling in tumors. To determine the impact of combination treatment on the survival of tumor-bearing mice, an independent mouse cohort that underwent the same tumor inoculation and treatment was monitored. We observed that coadministration of low-dose apatinib and anti–PD-L1 resulted in prolonged survival (P < 0.01; Fig. 6E).

We next tested the antitumor activity of this combination treatment in an experimental metastasis lung cancer model. Mice bearing tumors following tail-vein injection of LLC cells were treated by the same regimen as indicated above (Fig. 6F). As for the subcutaneous tumor model, we found that the metastases in lungs were reduced in the low-dose apatinib plus anti–PD-L1 group (Fig. 6G and H). Survival analysis confirmed that dual administration of anti–PD-L1 and low-dose apatinib improved OS (Fig. 6I). Collectively, these data supported our hypothesis that low-dose apatinib potentiated the antitumor effect when used in combination with anti–PD-L1 in vivo lung cancer models. A schematic diagram describing the potential mechanisms for the enhanced therapeutic efficacy delivered by combining low-dose apatinib and anti–PD-L1 blockade is shown in Fig. 6J.

**Combination treatment shows antitumor activity in patients with advanced NSCLC**

Based on our preclinical data from the animal experiments, a phase I/II trial to investigate the efficacy and toxicity of SHR-1210 combined with apatinib in patients with immuno-oncology naïve advanced NSCLC was initiated in our institute. At the time of cutoff, 9 patients were enrolled in part 1 to receive low-dose apatinib (250 mg/day, one third of the recommended 750 mg/dose in phase III trials) combined with SHR-1210 200 mg/q2w and to undergo response evaluation. We assessed the tumor PD-L1 expression of these patients using the 28-8 IHC assay (DAKO) on their archival tumor specimens collected before the treatment started. Among the 9 patients on the study, 6 were PD-L1 negative and 1 was positive (PD-L1 positive/negative was determined by cutoff value of 5% of tumor cells displaying membranous immunoreactivity). Two patients had no available tumor specimens for PD-L1 expression analysis (Supplementary Fig. S10A and S10B).

The minimum follow-up time for these patients was 26.7 weeks (the time elapsed from when the last patient was included to when data for all patients were locked). During the observation period, partial response was observed in 5 patients and an additional 3 patients had stable disease [ORR 55.6%] and disease control rate (DCR) 88.9%; Fig. 7A–C]. All the included patients experienced at least one treatment-related adverse event (AE; Fig. 7D). The majority of these AEs were mild and tolerable; no patient discontinued treatment due to AEs. When data were locked, 4 patients were still on treatment, 3 patients were alive after treatment failure, and 2 patients had died, 1 due to disease progression and 1 due to unknown causes (Fig. 7B).

**Discussion**

Optimized combinatorial strategies could extend the frontiers of anti–PD-1/PD-L1–based immunotherapy in lung cancer (11, 33, 34). The work presented here is a proof of concept that under optimal conditions, PD-1/PD-L1 blockade combined with antiangiogenic agents, such as apatinib, can induce enhanced therapeutic effect. This combination might be a promising strategy to...

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**Figure 6.** Antitumor effects from the combined administration of PD-L1 mAb and low-dose apatinib. **A,** Diagram depicting treated schedule for the subcutaneous LLC tumor model. Red arrow indicates administration of apatinib. Blue arrows indicate injections of PD-L1 mAb. **B,** Representative tumors isolated from each treatment group 32 days after apatinib–treatment initiation. **C,** Volume changes of the subcutaneously implanted tumors in each group beginning at the day of tumor inoculation (n = 8–10 per group). **D,** Body weight of the subcutaneous tumor-bearing mice in each group. The weight was measured every other day. **E,** Kaplan-Meier survival curves from an independent experiment of the subcutaneous tumor-bearing mice treated as indicated since the day of tumor inoculation (n = 6 per group). **F,** Diagram depicting treated schedule for metastasis LLC tumor model. Red arrow indicates administration of apatinib. Blue arrows indicate injections of PD-L1 mAb. **G,** Comparison of tumor burden in lungs. Left, representative micro-CT images of mice lungs from each treatment group 28 days after apatinib–treatment initiation. Right, comparison of lung weight 28 days after apatinib–treatment initiation (n = 5–7 per group). **H,** Comparison of tumor metastasis. Left, lung sections with H&E. Scale bars, 5,000 μm. **I,** Right, quantification of tumor areas per lung section 28 days after apatinib–treatment initiation (n = 5–7 per group). **J,** Kaplan–Meier survival curves from an independent experiment of the metastasis tumor-bearing mice treated as indicated since the day of tumor inoculation (n = 6 per group). **K,** An overview schematic illustrating interactions between low-dose VEGFR2-TKI, tumor microenvironment, and the antitumor effect of PD-L1/PD-L1 blockade. All the data in this figure are from one experiment representative of two independent experiments.
extend the benefit of anti–PD-1/PD-L1 treatment to a larger population of lung cancer patients. We compared various immune components in the TME after different doses of apatinib monotherapy and observed that apatinib, when administrated in a lower dose, could alleviate tumor hypoxia, increase CD8^+ cell infiltration, hinder recruitment of TAMs and MDSCs, and decrease TGFβ at certain time points, suggesting an immune modulating effect of angiogenesis inhibitors. Based on these findings, we found that low-dose apatinib potentiated antitumor activity when used in combination with PD-1/PD-L1 blockade in both local and metastatic LLC mouse models. This strategy of anti–PD-1 combined with low-dose apatinib also showed promising results in a preliminary clinical setting.

