Preclinical Evidence That Trametinib Enhances the Response to Antiangiogenic Tyrosine Kinase Inhibitors in Renal Cell Carcinoma

Victoria L. Bridgeman1, Elaine Wan1,2, Shane Foo1, Mark R. Nathan1, Jonathan C. Welt1, Sophia Frentzas1,2, Peter B. Vermeulen1,3, Natasha Preece4, Caroline J. Springer2, Thomas Powles5, Paul D. Nathan6, James Larkin2, Martin Gore2, Naveen S. Vasudev1,2, and Andrew R. Reynolds1

Abstract

Sunitinib and pazopanib are antiangiogenic tyrosine kinase inhibitors (TKI) used to treat metastatic renal cell carcinoma (RCC). However, the ability of these drugs to extend progression-free and overall survival in this patient population is limited by drug resistance. It is possible that treatment outcomes in RCC patients could be improved by rationally combining TKIs with other agents. Here, we address whether inhibition of the Ras-Raf-MEK-ERK1/2 pathway is a rational means to improve the response to TKIs in RCC. Using a xenograft model of RCC, we found that tumors that are resistant to sunitinib have a significantly increased angiogenic response compared with tumors that are sensitive to sunitinib in vivo. We also observed significantly increased levels of phosphorylated ERK1/2 in the vasculature of resistant tumors, when compared with sensitive tumors. These data suggested that the Ras-Raf-MEK-ERK1/2 pathway, an important driver of angiogenesis in endothelial cells, remains active in the vasculature of TKI-resistant tumors. Using an in vitro angiogenesis assay, we identified that the MEK inhibitor (MEKI) trametinib has potent antiangiogenic activity. We then show that, when trametinib is combined with a TKI in vivo, more effective suppression of tumor growth and tumor angiogenesis is achieved than when either drug is utilized alone.

In conclusion, we provide preclinical evidence that combining a TKI, such as sunitinib or pazopanib, with a MEKI, such as trametinib, is a rational and efficacious treatment regimen for RCC.

Introduction

Kidney cancer is the 13th most common cancer worldwide with >300,000 new cases diagnosed each year. In the United States alone, there are approximately 60,000 new cases of kidney cancer diagnosed each year and approximately 14,000 deaths from kidney cancer each year (1). The majority of kidney cancers (90%) are renal cell carcinomas (RCC). Approximately 20% of patients presenting with a primary RCC have synchronous metastatic RCC (mRCC). A further 30% of patients will develop mRCC following surgery for the primary.

Tumor angiogenesis stimulated by VEGF is considered to be an essential driver in mRCC and angiogenesis inhibitors are efficacious in mRCC patients (2–4). Current standard of care in the first-line treatment is with one of two different antiangiogenic tyrosine kinase inhibitors (TKI): sunitinib or pazopanib. Both drugs inhibit receptors involved in angiogenesis, including VEGFR1-3 and PDGFRα/β (5, 6), and have equal potency in their ability to extend progression-free and overall survival in mRCC (7–10).

However, approximately 20% of mRCC patients do not respond to these drugs (which is termed “intrinsic” or “early” resistance to treatment). Moreover, most patients that respond initially will typically progress within 12 months of starting therapy (described as “acquired” or “late” resistance to treatment). Median overall survival in mRCC patients treated with these agents remains in the region of 24 months (9). There is, therefore, a pressing need to find more effective treatment strategies for mRCC patients (2, 11). Importantly, functional imaging in mRCC patients showed that early resistance to TKIs can be correlated with incomplete suppression of angiogenesis, whereas acquired resistance is associated with tumor revascularization after an initial period of response (12). These and other data (13, 14) suggest that a strong and sustained suppression of the tumor vascularization process is key for ensuring the best response to TKIs in mRCC.

Numerous mechanisms have been proposed to explain both early and late resistance to antiangiogenic therapy.
mechanism that has received considerable attention is redundancy in proangiogenic growth factor signaling (2, 11, 15, 16).

Human cancers, including RCC, express numerous additional proangiogenic factors, including fibroblast growth factor 2 (FGF2), hepatocyte growth factor (HGF), and IL8, which may stimulate the growth and survival of tumor blood vessels even when the VEGF-pathway is blocked (17–25). It may therefore be necessary to develop therapies that block the activity of multiple proangiogenic factors in these tumors.

