



UNIVERSITY OF LEEDS

This is a repository copy of *Preclinical Evidence That Trametinib Enhances the Response to Antiangiogenic Tyrosine Kinase Inhibitors in Renal Cell Carcinoma*.

White Rose Research Online URL for this paper:
<http://eprints.whiterose.ac.uk/94472/>

Version: Accepted Version

Article:

Bridgeman, VL, Wan, E, Foo, S et al. (12 more authors) (2016) Preclinical Evidence That Trametinib Enhances the Response to Antiangiogenic Tyrosine Kinase Inhibitors in Renal Cell Carcinoma. *Molecular Cancer Therapeutics*, 15 (1). pp. 172-183. ISSN 1535-7163

<https://doi.org/10.1158/1535-7163.mct-15-0170>

Reuse

Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.



eprints@whiterose.ac.uk
<https://eprints.whiterose.ac.uk/>

Preclinical evidence that trametinib enhances the response to anti-angiogenic tyrosine kinase inhibitors in renal cell carcinoma

Victoria L. Bridgeman¹, Elaine Wan^{1,2}, Shane Foo¹, Mark Nathan¹, Jonathan C. Welti¹, Sophia Frentzas^{1,2}, Peter B. Vermeulen^{1,3}, Natasha Preece⁴, Caroline Springer⁴, Thomas Powles⁵, Paul Nathan⁶, James Larkin², Martin Gore², Naveen S. Vasudev^{1,2,7}, Andrew R. Reynolds¹

¹Tumour Biology Team, Breakthrough Breast Cancer Research Centre, The Institute of Cancer Research (ICR), London, SW3 6JB, United Kingdom

²The Royal Marsden (RM), London SW3 6JJ, United Kingdom

³Translational Cancer Research Unit, GZA Hospitals St. Augustinus, Antwerp, Belgium

⁴Cancer Research UK Cancer Therapeutics Unit, The Institute of Cancer Research, London SM2 5NG, United Kingdom

⁵Experimental Cancer Medicine Centre, Queen Mary University of London, Charterhouse Square, London, EC1A 7BE, United Kingdom

⁶Department of Medical Oncology, Mount Vernon Cancer Centre, Northwood, HA6 2RN, United Kingdom

⁷Current address: Cancer Research UK Centre, Leeds Institute of Cancer and Pathology, St James's University Hospital, Leeds LS9 7TF, United Kingdom

Running title: TKI and MEKI for renal cell carcinoma

Word count: 5140

Conflict of interest: the authors have no relevant conflict of interest to declare

Co-corresponding authors:

Andrew R. Reynolds

E-mail: andrew.reynolds@icr.ac.uk

Naveen S. Vasudev

E-mail: N.Vasudev@leeds.ac.uk

Abstract

Sunitinib and pazopanib are anti-angiogenic tyrosine kinase inhibitors (TKIs) 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 which are resistant to sunitinib have a significantly increased angiogenic response compared to tumors which are sensitive to sunitinib *in vivo*. We also observed significantly increased levels of phosphorylated ERK1/2 in the vasculature of resistant tumors, when compared to 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 anti-angiogenic 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 ~60,000 new cases of kidney cancer diagnosed each year and ~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 vascular endothelial growth factor (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 is treatment with one of two different anti-angiogenic tyrosine kinase inhibitors (TKIs): sunitinib or pazopanib. Both drugs inhibit receptors involved in angiogenesis, including VEGFR1-3 and PDGFR α/β (5-7), and have equal potency in their ability to extend progression free and overall survival in mRCC (8-11).

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 (10). There is, therefore, a pressing need to find more effective treatment strategies for mRCC patients (2, 12). Importantly, functional imaging in mRCC patients showed that early resistance to TKIs can be correlated with incomplete suppression of angiogenesis, whilst acquired resistance is associated with tumor revascularisation after an initial period of response (7). These data suggest that a strong and sustained suppression of the tumor vascularisation 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 anti-angiogenic therapy. One mechanism that has received considerable attention is redundancy in pro-angiogenic growth factor signalling (2, 12-14). Human cancers, including RCC, express numerous additional pro-angiogenic factors, including fibroblast growth factor 2 (FGF2), hepatocyte growth factor (HGF) and interleukin-8 (IL-8), which may stimulate the growth and survival of tumor blood vessels even when the VEGF-pathway is blocked (15-23). It may therefore be necessary to develop therapies that block the activity of multiple pro-angiogenic factors in these tumors.

