Dual Inhibition of Angiopoietin-TIE2 and MET Alters the Tumor Microenvironment and Prolongs Survival in a Metastatic Model of Renal Cell Carcinoma

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ABSTRACT

Receptor tyrosine kinase inhibitors have shown clinical benefit in clear cell renal cell carcinoma (ccRCC), but novel therapeutic strategies are needed. The angiopoietin/Tie2 and MET pathways have been implicated in tumor angiogenesis, metastases, and macrophage infiltration. In our study, we used trebananib, an angiopoietin 1/2 inhibitor, and a novel small-molecule MET kinase inhibitor in patient-derived xenograft (PDX) models of ccRCC. Our goal was to assess the ability of these compounds to alter the status of tumor-infiltrating macrophages, inhibit tumor growth and metastases, and prolong survival. Seven-week-old SCID mice were implanted subcutaneously or orthotopically with human ccRCC models. One month postimplantation, mice were treated with angiopoietin 1/2 inhibitor trebananib (AMG 386), MET kinase inhibitor, or combination. In our metastatic ccRCC PDX model, RP-R-02LM, trebananib alone, and in combination with a MET kinase inhibitor, significantly reduced lung metastases and M2 macrophage infiltration ($P = 0.0075$ and $P = 0.0205$, respectively). Survival studies revealed that treatment of the orthotopically implanted RP-R-02LM tumors yielded a significant increase in survival in both trebananib and combination groups. In addition, resection of the subcutaneously implanted primary tumor allowed for a significant survival advantage to the combination group compared with vehicle and both single-agent groups. Our results show that the combination of trebananib with a MET kinase inhibitor significantly inhibits the spread of metastases, reduces infiltrating M2-type macrophages, and prolongs survival in our highly metastatic ccRCC PDX model, suggesting a potential use for this combination therapy in treating patients with ccRCC.

Introduction

Kidney cancer remains among the top ten most diagnosed cancers in both men and women, with >62,000 new cases of kidney cancer and >14,000 deaths anticipated in 2019. Of those cases, approximately 15% will develop aggressive metastatic disease (1, 2). Clear cell renal cell carcinoma (ccRCC) is the most commonly diagnosed (70%) form of kidney cancer (1, 2). Among those patients diagnosed with sporadic ccRCC, the majority has loss-of-function mutations in the von Hippel-Landau tumor suppressor gene (3) resulting in an inability to provide factors (HIF). This loss leads to overexpression of HIF target genes, including VEGF, platelet-derived growth factor, hepatocyte growth factor (HGF), and the receptor tyrosine kinase mesenchymal-epithelial transition factor (MET), which drive tumor progression, metastases, and hypervascularization (4).

Tumor-associated macrophages (TAM) play a supporting role for kidney cancer. TAMs directly stimulate tumor growth, promote angiogenesis, support escape of immune surveillance, and assist in tumor cell dissemination (4–8). Furthermore, increased presence of TAMs in the tumor microenvironment (TME) is correlated with poor prognosis in patients with ccRCC (3). Production of immunosuppressive cytokines and extracellular matrix remodeling enzymes, that is, fibronectin, tenasein-c, and matrix metalloproteinases by TAMs allow the tumor immune escape and assist in the epithelial to mesenchymal transition and dissemination of metastatic cells (4–6). TAMs are attracted to the TME by CCR2 signaling and subsequently differentiate into perivascular macrophages by help of CXCL12 and CXCR4, which are released by tumor cells and perivascular fibroblasts, respectively (9). Anchorage of Tie2-expressing macrophages to the perivascular space is driven by Tie2-angiopoietin signaling (7, 10–12).

Metastatic disease remains the leading cause of ccRCC-related deaths with the most common sites being the lungs, bones, and lymph nodes (2, 13). Vascular stabilization and enhanced pericyte recruitment, while often reported to enhance tumor growth, have been recently linked to inhibition of tumor metastases, suggesting a dual potential role for the inhibition and stabilization of vascularization by pericytes (8, 14–17).

The angiopoietin/Tie2 axis plays a significant role in the anchorage of TAMs to the perivascular space, which significantly contributes to the maturation/disruption of tumor vasculature (18–20). Angiopoietin 1 (Ang1) activates the tyrosine kinase receptor, TIE2, and affects the
response of endothelial cells to VEGF (13, 19). Ang1/Tie2 interaction leads to blood vessel maturation and stabilization in both normal and tumor tissues (21, 22). In the TME, Ang1 has been shown in few studies to both promote tumor growth and inhibit metastasis (23, 24). Conversely, Tie2 receptor expressed by perivascular TAMs plays a key role in regulating Ang2-mediated vascular destabilization and sprouting in tumors (12).