Most proangiogenic factors activate the Ras-Raf-MEK-ERK1/2 pathway in endothelial cells, a signaling pathway that promotes angiogenesis by triggering the proliferation, survival, and migration of endothelial cells (26–29). In recent years, there has been great interest in developing clinically effective small-molecule inhibitors of MEK, the kinase that sits upstream of ERK1/2, as a means to inhibit the Ras-Raf-MEK-ERK1/2 pathway in cancer (30). Moreover, impressive results have been obtained in metastatic melanoma when the MEK inhibitor trametinib is combined with the B-Raf inhibitor dabrafenib; with increased progression-free survival and overall survival observed in the combination arm compared with B-Raf inhibition alone (31–33). Similarly, combining the MEK inhibitor cobimetinib with the B-Raf inhibitor vemurafenib has also been shown to extend progression-free survival and overall survival in metastatic melanoma when compared with vemurafenib alone (34). These data provide compelling evidence that certain MEK inhibitors can be safely and effectively combined with other agents in patients.

However, the efficacy of MEK inhibition has yet to be tested in patients with mRCC. In the current manuscript, we present preclinical evidence that trametinib enhances the response to antiangiogenic TKIs in RCC.

Materials and Methods

Reagents

Cell culture reagents: RPMI-1640, DMEM and M199 medium, FCS, collagcnase-1 (Invitrogen), ERM-2 media, EGM-2 Bulletkit (Lonza Biologics), and bovine brain endothelial mitogen (Serotech). Primary antibodies for Western blotting: phospho-Thr-202/Tyr-204-ERK1/2 (20G11, Cell Signaling Technology). Secondary antibodies: HRP- and biotin-conjugated (DAKO)

Reagents

Materials and Methods

Cell culture

The 786-0 cell line was obtained from the ATCC. Parental 786-0 cells, and the sublines 786-0-R and 786-0-S, were all cultured in RPMI-1640 plus 10% FCS. All three cell lines were confirmed as being 786-0 in origin by short tandem repeat (STR) typing, whereas Sanger sequencing confirmed that they all carried the VHL mutation reported for this cell line, 310delG (see below for Methods). Human umbilical vein endothelial cells (HUVECs; TCS Cell Works) were cultured on gelatin-coated flasks in M199 supplemented with 20% FCS, 0.1 µg/ml bovine brain endothelial mitogen and 1 ng/ml heparin. Human angiogenic fibroblasts (HAF; TCS Cell Works) were cultured in DMEM plus 10% FCS. Cells were regularly tested for mycoplasma and shown to be contamination free.

STR typing and VHL sequencing

For the purposes of STR typing and VHL sequencing, DNA was isolated from cells using the DNeasy Blood and Tissue Kit (Qiagen) or from FFPE tissue sections using the QIAamp DNA Extraction Kit (Qiagen) as per the manufacturer’s instructions. DNA concentrations were then quantified by using a Nanodrop instrument (Thermo Scientific). For STR typing, the Geneprint 10 Kit (Promega) was used to coamplify specific human loci that allow the cell lineage to be identified. The PCR products were run on a 3730xl DNA Analyzer (Life Technologies). Genemapper 4.1 software (Life Technologies) was used to interpret the results, calling peaks using the geneprint sizing/bin standards.

For sequencing of VHL, the following previously published (35) primer sequences were used to amplify the entire coding sequence of the VHL gene: exon 1 forward primer (GAAGACTACCGAGGTTCGA), exon 1 reverse primer (GATGTGGCTCGCTCAG), exon 2 forward primer (GATGTGCTCGCTCAG), exon 2 reverse primer (CATCACTACCATACACATCTCC), exon 3 forward primer (CATCAGTACACGTAGTGTC), exon 3 reverse primer (CACAATGGCTGATGACG).

The PCR was run using a Kapa2G Robust Hotstart kit (Kapa BioSystems) with annealing temperature of 56°C. PCR products were cleaned up using the QIAquick PCR Purification Kit (Qiagen) as per the manufacturer’s instructions and then sent for Sanger Sequencing (Eurofins UK). The sequences were aligned with the human VHL gene to identify mutations.

Xenograft models and treatment

Female CB17 SCID mice (CB17/lcr-Pbrd1<sup>cro</sup>/lcrcoCrl) were obtained from Charles River UK. For 786-0 xenografts, mice were shaved on the flank and then injected subcutaneously with 3 × 10<sup>6</sup> 786-0 cells. Once tumors reached 100 to 200 mm<sup>3</sup> in size, the mice were randomized to receive treatment with sunitinib or vehicle. Tumors were measured using calipers and tumor volumes were calculated using the formula: (length × width<sup>2</sup>) / 2. In order to generate the 786-0-R and 786-0-S sublines, cells were isolated from sunitinib-treated 786-0 xenografts as follows: the relevant tumor was harvested, minced, and digested in collagenase (1 mg/mL collagenase-1 in PBS supplemented with 1 mmol/L MgCl<sub>2</sub> and 1 mmol/L CaCl<sub>2</sub>) at 37°C for 90 minutes. Digested tissue was filtered and the cells collected by centrifugation. Cells were plated onto tissue culture flasks in RPMI-1640 plus 10% FCS and expanded in culture. To establish 786-0-R or 786-0-S xenografts, CB17 SCID mice were shaved on the flank and then injected subcutaneously with 3 × 10<sup>6</sup> cells. Once tumors reached 100 to 200 mm<sup>3</sup> in size, the mice were randomized to the treatment groups.