Most pro-angiogenic factors activate the Ras-Raf-MEK-ERK1/2 pathway in endothelial cells, a signalling pathway that promotes angiogenesis by triggering the proliferation, survival and migration of endothelial cells (24-27). In recent years there has been great interest in developing clinically effective small molecule inhibitors of MEK, the kinase which sits upstream of ERK1/2, as a means to inhibit the Ras-Raf-MEK-ERK1/2 pathway in cancer (28). 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 and overall survival observed in the combination arm compared to B-Raf inhibition alone (29-31). These data provide compelling evidence that the MEK inhibitor trametinib can be safely and effectively combined with other kinase inhibitors 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 anti-angiogenic TKIs in renal cell carcinoma.

Materials and Methods

Reagents

Cell culture reagents: RPMI-1640, DMEM and M199 medium, fetal calf serum, collagenase-1 (Invitrogen), EBM-2 media, EGM-2 Bulletkit (Lonza Biologics), bovine brain endothelial mitogen (Serotech). Primary antibodies for western blotting: phospho-Thr-202/Tyr-204-ERK1/2 (M8159, Sigma-Aldrich, St Louis, MO, USA), total ERK1/2 (#9102, Cell Signalling Technology), HSC-70 (sc-7298, Santa Cruz Biotechnology). Primary antibodies for immunohistochemistry: endomucin (sc-65495, Santa Cruz Biotechnology), CD31 (550274, BD Biosciences), Ki67 (Ab15580, Abcam), phospho-Thr-202/Tyr-204-ERK1/2 (20G11, Cell Signaling Technology). Secondary antibodies: HRP- and biotin-conjugated (DAKO), fluorescently-conjugated (Life Technologies). Growth factors and inhibitors: VEGF-A (R&D Systems), FGF2 (Peprotech), sunitinib, sorafenib, pazopanib (LC Laboratories), trametinib, selumetinib (Selleck Chemicals), PD184352 (Sigma-Aldrich). Unless otherwise stated, all other reagents were obtained from Sigma-Aldrich.

Cell culture

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

Xenograft models and treatment

Female CB17 SCID mice (CB17/lcr-*Prkdc*^{scid}/lcrIcoCrl) were obtained from Charles River UK. For 786-0 xenografts, mice were shaved on the flank and then injected subcutaneously with 3×10^6 786-0 cells. Once tumors reached 100 – 200 mm³ 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 x width²) x 0.5.

In order to generate the 786-0-R and 786-0-S sub-lines, 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 mM MgCl₂ and 1 mM CaCl₂) at 37°C for 90 mins. 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 3×10^6 cells. Once tumors reached 100 – 200 mm³ in size, the mice were randomized to the treatment groups.

The patient-derived xenograft (PDX) model was established from the tumour of a 71 year old male who underwent nephrectomy for clear cell RCC at the Royal Marsden hospital. Tumour fragments of ~2 mm³ in size were implanted subcutaneously into CB17 SCID mice through a small incision under anaesthesia. Mice were observed every week for the presence of palpable tumors. Subsequent 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 mM 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, 32, 33). 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 – 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.

Immunohistochemistry

Immunohistochemistry was performed essentially as described (32). In brief, tumors were harvested from mice and bisected. Half the tumor was fixed in 4% w/v formalin, whilst 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 heamatoxylin prior to 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.

Co-staining for CD31 and Ki67 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 Ki67 or pERK was calculated by manually counting the number of cell nuclei which stained positive per field and then dividing this by the total number of cells per field (5 fields per tumor sample were used).

Western blotting

Western blotting was performed essentially as described (27), see Supplementary Methods.

***In vitro* angiogenesis assays**

Endothelial tubule formation assays were performed essentially as described (16), see Supplementary Methods.

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's *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 100-200 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 to the start of treatment (Figure 1A). 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 (18). We took advantage of this heterogeneity and allocated tumors into three categories based on their response to treatment: 'sensitive,' 'early resistance' or 'late resistance' (Figure 1B). 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 to the start of treatment) were allocated to the sensitive category (Figure 1B, left). Tumors that underwent ≥ 5 -fold increase in volume after 42 days (compared to the start of treatment) were allocated to the early resistance category (Figure 1B, middle). Finally, tumors that underwent ≤ 2.5 -fold increase in volume by 50 days (compared to the start of treatment) with progression to ≥ 5 -fold increase in volume during the following 50-100 days (compared to the start of treatment) were allocated to the late resistance category (Figure 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.