Preclinical studies in colorectal cancer, breast cancer, and melanoma models have shown that inhibition of Ang2 resulted in reduced blood vessels, increased pericyte coverage, blood vessel stabilization, and altered EMT pathway activation (12, 14, 15, 22). On the other hand, studies on Ang1 inhibition draw conflicting results on whether it induces or suppresses postnatal angiogenesis, which suggests a context-dependent function of Ang1 that require further investigation to be therapeutically harnessed (25). Therefore, although the regulation of angiogenesis through the Ang–Tie2 pathway has been well characterized, there is a lack of understanding of the crucial role that this pathway plays in metastatic disease.

The MET/HGF (c-MET) pathway is upregulated in 60%–70% of ccRCC tumors leading to increased cell proliferation and metastatic potential (26). Recent reviews of MET-addicted tumors indicate that MET/HGF inhibitors impact both the stroma and cancer cells. While MET/HGF inhibitors can directly inhibit tumor growth; these targeted agents may attenuate the efficacy of stromal cells, such as tumor-associated neutrophils and cytotoxic T cells, which inhibit tumor growth through upstream MET signaling (27, 28). HGF also plays a key role downstream of the Ang/Tie2 axis by inducing pericyte recruitment and vascular maturation (19). The MET pathway is also implicated in epithelial-to-mesenchymal transition (EMT), which is involved in tumor metastases (26, 29). MET has also been reported to play a crucial role in the disruption of E-cadherin–based cell–cell contacts, thus affecting the TME in addition to the tumor niche (3, 27).

The concept of combining inhibitors of both the MET and Ang/Tie2 pathways as treatment for metastatic ccRCC is postulated to be efficacious on both the tumor niche and the TME (1, 17, 30–32). The purpose of this study was to examine the efficacy of this novel combination in preclinical ccRCC models. Overall, our data suggest that combination of dual Ang1/2 inhibitor (trebananib; ref. 25) and a MET kinase inhibitor (compound 22, re; ref. 33) increases trebananib-induced antitumoral activity in a metastatic ccRCC model.

Materials and Methods

Cell lines

The murine renal cell carcinoma cell line RENCA was initially purchased from ATCC (National Cancer Institute, Bethesda, MD) and stably tagged with a luciferase reporter in R. Pili’s laboratory. Cells were cultured in RPMI1640 (Corning) with 10% FBS (Corning) and 1% nonfetal bovine serum (Corning) and stably tagged with a luciferase reporter in R. Pili’s laboratory. Cells were cultured in RPMI1640 (Corning) with 10% FBS (Corning) and 1% nonfetal bovine serum (Corning). Nonconfluent cells were harvested using 0.25% Trypsin (Corning) and suspended in Matrigel (Corning) in a 1:1 ratio, 10 μL containing 1 × 10⁴ cells was injected under the renal capsule. Mice were serially imaged using a bioluminescent IVIS imaging machine. Trebananib and compound 22 were provided by Amgen.

In vivo tumor growth (RENSA)

Five- to six-week-old Balb/c mice (National Cancer Institute, Bethesda, MD) were kept in a temperature controlled room on a 12/12-hour light/dark schedule with food and water ad libitum. Nonconfluent RENCA-Luc cells were harvested using 0.25% Trypsin (Corning) and suspended in Matrigel (Corning) and DPBS (Gibco) in a 1:1 ratio, 10 μL containing 1 × 10⁴ cells was injected under the renal capsule. Animals were randomly distributed into four groups: vehicle (soybean oil), trebananib (AMG386), a MET kinase inhibitor (compound 22), or combination. Mouse tumors were serially imaged using a bioluminescent IVIS imaging machine. Trebananib and compound 22 were provided by Amgen.

Xenograft models

Wild-type male ICR SCID mice, ages 6–8 weeks, were purchased from Charles River and housed in a sterile, pathogen-free facility and maintained in a temperature controlled room under a 12-hour light/dark schedule with food and water ad libitum. Upon arrival and acclimation, mice were implanted orthotopically or subcutaneously (~1 mm³ tumor piece) with RP-R-01, RP-R-02, or RP-R02LM, which were established from the skin metastasis of a patient with sporadic ccRCC, which developed sunitinib resistance and skin metastasis from a patient with hereditary VHL syndrome ccRCC, respectively (34, 35).