The patient-derived xenograft (PDX) model was established from the tumor of a 71-year-old male who underwent nephrectomy for clear cell RCC at The Royal Marsden hospital (London, United Kingdom). Tumor fragments of approximately 2 mm<sup>3</sup> in size were implanted subcutaneously into CB17 SCID mice through a small incision under anesthesia. Mice were observed every week for the presence of palpable tumors. Subsequent
Figure 1.
Heterogeneous response to sunitinib in a preclinical model of RCC. A and B, mice with established subcutaneous 786-0 tumors were treated with 40 mg/kg/day sunitinib or vehicle alone. Growth kinetics are shown for three representative tumors belonging to each category: vehicle-treated control tumors (A) and sunitinib-treated tumors from the “sensitive,” “early resistance,” or “late resistance” categories (B). Graphs show the change in tumor volume over time relative to start of treatment. Growth kinetics for all tumors included in the study are shown in Supplementary Fig. S1. C, tumor vessel density in the control, sensitive, early resistance, and late resistance categories. Graph shows the density of endomucin-positive tumor vessels per mm² ± SEM (n = 6 tumors from each category). Example staining of endomucin is shown. (Continued on the following page.)
passaging of tumors was achieved by harvesting the subcutaneous tumors and implanting tumor fragments subcutaneously into further recipient mice.

Sunitinib, trametinib, and pazopanib were prepared in vehicle solutions suitable for oral dosing (sunitinib: 0.5% carboxymethyl cellulose, 300 mmol/L NaCl, 0.4% Tween-80, 0.9% benzyl alcohol adjusted to pH 6.0; trametinib: 0.5% hydroxypropylmethyl cellulose and 0.2% Tween-80; pazopanib: 0.5% hydroxypropylmethyl cellulose and 0.1% Tween-80) as previously described (5, 36, 37). Mice were administered 0.2 mL of drug, or vehicle alone, by oral gavage at 40 mg/kg/day (sunitinib), 1 mg/kg/day (trametinib), or 30 mg/kg/day (pazopanib). In experiments where mice were dosed with sunitinib or vehicle only, mice received one dose by oral gavage each day. In experiments where mice received combination therapy, all mice in the experiment were dosed twice daily: vehicle, sunitinib, or pazopanib was dosed first, followed by vehicle or trametinib 3 to 4 hours later that same day. No overt signs of toxicity (such as weight loss) were observed in any treatment group, including mice treated with drugs in combination.

IHC

IHC was performed essentially as described (36). In brief, tumors were harvested from mice and bisected. Half the tumor was fixed in 4% w/v formalin, whereas the other half was embedded in OCT and frozen at −80°C. For endomucin staining, formalin-fixed paraffin-embedded sections were incubated with anti-endomucin antibody, followed by detection with a biotinylated secondary antibody and a DAB substrate kit (VECTOR). Slides were counterstained with hematoxylin before mounting in DEPEX. Slides were scanned using a Hamamatsu Nanozoomer and viewed using NDPi software (Hamamatsu Photonics). The number of endomucin-positive vessels in each section was counted manually. To calculate vessel density, the number of vessels present in the section was divided by the area of the section. Areas of necrosis were excluded from the quantification.

Costaining for CD31 and Ki-67 or CD31 and pERK1/2 was performed on frozen sections that were fixed in formalin and incubated at 4°C overnight with primary antibodies, followed by detection with appropriate fluorescently conjugated secondary antibodies and counterstaining with DAPI. Immunofluorescence images were captured using an SP2 confocal laser-scanning microscope (Leica). The proportion of cells (endothelial or cancer cells) positive for Ki-67 or pERK was calculated by manually counting the number of cell nuclei that stained positive per field and then dividing this by the total number of cells (endothelial or cancer cells) per field (5 fields per tumor sample were used).