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

Since sunitinib inhibits tumor growth in RCC principally by blocking tumor angiogenesis (34), 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 anti-angiogenic activity of sunitinib, tumor vessel density was significantly decreased in all sunitinib-treated categories compared to vehicle-treated tumors (Figure 1C). However, vessel density was significantly increased in both the early resistance category and late resistance category compared to the sensitive category (Figure 1C).

Proliferation of tumor endothelial cells can also be used as a marker of tumor angiogenesis. Therefore, we quantified the proportion of Ki67-positive endothelial cells present in tumor vessels. No significant difference in the proportion of Ki67-positive endothelial cells was observed between the vehicle group and the sensitive category (Figure 1D). However, the proportion of Ki67-positive endothelial cells was significantly increased in both the early resistance category and late resistance category when compared to the sensitive category (Figure 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 to the sensitive category (Figure 1E).

The MEK inhibitor trametinib has potent anti-angiogenic activity *in vitro*

We next examined the ability of small molecule MEK inhibitors (MEKIs) to inhibit ERK1/2 activation in endothelial cells. Three MEKIs were tested: trametinib (33), selumetinib (35) and PD184352 (36). Human umbilical vein endothelial cells (HUVECs) were stimulated with a combination of VEGF and FGF2 in the presence of MEKI or vehicle alone, followed by blotting for pERK1/2 (Figure 2A-F). Blots were

performed in triplicate and densitometry measurements used to determine IC₅₀s for the inhibition of ERK1/2 activation (Table 1). All three MEKIs suppressed ERK1/2 activation in stimulated endothelial cells, with IC₅₀s in the nanomolar range (Figure 2 and Table 1). In parallel, we tested three anti-angiogenic TKIs: sunitinib, pazopanib and sorafenib. The MEKIs were more effective at inhibiting ERK1/2 activation than the TKIs (Figure 2 and Table 1). Moreover, the most effective inhibitor of ERK1/2 activation in endothelial cells was trametinib (IC₅₀ = 1.3 nM).

We then examined the anti-angiogenic activity of all six drugs (three MEKIs and three TKIs) using a previously described *in vitro* angiogenesis assay (16, 37). 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 Figure 2A). To measure inhibition of new tubule formation, assays were performed in the presence of drug from the first day of the assay onwards (Supplementary Figure 2B). Within the range of concentrations tested (10 pM to 10 μM), all drugs exhibited anti-angiogenic activity, as measured by inhibition of tubule formation (Figure 3A and Table 1). The MEKI trametinib showed the most potent anti-angiogenic activity (Figure 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 pre-formed 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 Figure 2C). Only trametinib induced tubule regression at all concentrations tested (1, 10 and 100 nM) (Figure 3B). For all other drugs, a dose of at least 100 nM was required to induce tubule regression (Figure 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 sub-line (786-0-R) that we isolated from the parental 786-0 cell line (Supplementary Figure 2). Once 786-0-R tumors reached 100-200 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 were observed in any treatment group, including 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 (Figure 4A). However, the combination of sunitinib and trametinib was more effective than administering either drug alone (Figure 4A).

To evaluate effects on tumor angiogenesis, vessel density was quantified in treated tumors (Figure 4B,C). Trametinib alone did not induce a significant reduction in tumor vessel density compared to vehicle. However, both single agent sunitinib and the sunitinib plus trametinib combination led to a significant reduction in vessel density compared to vehicle (Figure 4B). Importantly, the strongest reduction in vessel density was observed with the combination therapy (Figure 4B).

To address why these regimens differ in their potential to suppress tumor angiogenesis *in vivo*, we evaluated ERK1/2 phosphorylation in 786-0-R tumors that were harvested after 2 weeks of treatment. Phosphorylated ERK1/2 was quantified in tumor blood vessels and cancer cells separately. A modest, yet significant reduction in ERK1/2 phosphorylation was observed in blood vessels from tumors treated with either trametinib alone or sunitinib alone (Fig 4D), but 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 inhibition of ERK1/2 phosphorylation in cancer cells was observed in any treatment group (Fig 4E).

To provide further evidence that the combination of sunitinib with trametinib can give rise to enhanced anti-angiogenic activity, we examined their activity again in the previously described *in vitro* angiogenesis assay. Importantly, we found that the combination of sunitinib with trametinib was more effective than either drug alone at inhibiting angiogenesis *in vitro* when the drugs were tested in this tubule formation assay at sub-threshold concentrations of drug (Supplementary Figure 4).

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

Since the anti-tumor activity of single agent trametinib was no more effective than single agent sunitinib within our *in vivo* model, in our next 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 – 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 (Figure 5A) and in suppressing tumor vessel density (Supplementary Figure 4A).