Trebananib and MET kinase inhibitor treatment

Mice in the vehicle group were given soybean oil daily (5 days/week) by oral gavage. Mice in the treatment groups were treated with 5.6 mg/kg of trebananib (AMG 386; ref. 25), twice a week intraperitoneally, and/or 30 mg/kg of MET kinase inhibitor (compound 22) (33), 5 days a week by oral gavage. In the sunitinib-resistant studies, RP-R-01 and RP-R-02, mice were treated with 40 mg/kg of sunitinib for three weeks, or until tumor progression. Following this, the mice were treated with trebananib, MET kinase inhibitor, or a combination of both.

Histologic and IHC analysis

Tumor tissue specimen from each treatment group was fixed for 24 hours in formalin, paraffin-embedded, and sectioned (5 μm). Slide sections were deparaffinized and rehydrated via gradient alcohol washes. Antigen unmasking was performed by boiling slides in sodium citrate buffer (pH = 6.0). Slides were subsequently incubated in hydrogen peroxide to reduce endogenous activity. For probing of the tissue for the proteins of interest, tissue sections were blocked in 2.5% horse serum (Vector Laboratories), and incubated overnight in primary antibodies against CD31 (1:100, Dianova). Following primary antibody incubation, slides were incubated with horseradish peroxidase (HRP)-conjugated anti-rat antibody according to the manufacturer’s protocol (Vector Laboratories). Following this, slides were subjected to enzymatic development in diaminobenzidine (DAB). Sections were then dehydrated and mounted with cytoesal 60 (Thermo Fisher Scientific). Stained sections were imaged under bright field (IHC) using the Zeiss Axio microscope. The positive fields were calculated in a blinded fashion by analyzing four random 20× fields per tissue and quantified using ImageJ software (36).

Lung tissues were paraffin embedded, sectioned, and stained with hematoxylin and eosin to quantify the presence of micrometastases. Lung tissues were sectioned from the front, middle, and back of the lungs to obtain an overall assessment of the presence of lung metastases. Measurements were then performed in a blinded manner using ImageJ, where the total number and the diameter of micrometastases were measured in 5–6 areas of each sectioned lung. Five images of each
section of the lungs were taken (~30 images/lung). There were six lungs in each group (~720 images used in analysis).

Western blot analysis
Tumors from each treatment group were lysed in RIPA buffer (Sigma–Aldrich) containing protease and phosphatase inhibitor cocktails (Pierce). Protein concentrations were assessed using a standard BSA assay (Bio-Rad). Fifty micrograms of protein from each sample was subjected to electrophoresis on 10% SDS-polyacrylamide gels (Bio-Rad) and transferred onto nitrocellulose membranes. Proteins of interest were detected with the following primary antibodies: E-cadherin (1:1,000; Cell Signaling Technology), NG2 – Chondroitin Sulfate Proteoglycan (1:1,000; EMD Millipore), phosphor-p44/42 MAPK (Erk1/2; Thr202/Tyr204; 1:1,000; Cell Signaling Technology), phosphor-AKT (Ser473; 1:1,000; Cell Signaling Technology), GAPDH (1:1,000; Cell Signaling Technology). Following incubation with primary antibody, the membranes were probed with HRP-conjugated secondary antibodies (Bio-Rad), exposed to chemiluminescence reagent (Pierce). Protein concentrations were assessed using a standard BCA assay (Pierce). Total RNA was isolated from tumor samples according to manufacturer's instructions using TRIzol (Life Technologies) and measured using nanodrop technology. Quantitative RT-PCR was performed with E-cadherin and Snail primers (IDT Technologies). Samples were denatured at 95 °C for 10 seconds, annealed at 60 °C for 30 seconds, and extended at 72 °C for 1 minute using the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems). Sequence Detection Systems software v2.3 was used to identify the cycle threshold (Ct) values and to generate gene expression curves. Data were normalized to GAPDH expression and fold change was calculated.