Western blotting

Western blotting was performed essentially as described (29). HUVECs were seeded at a density of 75,000 cells per well in 6-well plates that were precoated with 0.1% gelatin. Cells were cultivated for 48 hours to reach approximately 80% confluence before being used for experiments. Cells were then serum-deprived for 3 hours in serum-free M199, followed by 10-minute preincubation with the indicated small-molecule inhibitor or vehicle (0.1% DMSO) before stimulation with 100 ng/mL VEGF and 50 ng/mL FGF2. After 10-minute stimulation, plates were transferred to ice, washed twice with PBS-A, and lysed in 75 μL of lysis buffer (150 mmol/L NaCl, 20 mmol/L Tris pH 7.5, 10% glycrol, 1% IGEPAI CA-630, 1 mmol/L Na2VO4, 10 mmol/L NaF, 1 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.8 mmol/L aprotinin, 0.05 mmol/L bestatin, 0.015 mmol/L E-64, 0.02 mmol/L leupeptin and 0.01 mmol/L pepstatin). Lysates were mixed with reducing Laemmli sample buffer, separated on 10% SDS-Polyacryl-amide gels (Invitrogen) and transferred onto Hybond-ECL nitrocellulose membrane (GE Healthcare Life Sciences). Membranes were blocked at room temperature in TBS-T containing 5% milk, followed by primary antibody incubation either for 1 hour at room temperature or 16 hours at 4°C in TBS-T containing 5% BSA. After washing, incubation with horseradish peroxidase–conjugated secondary antibodies was performed for 1 hour at room temperature in TBS-T containing 5% milk. Signals were visualized using the enhanced chemiluminescence reagent (GE Healthcare Life Sciences). Densitometry was performed from scanned blots using ImageJ software and IC50 were determined using PRISM software.

In vitro angiogenesis assays

Endothelial tubule formation assays were performed essentially as described (18). In brief, Cytodex3 beads (GE Healthcare Life Sciences) were coated with HUVECs overnight in EGM-2 media (EGM-2 is composed of EBM-2 media supplemented with all EGM-2 bullet kit components). The beads were then washed and embedded in a fibrinogen gel. HAFs were seeded on top of the gel in EGM-2 media. After 24 hours, the media were removed and replaced with EBM-2 media supplemented with 2.5% FBS, 10 ng/mL VEGF-A, and 50 ng/mL FGF2. When the effect of inhibitors on tubule formation was assayed, the inhibitors or vehicle alone (0.1% DMSO) were also added at this time point. The cultures were left to allow sprouts to form, with media changed every 2 to 3 days. After 9 days, tubule formation was quantified by counting the number of sprouts coming from individual beads (30 beads were counted for each condition). IC50 were determined using PRISM software.

A modified version of the protocol was used to assess the effect of the inhibitors on preformed tubules. Here, HUVEC-coated beads were embedded in a fibrinogen gel and the tubules were allowed to form in the absence of any inhibitors. After 7 days, images of individual beads were captured using an inverted phase contrast microscope. Inhibitors or vehicle alone were then added and the cultures returned to the incubator. For 48 hours, images of the same individual beads were captured again. The change in

(Continued) D, tumor vessel proliferation in the control, sensitive, early resistant, and late resistant categories. Graph shows the percentage of endothelial cells with Ki-67-positive nuclei per field ± SEM (15 high power fields were analyzed from across three tumors in each category). Example staining of a Ki-67-positive endothelial cell is shown. E, phosphorylated ERK1/2 (pERK1/2) in tumor vessels from the control, sensitive, early resistance, and late resistance categories. Graph shows the percentage of endothelial cells with pERK1/2-positive nuclei per field ± SEM (15 high power fields were analyzed from across three tumors in each category). Example staining of a pERK1/2-positive endothelial cell is shown. nsd, no significant difference. *, P < 0.05; ***, P < 0.01; ****, P < 0.0001. Scale bar, 50 μm (C) or 5 μm (D and E).
tubule length between the first image and the second image was then quantified using ImageJ software.

Ethical approval

Ethical approval for animal experimentation was granted by the Institute of Cancer Research Animal Ethics Committee. All procedures were performed in accordance with UK Home Office regulations. Ethical approval for the use of human tissue collected from consented patients was obtained from the Royal Marsden Research Ethics Committee.

Statistical analysis

Analysis of statistical significance was performed using the Student t test (P values of less than 0.05 were considered to be statistically significant).