Although anti–PD-1/PD-L1–based immunotherapy has demonstrated durable clinical response in certain patients with advanced NSCLC, many patients get no benefit from anti–PD-1/PD-L1 monotherapy (35). Resistance to PD-1/PD-L1 blockade can be attributed to shifts in various aspects of the antitumor immunity cycle, such as poor capability to produce immunogenic neoantigens, invalid antigen processing, deficiency in infiltrating lymphocytes and enrichment of immunosuppressive components (8, 9, 36). From this perspective, enhancing particular steps in the antitumor immunity cycle via combinational strategy might help to overcome resistance to anti–PD-1/PD-L1. As indicated above, VEGF signaling not only promoted angiogenesis, but also mediated antitumor immunity by influencing lymphocyte trafficking and inducing expansion of immunosuppressive cell subsets (13, 15). Anti-VEGF/VEGFR2 enhances antitumor efficacy when combined with immunotherapy via altering the infiltration of lymphocytes or macrophages in a range of tumor types in preclinical studies (17, 19–21). Data on combining antiangiogenesis and anti–PD-1/PD-L1 in lung cancer remain scarce (37, 38). The current study investigated the impact of low-dose VEGFR2-TKI (apatinib) on the changes of immune components within the TME and estimated the antitumor activity of combining PD-1/PD-L1 blockade with apatinib in both NSCLC animal models and the clinical setting. Our work indicates that low-dose apatinib can enhance the therapeutic efficacy of anti–PD-1/PD-L1.

Several challenges involving the selection of optimal dosing and treatment schedules need to be resolved for the clinical application of the anti–PD-1/PD-L1 and antiangiogenic combination (39). Angiogenesis inhibitors are conventionally used to prune tumor blood vessels and starve cancer cells by using the highest tolerable doses in the treatment of lung cancer, which may result in aggravating hypoxia and promote tumor immunosuppression (16). Based on the notion that antiangiogenic agents demonstrate dose- and time-dependent influence on tumor vasculature (17, 18), we investigated the effects of distinct doses of apatinib on intratumoral vessels and immune components ahead of the experiments for combination treatment. We observed a more favorable proinflammatory microenvironment for immunotherapy 2 weeks after low-dose apatinib monotherapy instead of high-dose treatment. We thus hypothesized that a low dose of apatinib might be more effective than higher doses when combined with anti–PD-L1 immunotherapy. Our results from in vivo combination treatment experiments confirmed this hypothesis. Our findings were congruent with several previous reports. Tian and colleagues (40) demonstrated the mutual regulation between tumor vessel-normalization and immune-stimulatory pathways, and they speculated that combinatory interventions of checkpoint inhibitors and vessel-normalization treatment might present promising strategies for synergy. The studies from Schmittnagel and colleagues (41) and Allen and colleagues (21) provided evidence that antiangiogenic agents could improve anti–PD-1/PD-L1 therapy when it facilitated an immunostimulatory TME and normalized tumor vessels in various tumor models. Data from the phase III IMpower150 study (38) suggested that the addition of atezolizumab to chemotherapy plus bevacizumab resulted in improved OS (median 19.2 vs. 14.7 months, P = 0.0164) in untreated nonsquamous NSCLC patients. Bevacizumab is administered at a high therapeutic dose (15 mg per kilogram) in this trial. However, as both of bevacizumab and chemotherapy can show immunomodulatory effects that may augment the efficacy of atezolizumab, this study is insufficient to provide information in regard to the optimal regimens when combining anti–PD-1/PD-L1 with antiangiogenesis alone. A direct comparison between the atezolizumab plus bevacizumab plus chemotherapy arm (arm B) and the atezolizumab plus chemotherapy arm (arm A) was not conducted, and a median OS of 19.4 months was observed in arm A, suggesting the survival benefit in arm B might be mainly attributed to PD-1/PD-L1 blockade and the effect of adding high-dose bevacizumab to chemoimmunotherapy remained doubtful. Collectively, there is merit to investigating the efficacy of combining lower-dose bevacizumab with anti–PD-1/PD-L1 therapy in clinical settings.