Immunofluorescence staining
Tissue sections were prepared as described previously and stained for anti-rat CD31 (Dianova) at a 1:50 dilution and anti-rabbit NG2 at a 1:50 dilution overnight at 4 °C. Following the primary incubation, slides were incubated with conjugated secondary antibodies—anti-rabbit FITC and anti-rat Cy5,ostained with DAPI, and mounted using Vectashield immunofluorescence mounting reagent. At least 5 images/tumor (referred to as fields of view) were acquired for at least 5 tumor pieces per group. High-power fields were randomly and blindly taken to reflect the entire tumor. Analysis of the NG2 CD31 costaining was performed in a blinded manner utilizing ImageJ software.

Confocal microscopy
CD31 and NG2 costaining was performed on thicker sections of tumor tissue (μm) as described above. The Indiana Center for Biological Microscopy at Indiana University Purdue University Indianapolis (IUPUI) assisted in acquiring confocal images of the costained sections.

Statistical analysis
All statistical analyses were performed using GraphPad Prism7 software for Windows. Analysis of survival was conducted using the Kaplan–Meier method. Differences in treatment group survival were assessed with the log-rank test. All other statistical analyses in this study were performed between experimental groups using the Student t test with Welch correction. A P < 0.05 was considered statistically significant.

Results
Combined inhibition of Ang1/Ang2 and MET significantly reduces spontaneous lung metastases in the patient-derived xenograft model RP-R-02LM
To assess the efficacy of combining Ang1/Ang2 inhibition and MET kinase inhibition on primary tumor growth, we utilized two patient-derived xenograft (PDX) models, RP-R-01 and RP-R-02 (29), in addition to the luciferase-tagged syngeneic orthotopic murine model of renal cell carcinoma RENCA-Luc (37). As a MET inhibitor, we utilized compound 22, which was previously reported to have selective kinase inhibitory activity (Supplementary Fig. S1A and S1B; ref. 33). The PDX models have been detailed previously as maintaining their original clear cell morphology, VHL-negative status, human Alu-positive status, and containing common ccRCC gene mutations (34). Clinically effective first-line therapeutics such as VEGF-targeting sunitinib elicit antitumor responses in patients with ccRCC. However, the majority of patients who initially respond to sunitinib develop resistance and their tumors progress on treatment (34, 38). Thus, we examined the treatment of sunitinib-sensitive and sunitinib-resistant models with trebananib and cMET inhibition to assess the efficacy of this novel combination.

Our preliminary experiments assessed the primary tumor growth of RP-R-01, RP-R-02LM (intrinsically sunitinib resistant), and RENCA-Luc tumors. When the tumors reached a detectable size, 100–150 mm³ for the subcutaneous PDX models approximately or 1–3 × 10⁵ average radiance for RENCA mice were randomized into four groups: vehicle (soybean oil), trebananib, MET kinase inhibitor (compound 22), and combination. Mice were treated with 5.6 mg/kg subcutaneous injections of trebananib twice a week and/or 30 mg/kg MET kinase inhibitor by oral gavage 5 days a week. In the RP-R-02LM model (35), which spontaneously metastasizes to the lungs when implanted subcutaneously or orthotopically, we found that although MET kinase inhibitor–treated tumors trended toward a decrease in end of treatment size in comparison with vehicle and combination groups, this trend did not mount to statistical significance and overall there was no significant difference in the growth of the primary tumors with any of the treatments (Fig. 1A and B; Supplementary Fig. S2). Similarly, no significant inhibition of primary tumor growth was observed in the sunitinib-sensitive or -resistant RP-R-01 PDX models (Supplementary Fig. S3A–S3C). In the RENCA model, however, we observed a significant reduction in final tumor weights in the trebananib-treated group compared with vehicle, but not in the combination group (Supplementary Fig. S3D), which we speculate could be attributed to the distinct TME of a mouse tumor. Importantly, while the MET kinase inhibitor modestly restricted tumor growth in our spontaneously metastatic RP-R-02 LM model, the mice in this group had similar accumulation and distribution of lung metastases as the untreated cohort (Fig. 1C). Strikingly, the overall number (Fig. 1D) and diameter of lung metastases (Fig. 1E) were significantly reduced after treatment with trebananib as compared with the vehicle. Furthermore, the combination group had a significant reduction in the size of metastases as compared with vehicle and both single-agent treatments, while reduction in metastases count was significantly reduced compared with vehicle and single-agent MET kinase inhibitor but not trebananib (Fig. 1C–E). In summary, these data suggest that inhibition of the angiopoietin–TIE2–MET kinase axis may result in tumor microenvironment alterations, which may hinder tumor metastasis.
Combination treatment hinders the metastatic potential of orthotopically implanted RP-R-02LM tumors