Results

Heterogeneous response to sunitinib in a preclinical model of renal cell carcinoma

We established subcutaneous 786-0 xenografts in mice and once tumors reached 100 to 200 mm³ in volume, mice were randomized to treatment with either 40 mg/kg sunitinib or vehicle. Tumors in the vehicle group (control) progressed rapidly, undergoing ≥5-fold increase in tumor volume by 42 days compared with the start of treatment (Fig. 1A and Supplementary Fig. S1). In contrast, the progression of tumors in the sunitinib-treated group was heterogeneous. This was not unexpected, since a heterogeneous response to sunitinib in 786-0 xenografts has been described before (20). We took advantage of this heterogeneity and allocated tumors into three categories based on their response to treatment: "sensitive," "early resistance," or "late resistance" (Fig. 1B and Supplementary Fig. S1). Individual tumors were allocated to these categories based on the following criteria. Tumors undergoing ≤2.5-fold increase in tumor volume after 42 days (compared with the start of treatment) were allocated to the sensitive category (Fig. 1B, left). Tumors that underwent ≥5-fold increase in volume after 42 days (compared with the start of treatment) were allocated to the early resistance category (Fig. 1B, middle). Finally, tumors that underwent ≤2.5-fold increase in volume by 50 days (compared with the start of treatment) with progression to ≥5-fold increase in volume during the following 50 to 100 days (compared with the start of treatment) were allocated to the late resistance category (Fig. 1B, right). However, it should also be stated that tumors in the sensitive category most likely represent the "sensitive phase" of tumor growth seen in the late resistance category.

Characterization of the tumor vasculature in control, sensitive, and resistant tumors

Because sunitinib inhibits tumor growth in RCC principally by blocking tumor angiogenesis (38), we examined the vasculature in control, sensitive, and early resistance tumors (all harvested after 42 days of treatment) and late resistance tumors (harvested after 99–101 days of treatment). Consistent with the potent antiangiogenic activity of sunitinib, tumor vessel density was significantly decreased in all sunitinib-treated categories compared with vehicle-treated tumors (Fig. 1C). However, vessel density was significantly increased in both the early resistance category and late resistance category compared with the sensitive category (Fig. 1C).

Proliferation of tumor endothelial cells can also be used as a marker of tumor angiogenesis. Therefore, we quantified the proportion of Ki-67-positive endothelial cells present in tumor vessels. No significant difference in the proportion of Ki-67-positive endothelial cells was observed between the vehicle group and the sensitive category (Fig. 1D). However, the proportion of Ki-67-positive endothelial cells was significantly increased in both the early resistance category and late resistance category when compared with the sensitive category (Fig. 1D).

Finally, we quantified the presence of phosphorylated ERK1/2 (pERK1/2) in tumor endothelial cells. The proportion of endothelial cells positive for pERK1/2 was significantly increased in both the early resistance category and late resistance category compared with the sensitive category (Fig. 1E).

The MEK inhibitor trametinib has potent antiangiogenic activity in vitro

We next examined the ability of small-molecule MEK inhibitors (MEKI) to inhibit ERK1/2 activation in endothelial cells. Three MEKIs were tested: trametinib (37), selumetinib (39), and PD184352 (40). Human umbilical vein endothelial cells (HUVEC) were stimulated with a combination of VEGF and FGF2 in the presence of MEKI or vehicle alone, followed by blotting for pERK1/2 (Fig. 2A–C). Blots were performed in triplicate and densitometry measurements used to determine IC₅₀ for the inhibition of ERK1/2 activation (Table 1). All three MEKIs suppressed ERK1/2 activation in stimulated endothelial cells, with IC₅₀ in the nanomolar range (Fig. 2 and Table 1). In parallel, we tested three antiangiogenic TKIs: sunitinib, pazopanib, and sorafenib (Fig. 2D–F). The MEKIs were more potent at inhibiting ERK1/2 activation compared with the TKIs (Fig. 2 and Table 1). Moreover, the most effective inhibitor of ERK1/2 activation in endothelial cells was trametinib (IC₅₀ = 1.3 mmol/L).

We then examined the antiangiogenic activity of all six drugs (three MEKIs and three TKIs) using a previously described in vitro angiogenesis assay (18, 41). In brief, latex beads coated with HUVECs were embedded in a fibrinogen gel and incubated in the presence of VEGF and FGF2 in order to induce the formation of endothelial tubules (Supplementary Fig. S2A). To measure inhibition of new tubule formation, assays were performed in the presence of drug from the first day of the assay onwards (Supplementary Fig. S2B). Within the range of concentrations tested (10 pmol/L to 10 µmol/L), all drugs exhibited antiangiogenic activity, as measured by inhibition of tubule formation (Fig. 3A and Table 1). The MEKI trametinib showed the most potent antiangiogenic activity (Fig. 3A and Table 1).

These results measure the ability of the tested drugs to block the formation of new tubules. We next tested their ability to regress preformed tubules. To do this, drugs were added to the assay at a time point after tubules were formed (7 days), and the extent of tubule regression was measured 48 hours later (Supplementary Fig. S2C). Only trametinib induced tubule regression at all concentrations tested (1, 10, and 100 nmol/L; Fig. 3B). For all other drugs, a dose of at least 100 nmol/L was required to induce tubule regression (Fig. 3B).