Because PD-L1 expression was associated with the response to anti–PD-1/PD-L1 immunotherapy (7), we also investigated the impact of apatinib treatment on PD-L1 expression in tumor. We found that PD-L1 expression in all treated groups had a transient reduction on day 7 after apatinib treatment, and then elevated to rates comparable with the control group on days 14 and 21, suggesting that the enhanced antitumor effect of low-dose apatinib plus anti–PD-1/PD-L1 therapy was not induced by upregulated PD-L1 expression. Antiangiogenesis can have immune modulatory effects (13, 15). In our study, we detected PD-L1 expression in whole viable cells in tumor tissue. Because we did not observe an effect of apatinib on apoptosis and PD-L1 expression of LLCs in vitro, we speculated that the dynamic changes of PD-L1 expression could be due to the effects of apatinib on host cells such as endothelial cells, TAMs, and MDSCs. Nevertheless, the modulation of antiangiogenic therapy on intratumoral PD-L1 expression remains controversial. For example, Zheng and colleagues (42) found that apatinib attenuated PD-L1 expression in osteosarcoma mouse model. However, Allen and colleagues (21) observed that PD-L1 expression was upregulated in tumors following DC101 (anti-VEGFR2) treatment in certain tumor models. Schmittnagel and colleagues (41) also found that endothelial PD-L1 was upregulated in response to increased IFNγ released by perivascular CD8^+ T cells after combined ANGPT2 and VEGFA blockade in several cancer models. Thus, we believe further work is needed to elucidate the mechanism for the modulation of PD-L1 expression via antiangiogenesis. Our findings have clinical implications. Because elimination of immunosuppressive cells supports PD-1/PD-L1 blockade (43, 44), and we observed that low-dose apatinib reduced immunosuppressive components and that PD-L1 expression was regained at day 14, we suggest that initiating anti–PD-1/PD-L1 therapy after 14 days of...
Figure 7.
Efficacy and safety of apatinib and SHR-1210 combinational therapy in patients with NSCLC. 
A, Radiologic evidence of the 5 patients (patient numbers #1, #2, #3, #6, and #8) who achieved PR. CT scans were performed at the baseline and subsequent treatment cycles. The red arrows denote the target lesions that show size decrease with treatment. 
B, Individual clinical outcomes and follow-up of the 9 patients enrolled grouped by therapeutic responses (assessed via RECIST v1.1). 
C, Best change from baseline in size of target lesions (n = 9). Bar color represents best overall response. Bar length represents increase or decrease in target lesion size. 
D, The summary of safety and toxicity results for all treated patients. AE, adverse event; ALP, alkaline phosphatase; Pt No, patient number; y, years old; PY, pack-years; NA, not available; PR, partial response; SD, stable disease; PD, progressive disease; AEs, adverse events.
low-dose apatinib therapy might amplify the antitumor effect. Indeed, a combination of azezolizumab and bevacizumab (azezolizumab started on cycle 2) achieved a response rate of 40% in previously treated patients with metastatic renal cell carcinoma (20).

In the apatinib monotherapy experiments, we observed that TGFβ expression varied with different doses of apatinib treatment. The greatest reduction in TGFβ expression was observed in the 60 mg group. Expression of TGFβ in the 60 mg plus anti-PD-L1 group was the lowest compared with other combination groups. Multiple mechanisms could be involved in affecting intratumoral TGFβ expression (45). Reduced TGFβ expression after low-dose apatinib treatment might be attributed to the alleviation of hypoxia and decreased recruitment of myeloid suppressor cells in tumor. TGFβ, as a pleiotropic cytokine, plays a role during immune response in the TME and contributes to antitumor immunosuppression (46, 47). In addition, TGFβ is thought to confer resistance to antiangiogenic therapy (48). TGFβ is capable of inducing resistance to anti–PD-L1 blockade (49). Collectively, the decreased expression of TGFβ after low-dose apatinib treatment supported our logic to combine low-dose apatinib with anti–PD-1/PD-L1 immunotherapy.

The preliminary results from our phase II clinical trial further support our hypothesis. We found an ORR of 55.6% and DCR of 88.9% in the enrolled cohort with a low incidence of PD-L1 expression, which are better than those of anti–PD-1 monotherapy in the same clinical setting. In addition, toxicity was mild with no severe AEs observed so far. Thus, both the preclinical data and preliminary phase II trial results provide a rationale to initiate a phase II study to investigate the efficacy and toxicity of SHR-1210 combining with low-dose apatinib in patients with advanced NSCLC in a larger cohort (NCT03083041).

In summary, our study suggested that apatinib, a VEGFR2-TKI, when administrated at a lower dose, could optimize the immunosuppressive TME and increase the therapeutic response to immunotherapy both in vivo lung cancer models and in a preliminary clinical setting in NSCLC. Our work is limited by the capacity of animal models to mimic the human TME and the hypothesis-generating nature of the study. Further evaluation in more mouse experimental systems and randomized trials is warranted. We are waiting for the confirmatory results from the ongoing phase II clinical trial (NCT03083041) to validate the strategy developed in this study.

Disclosure of Potential Conflicts of Interest

F.R. Hirsch is on the scientific advisory boards of Bristol-Myers Squibb, Merck, Genentech, Loxo, Bayer, Astra Zeneca, Ventana, Novartis, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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Apatinib Potentiates Anti-PD-1/PD-L1 Effect in Lung Cancer


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