To determine the effect of combination treatment on hindering tumor metastasis, we assessed the survival and metastatic burden of mice in vehicle, single agent, and combination groups. We implanted RP-R-02LM tumor pieces orthotopically, under the kidney capsule, and began treatment with AMG 386 at 5.6 mg/kg subcutaneously twice a week and/or 30 mg/kg MET kinase inhibitor at 4.5 weeks postimplantation—a time-point at which we have shown metastases have begun to shed from the primary tumor to the lungs (35). We observed a significant increase in survival of mice in the combination group as compared with the vehicle cohort (P < 0.0005) while the single-agent MET kinase cohort performed worse (median survival: vehicle—183.5 days, trebananib—161.5 days, MET kinase inhibitor 142 days, combination 218 days; Fig. 1F). These data suggest that, although in our initial studies (Fig. 1A and B) we did not see a significant change in primary tumor growth, the significant decrease in metastatic burden that we observed in the trebananib and combination treatment (Fig. 1D and E) could potentially be translated into a significant survival benefit as observed in the orthotopically implanted tumors.

Combination treatment hinders the metastatic potential of RP-R-02LM post tumor resection

Clinically, patients with RCC may have their primary tumor resected, but they are still at risk to develop metastatic disease, most commonly to the lungs (39). To study the efficacy of trebananib in...
Inhibition of c-MET and Ang 1/2 Inhibits ccRCC Metastases

Pathway activation downstream of Tie2 and c-MET is altered

To elucidate whether we were hitting our target in the TME, we assessed the treatment effects on downstream effectors of the MET and Tie2 signaling pathways including AKT, STAT3 by Western blot analysis (Supplementary Fig. S7A). We observed decreased AKT phosphorylation with single-agent treatment as well as combination treatment (Supplementary Fig. S7B and S7C). Decreased AKT phosphorylation upon treatment is indicative of successful target modulation; however, associated changes in total AKT points toward more complicated mechanism of action downstream of hitting both Tie2 and MET signaling pathways. We also examined p-STAT3, a marker of aggressiveness and proliferation (47), and noted that while there was an overall trend of p-STAT3 inhibition in the single agent and combination groups, there was no significant change from the vehicle (Supplementary Fig. S7D). Previous studies have reported lack of correlation between Tie phosphorylation levels and targeting the angiopeptin/Tie2 axis (48). Similarly, we did not detect a consistent trend in Tie phosphorylation changes in relation to neither type nor duration of treatment (Supplementary Fig. S8). Taken together, these results suggest that we were capable of modulating signaling downstream of Tie2 and MET-targeted inhibition both as single agents and when combined. However, modulation of AKT phosphorylation in treatment groups was not significantly different from vehicle control group when taking into account changes in total AKT. This was also true for changes in STAT phosphorylation. This may explain why, in the RP-R-02LM model, no significant difference was observed in the size of tumors in each treatment group at the end of treatment (Fig. 1A and B). However, the benefit attained with the combination was limited to hindering tumor metastasis and prolonging survival that could be driven by alternative signaling mechanisms.

Combination treatment of the patient-derived xenograft RP-R-02LM leads to enhanced pericyte coverage

In addition to examining the EMT markers and downstream constituents of the MET and Tie2 pathways, we were interested in assessing the mechanism by which the combination treatment inhibits tumor metastases, as we were not seeing the expected inhibition of downstream markers in the combination treatment group. A possible explanation for inhibiting tumor metastasis is inducing vascular normalization. Vascular normalization is a multifactorial process that is assessed by examining vascular permeability, vascular sprouting, and pericyte coverage (49, 50). Therefore, we analyzed the presence of pericytes (NG2 staining) cosituated with endothelial cells (CD31 staining). Because the combination treatment of trebananib and MET kinase inhibitor may inhibit the dissemination of RP-R-02LM cells from the primary tumor to the lungs as well as decreases intratumor hypoxia despite continued growth of the primary tumor, we postulated that we might observe an alteration in the vasculature of the primary tumor. Indeed, we found that in the combination group there was a significant increase in the presence of NG2-positive pericytes colocalized with CD31 endothelial staining with an average Pearson coefficient (R) of 0.842 in the combination compared with 0.60562 in the vehicle (P < 0.05; Fig. 3A–C). This increase of pericyte expression was verified by Western blot and densitometry analysis using ImageJ software (Fig. 3D). These results indicate that the combination treatment of trebananib and MET kinase inhibitor may be strengthening the vasculature in a manner that functions as an antimetastatic modulation of the tumor microenvironment. Taken together, these data indicate that inhibition of the angiopeptin–TIE2–MET axis in our PDX model stabilizes blood vessels in the primary tumor and potently inhibits tumor cells metastases.
Tumor-associated macrophage presence is significantly decreased with inhibition of the angiopoietin–Tie2–cMET axis