Combination of sunitinib with trametinib is an effective treatment regimen in vivo

We then examined the ability of trametinib, sunitinib, or a combination of both drugs, to inhibit tumor growth in vivo. We...
used a sunitinib-refractory subline (786-0-R) that we isolated from the parental 786-0 cell line (Supplementary Fig. S3). Once 786-0-R tumors reached 100 to 200 mm³ in volume, mice were randomized to receive vehicle alone, trametinib alone, sunitinib alone, or a combination of sunitinib and trametinib. No overt signs of toxicity (such as weight loss) were observed in any treatment group during the experiment, which included mice treated with the drug combination. Treatment with single-agent trametinib was no more effective than single-agent sunitinib in suppressing tumor growth in this model (Fig. 4A). However, the combination of sunitinib and trametinib was more effective than administering either drug alone (Fig. 4A).

To evaluate effects on tumor angiogenesis, vessel density was quantified in treated tumors (Fig. 4B and C). Trametinib alone did not induce a significant reduction in tumor vessel density compared with vehicle (Fig. 4B). Importantly, the strongest reduction in vessel density was observed with the combination therapy (Fig. 4B).

To address why the combination was more effective at suppressing tumor angiogenesis in vivo (compared with single-agent therapy), we evaluated ERK1/2 phosphorylation in treated 786-0-R tumors. Phosphorylated ERK1/2 was quantified in the endothelium of tumor blood vessels and in the cancer cells separately. Although a significant reduction in ERK1/2 phosphorylation was observed in blood vessels in tumors treated with either trametinib alone or sunitinib alone (Fig. 4D), the strongest inhibition of ERK1/2 phosphorylation in tumor blood vessels was observed in tumors treated with the sunitinib plus trametinib combination (Fig. 4D). In contrast, no significant difference in ERK1/2 phosphorylation was observed in cancer cells when comparing the treatment groups (Fig. 4E).

We also examined the combination of sunitinib plus trametinib within the previously described in vitro angiogenesis assay.

**Figure 2.**

Inhibition of ERK1/2 activation in endothelial cells by MEKIs and TKIs. A–F, endothelial cells were serum deprived and lysed without stimulation (no stim) in the presence of vehicle (veh) alone or stimulated with VEGF and FGF2 in the presence of vehicle alone or trametinib (A), selumetinib (B), PD184352 (C), sunitinib (D), pazopanib (E), or sorafenib (F) at the indicated concentration (concn). Cell lysates were then probed for phosphorylated ERK1/2 or total ERK.
Importantly, when sunitinib and trametinib were tested in the angiogenesis assay at subthreshold concentrations, we found that the combination of sunitinib with trametinib was more effective than either drug alone at inhibiting angiogenesis (Supplementary Fig. S4).

Efficacy of trametinib in a discontinuous schedule or when added after progression

Because the antitumor activity of single-agent trametinib was no more effective than single-agent sunitinib within our in vivo model, in the next two experiments we focused on comparing the sunitinib–trametinib combination with sunitinib monotherapy. Sunitinib is typically administered in a discontinuous schedule in the clinic for 2 weeks on/1 week off (2/1 schedule) or for 4 weeks on/2 weeks off (4/2 schedule). Therefore, we examined discontinuous scheduling of treatment. Once 786-0-R tumors reached 100 to 200 mm³ in volume, mice were randomized to receive a 2/1 schedule of sunitinib or a 2/1 schedule of sunitinib plus trametinib. Again, we found that the combination was more effective than sunitinib alone in suppressing tumor growth (Fig. 5A) and in suppressing tumor vessel density (Supplementary Fig. S5A).

We then tested the efficacy of switching to a combination of sunitinib plus trametinib in mice whose tumors had already progressed whilst on treatment with sunitinib. Mice bearing established 786-0-R tumors were treated with sunitinib alone for 28 days, during which time their tumors increased in volume approximately 2-fold compared with the start of treatment (Fig. 5B). The same mice were then randomized to either continue on sunitinib alone for 14 days or switch to treatment with sunitinib plus trametinib for 14 days. Importantly, although tumors in the group that continued on sunitinib progressed,

<table>
<thead>
<tr>
<th>Drug</th>
<th>Inhibition of ERK activation determined from Western blot analyses (IC₅₀ ± SEM)</th>
<th>Inhibition of endothelial cell tubule formation (IC₅₀ ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trametinib</td>
<td>1.33 ± 0.05 nmol/L</td>
<td>0.09 ± 0.02 nmol/L</td>
</tr>
<tr>
<td>PD184352</td>
<td>20.35 ± 13.21 nmol/L</td>
<td>6.34 ± 3.73 nmol/L</td>
</tr>
<tr>
<td>Selumetinib</td>
<td>6.60 ± 2.88 nmol/L</td>
<td>7.33 ± 0.76 nmol/L</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>3.174.33 ± 1.564.04 nmol/L</td>
<td>39.60 ± 8.35 nmol/L</td>
</tr>
<tr>
<td>Pazopanib</td>
<td>640.75 ± 329.27 nmol/L</td>
<td>2.75 ± 0.25 nmol/L</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>Not determined</td>
<td>199.80 ± 11.40 nmol/L</td>
</tr>
</tbody>
</table>

Table 1. Activity of MEKIs and TKIs for inhibition of ERK1/2 activation in endothelial cells and inhibition of endothelial cell tubule formation.
tumors that switched to the combination regressed (Fig. 5B) and had a significantly lower vessel density than tumors that remained on sunitinib (Supplementary Fig. S5B).

Efficacy of trametinib when combined with pazopanib

Because our experiments thus far were limited to testing trametinib in combination with sunitinib, we also tested whether trametinib would be effective when combined with pazopanib. Mice bearing established 786-O-R tumors were randomized to receive trametinib alone, pazopanib alone, or a combination of pazopanib plus trametinib. Importantly, the combination of pazopanib plus trametinib was more effective than administering either agent alone (Fig. 5C and Supplementary Fig. S5C).
Efficacy of trametinib in a PDX model of RCC

We established a PDX model of RCC from the primary renal cancer of a 71-year-old male. Samples from four different viable tumor regions (regions 1–4) were grafted subcutaneously into mice (Fig. 6A). In this first generation of the PDX, the only graft that took was from region 4 (Fig. 6A). This tumor was passaged into 5 further mice to establish the second generation of the PDX (Fig. 6A). Established tumors from these 5 mice were then grafted into a further 20 mice to establish the third generation of the PDX (Fig. 6A). The clear cell histology of the primary cancer was retained at each generation of the PDX (Fig. 6B–E) and STR typing confirmed good concordance between the genotype of the original patient tumor and the genotype of the PDX (Supplementary Table S1). Sequencing of the PDX model confirmed the presence of a mutation in exon 3 of the VHL gene (464delT). This mutation has been previously reported in RCC and is predicted to generate a truncated version of VHL (42). We called this model ICR-RCC-01.

Mice bearing third-generation ICR-RCC-01 tumors were randomized to treatment. Trametinib alone was not effective at suppressing tumor growth compared with vehicle (Fig. 6F). However, treatment with sunitinib alone suppressed tumor growth compared with vehicle (Fig. 6F) and the
combination of sunitinib with trametinib was more effective at suppressing tumor growth than administering sunitinib alone (Fig. 6F).

Discussion

Treatment outcomes in mRCC patients might be improved by rationally combining TKIs with other agents (2, 11, 43). In the current preclinical study, we addressed whether inhibition of the Ras-Raf-MEK-ERK1/2 pathway is a rational means to improve the response to antiangiogenic TKIs in RCC. We found that 786-0 xenografts responded heterogeneously to treatment with sunitinib, allowing us to define tumors as being “sensitive,” “early resistant,” or “late resistant” to sunitinib. Both early and late resistant tumors showed significantly increased vessel density and significantly increased endothelial cell proliferation compared with sensitive tumors. This may mirror the clinical scenario in RCC, where early resistance to TKIs can be correlated with incomplete suppression of angiogenesis and where late resistance can be correlated with tumor revascularization after an initial period of response (12). Importantly, we also found elevated levels of phosphorylated ERK1/2 in the vessels of these early and late resistant tumors.
late resistant tumors compared with sensitive tumors, showing that the Ras-Raf-MEK-ERK1/2 pathway remains active in the vascular endothelium of tumors that are resistant to TKI treatment. We then showed that combination treatment with the TKI sunitinib plus the MEKI trametinib is significantly more effective at inhibiting tumor growth and tumor angiogenesis than utilizing either drug as a single agent.

In order to address why the TKI plus MEKI combination was more effective at limiting tumor angiogenesis and tumor growth in vivo, we quantified phosphorylated ERK1/2 in both tumor blood vessels and cancer cells. We found that the TKI plus MEKI combination resulted in more effective inhibition of ERK1/2 phosphorylation in tumor endothelial cells compared with treatment with either drug alone. From these data, we conclude that the superior antiangiogenic activity of the combination stems from its superior inhibition of the Ras-Raf-MEK-ERK1/2 pathway in tumor endothelial cells in vivo. Moreover, because no significant effect on ERK1/2 phosphorylation was observed in cancer cells, we conclude that the superior antitumor effect of the combination stems primarily from this superior antiangiogenic effect rather than a direct effect on the cancer cells themselves (at least in the model system studied here).