Tumor-associated macrophages have been shown to facilitate tumor metastases via promotion of tissue remodeling, angiogenesis, and production of extracellular matrix–remodeling enzymes, such as matrix metalloproteinases (5, 6). We hypothesized that perivascular, tumor-associated macrophages, angiopoietin receptor Tie2$^+$ cells, which are known to mediate tumor metastases (4), may be reduced in the tumor microenvironment of our combination group. To test this hypothesis, we examined tumors from our in vivo subcutaneous study of RP-R-02LM, for Tie2, F4/80 pan-macrophage marker, and CD206 M2-like macrophage marker. In the combination cohort, there was a significant decrease in the presence of TAMs (F480$^+$Tie2$^+$CD206$^+$) in the tumor microenvironment in comparison with vehicle control (Fig. 4A and B; Supplementary Fig. S9 and 10). We found that in both control, single agent, and combination treated groups, there was a significant colocalization of Tie2/CD206 (Pearson coefficient = 0.9; Fig. 4C), confirming the presence of Tie2$^+$ macrophages in our tumors. These data show that combination treatment of trebananib and MET kinase inhibitor inhibits the infiltration of M2-like TAMs into the tumor microenvironment, suggesting a potential role for these TAMs in the metastases of the PDX model RP-R-02LM.
Discussion

Successful treatment of patients with cancer with metastatic disease remains one of the most daunting tasks for clinicians managing solid tumors, including ccRCC (17, 30). Despite the recent surge of research contributing to the understanding of metastatic disease and its interaction with stroma, there is much work to be done to translate these advances into the clinic (17, 30). In our study, we assessed the effect of the Ang1/Ang2 peptibody, trebananib, as a single agent and in combination with inhibition of the MET kinase pathway. Our data show evidence that this combination may alter both the tumor niche and microenvironment through the alteration of pericytes and inhibition of metastases-promoting macrophages in a metastatic ccRCC PDX model (4–8, 51).

The MET kinase pathway is often overactivated in VHL-negative ccRCC tumors, as reflected also in our RP-R-02LM model compared with the parental nonmetastatic RP-R-02 PDX (51). MET kinase overactivation leads to worse patient survival and a more aggressive and invasive phenotype (3, 32, 52, 53). In addition, inhibition of MET has been shown to be sufficient to subdue HGF-dependent migration and downstream targets, such as ERK and AKT (32). Thus, MET kinase inhibition is a promising target for metastatic ccRCC and cabozantinib, a multikinase inhibitor with activity against MET, has recently been approved for patients with RCC (54). Trebananib, a recombinant peptide-Fc fusion protein, which negates the receptor/ligand interaction of Ang1/Ang2 with the Tie2 receptor, has been reported to improve progression-free survival (PFS) in patients with ovarian cancer in a phase III trial as compared with paclitaxel (55). In our studies, we assessed the combination of MET kinase inhibition with trebananib in both murine and patient-derived xenograft models of RCC. Although our combination treatment studies did not yield a significant difference in primary tumor growth compared with the single-agent treatments, we observed a significant inhibition of metastases, a trend of increased expression of the epithelial marker E-
cadherin albeit nonstatistically significant, a reduction of mesenchymal marker Snail, upregulated pericyte coverage, improved intratumor oxygenation, and reduced presence of metastases-promoting M2-like macrophages in the primary tumor of our metastatic ccRCC PDX model, RP-R-02LM. These results suggest that in our models combination of MET kinase inhibition and angiopoietin inhibition may not provide an advantage in inhibition of primary tumor growth as compared with single-agent treatments alone. In contrast, trebananib treatment combined with MET kinase inhibition in our metastatic PDX model, RP-R-02LM, not only decreased the presence of tumor metastases to the lungs but also significantly enhanced survival. In this highly metastatic model, we observed the need to inhibit both the angiopoietin/Tie2 axis and the MET axis concomitantly to significantly affect the metastatic burden and survival.