However, the combination of TKI plus MEKI could have additional antitumor activity arising through other mechanisms. For example, MEK-dependent recruitment of proangiogenic neutrophils to tumors has been shown to mediate resistance to VEGF-inhibition in preclinical models and can be overcome by inhibiting the MEK pathway in vitro (44). Moreover, RCC cell lines resistant to sorafenib or sunitinib in vitro have increased expression of pERK1/2, suggesting that an activated Ras-Raf-MEK-ERK1/2 pathway in cancer cells may directly promote cell survival in TKI-resistant cancer cells (45, 46). Taken together, these data suggest that targeting of the VEGF pathway and the Ras-Raf-MEK-ERK1/2 pathway in RCC could potentially lead to improved responses due to multiple mechanisms, including enhanced inhibition of angiogenesis in endothelial cells (current study), inhibition of proangiogenic neutrophil recruitment (44), and suppression of prosurvival signaling in cancer cells (45, 46).

Although our data provide preclinical evidence that trametinib enhances the response to TKIs in RCC, translating combination treatment strategies such as this to the clinic can be challenging (43) because combining a TKI with a MEKI in patients could lead to unmanageable levels of toxicity. One strategy utilized to minimize toxicity in the clinic is to include planned treatment breaks in the dosing schedule. Importantly, we showed that the combination of sunitinib and trametinib was still more effective than sunitinib monotherapy when administered in a schedule that incorporated a planned treatment break.

We also examined the efficacy of combining pazopanib with trametinib. We found that the combination of pazopanib plus trametinib was more effective than pazopanib alone. Moreover, a recent phase I trial showed that the pazopanib plus trametinib combination is tolerable in patients with solid tumors at full dose of both drug (47). Although no patients with mRCC were included in that study, the results indicate that combining a TKI with a MEKI is feasible in cancer patients.

Taken together, these data support the concept of testing MEK inhibition in combination with TKIs in patients with mRCC. A randomized phase II clinical trial of TKI versus TKI plus MEKI in patients with mRCC can be envisaged. The question also arises as to whether adding a MEKI to TKI therapy could improve outcome in patients who have already progressed on prior TKI therapy. Importantly, here we also showed that adding trametinib to single-agent TKI can suppress tumor growth even in tumors that already progressed on prior TKI therapy. Therefore, it may also be appropriate to test this combination in the second line.

Disclosure of Potential Conflicts of Interest
T. Powles is a consultant/advisory board member for Roche and Novartis. P.D. Nathan has received speakers bureau honoraria from GSK, Novartis, and BMS and is a consultant/advisory board member for GSK, Novartis, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: V.L. Bridgeman, T. Powles, N. Vasudev, A.R. Reynolds
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V.L. Bridgeman, E. Wan, S. Foo, M.R. Nathan, J.C. Welii, C.J. Springer, M. Gore, N. Vasudev, A.R. Reynolds
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V.L. Bridgeman, M.R. Nathan, P.B. Vermeulen, N. Preece, T. Powles, J. Larkin, M. Gore, N. Vasudev, A.R. Reynolds
Writing, review, and/or revision of the manuscript: V.L. Bridgeman, J.C. Welii, N. Vasudev, P.B. Vermeulen, C.J. Springer, T. Powles, P.D. Nathan, J. Larkin, M. Gore, N. Vasudev, A.R. Reynolds
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Frentzas
Study supervision: P.D. Nathan, A.R. Reynolds

Acknowledgments
The authors thank the staff of the ICR biological services unit and the Breakthrough Breast Cancer histopathology facility for technical assistance.

Grant Support
This work was supported by Breakthrough Breast Cancer (recently merged with Breast Cancer Campaign forming Breast Cancer Now) and by NHS funding to the NIHR Biomedical Research Centre at The Royal Marsden/Institute of Cancer Research.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 27, 2015; revised August 19, 2015; accepted September 17, 2015; published OnlineFirst October 20, 2015.

References
Molecular Cancer Therapeutics

Preclinical Evidence That Trametinib Enhances the Response to Antiangiogenic Tyrosine Kinase Inhibitors in Renal Cell Carcinoma

Victoria L. Bridgeman, Elaine Wan, Shane Foo, et al.

Mol Cancer Ther  Published OnlineFirst October 20, 2015.

Updated version  Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-15-0170

Supplementary Material  Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2015/10/20/1535-7163.MCT-15-0170.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.