In our initial short-term treatment study with the highly metastatic RP-R-02LM model, we noted a significant decrease in metastases to the lungs, indicating that we were impairing the metastatic potential of the tumor. In our orthotropic study, we noted that when RP-R-02LM tumor pieces were implanted into the kidney and treated for at least 4 months, a significant improvement in survival in the combination groups was observed. The mice in the combination group died of their primary tumor growth and not their metastatic burden while those in the vehicle and c-MET inhibition group died of metastatic burden. In our subcutaneous study, we removed the primary tumors at 3 months postimplantation, a time which we have shown that these tumors have already shed metastatic cells (35), continued to treat the mice, and assessed for metastatic burden. We found that the combination group had a striking increase in survival compared with both single agent and vehicle cohorts. These findings together suggest that combining Ang1/ Ang2 inhibition with c-MET inhibition results in significant decrease in metastasis and thus prolonged survival.

Blood vessel normalization and increased pericyte coverage have traditionally been viewed as tumor promoting. However, recent literature has suggested a dual role (22, 56). Studies have shown that increased blood vessel stabilization, E-cadherin restoration, reduced presence of perivascular macrophages, and pericyte coverage in multiple cancer types contribute to reduced metastases and prolonged survival (4, 5, 8, 15, 56). These studies elucidate the vital role of

### Figure 4.
Concomitant inhibition of the angiopoietin–TIE2 axis and the MET kinase pathway in RP-R-02LM tumors (orthotopic study) yields significant alterations in protumor macrophages. A. Single stain and merge of ‘TIE2’ (blue) M2-like, tumor-promoting macrophages (CD206` F4/80`, green/red) reveals a significant reduction in the presence of M2-like tumor–associated macrophages in the combination treatment cohort. Scale bar, 400 μm. B, TIE2` CD206` colocalization indicates the potential for combination treatment to directly impact the presence of TAMs in the tumor microenvironment. Spearman correlation coefficient for all treatment groups = 0.9. Spearman correlation coefficient was calculated using ImageJ color2 across different treatment conditions. C–E, Blinded quantitation of the immunofluorescence staining reveals a statistically significant reduction in TAMs (TIE2` F4/80` CD206`) in the tumor microenvironment (n = 30–40 images per group). *, P < 0.05; **, P < 0.001; ***, P < 0.0001; ns, not significant.
Inhibition of c-MET and Ang 1/2 Inhibits ccRCC Metastases

macrophage and pericyte recruitment on blood vessel normalization in the tumor microenvironment (4, 5, 7, 8, 15). In addition, clinical evidence shows in patients with colorectal cancer and RCC that reduced macrophage and increased microvesSEL pericyte coverage correlates with increased overall patient survival and a reduction in metastases (5, 13, 37, 58). Our results indicate that pericyte coverage, reduced macrophage presence, and stabilization of tumor vasculature in combination cohort may contribute to prolonged survival and reduced lung metastases in ccRCC. These results further emphasize the potential of MET kinase and angiopoietin inhibition to affect the tumor microenvironment in a manner that significantly inhibits the metastatic phenotype of ccRCC.

Therapeutic treatments for patients with ccRCC are rapidly evolving. Recently, two RTKI (receptor tyrosine kinase inhibitors), cabozantinib and lenvatinib, have been approved for use in the treatment of metastatic, RTKI-resistant disease. However, to date, the use of RTKIs in the adjuvant setting remains controversial as studies have shown discordant results (59, 60). Our preclinical studies suggest a selective antitumorigenic effect of concomitant angiopoietin1/2 and c-MET inhibition. This observation provides the rationale for developing selective inhibitors for these pathways to be tested in the adjuvant setting. This is clinically relevant as small molecules that inhibit both Tie2 and MET are currently in clinical testing.

In conclusion, targeting the tumor microenvironment and the tumor niche via combination of a small-molecule MET kinase inhibitor with the peptide inhibitor of angiopoietin 1/2, trebananib has striking biological effects. In our metastatic patient-derived xenograft ccRCC model, RP-R-02LM, this combination suppressed statistically significant inhibition of MET and Ang 1/2 and c-MET expression and reduced lung metastases in ccRCC. These results further emphasize the potential of MET kinase and angiopoietin inhibition to affect the tumor microenvironment in a manner that significantly inhibits the metastatic phenotype of ccRCC.

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