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 to the start of treatment (Figure 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, whilst tumors in the group that continued on sunitinib progressed, tumors that switched to the combination regressed (Figure 5B)

and had a significantly lower vessel density than tumors that remained on sunitinib (Supplementary Figure 4B).

We also tested whether trametinib would be effective when combined with pazopanib. Mice bearing established 786-0-R tumors were randomized to receive trametinib alone, pazopanib alone or a combination of pazopanib and trametinib. The combination of pazopanib and trametinib was more effective than administering either agent alone (Figure 5C and Supplementary Figure 4C).

Efficacy of trametinib combined with sunitinib 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 (Figure 6A). In this first generation of the PDX, the only graft which took was from region 4 (Figure 6A). This tumor was passaged into 5 further mice to establish the second generation of the PDX (Figure 6A). Established tumors from these 5 mice were then grafted into a further 20 mice to establish the third generation of the PDX (Figure 6A). The clear cell histology of the primary cancer was retained at each generation of the PDX (Figure 6B-E) and STR typing confirmed good concordance between the genotype of the original patient tumour and the genotype of the PDX (Supplementary Table 1). 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 (38). 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 to vehicle (Figure 6F). However, treatment with sunitinib alone suppressed tumor growth compared to vehicle (Figure 6F) and the combination of sunitinib with trametinib was more effective at suppressing tumor growth than administering sunitinib alone (Figure 6F).

Discussion

Treatment outcomes in mRCC patients might be improved by rationally combining TKIs with other agents (2, 12, 39). 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 anti-angiogenic 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 to 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 re-vascularization after an initial period of response (7). Importantly, we also found elevated levels of phosphorylated ERK1/2 in the vessels of these early and late resistant tumors compared to 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 sunitinib and the MEK inhibitor trametinib is significantly more effective at inhibiting tumor growth and tumour angiogenesis than utilizing either drug as a single agent.

In order to address why the MEKI plus TKI combination was more effective at limiting tumor growth and tumor angiogenesis *in vivo*, we quantified phosphorylated ERK1/2 in both tumor blood vessels and cancer cells in tumors harvested from treated mice. We found that the combination resulted in more effective inhibition of ERK1/2 phosphorylation in tumor endothelial cells than either drug alone. However, ERK1/2 phosphorylation in cancer cells was not significantly affected by the drug treatments. From these data we conclude that the superior anti-angiogenic activity of the combination *in vivo* is most likely due to the greater inhibition of the Ras-Raf-MEK-ERK1/2 pathway in tumor endothelial cells when the drugs are combined. This superior anti-angiogenic effect then translates into a superior inhibition of tumor growth.

However, we also acknowledge that the superior anti-tumor effect of the combination could arise through other mechanisms. For example, MEK-dependent recruitment of pro-angiogenic neutrophils to tumours has been shown to mediate resistance to VEGF-inhibitor in preclinical models and can be overcome by inhibiting the MEK pathway *in vivo* (40). 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 may directly promote cancer cell survival during resistance to TKI treatment (41, 42). Taken together, these data suggest that co-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 pro-angiogenic neutrophil recruitment (40) and suppression of pro-survival signalling in cancer cells (41, 42).

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 (39) because combining a TKI with a MEKI in patients could lead to unacceptable 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 and 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 (43). Although no patients with mRCC were included in that study, the results indicate 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.

Acknowledgments

We thank the staff of the ICR biological services unit and the Breakthrough Breast Cancer histopathology facility for technical assistance. We acknowledge funding from Breakthrough Breast Cancer (recently merged with Breast Cancer Campaign forming Breast Cancer Now) and NHS funding to the NIHR Biomedical Research Centre at RM / ICR.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin*. 2015;65:5-29.
2. Vasudev NS, Reynolds AR. Anti-angiogenic therapy for cancer: current progress, unresolved questions and future directions. *Angiogenesis*. 2014;17:471-94.
3. Rini BI, Campbell SC, Escudier B. Renal cell carcinoma. *Lancet*. 2009;373:1119-32.
4. Fisher R, Gore M, Larkin J. Current and future systemic treatments for renal cell carcinoma. *Semin Cancer Biol*. 2013;23:38-45.
5. Kumar R, Knick VB, Rudolph SK, Johnson JH, Crosby RM, Crouthamel MC, et al. Pharmacokinetic-pharmacodynamic correlation from mouse to human with pazopanib, a multikinase angiogenesis inhibitor with potent antitumor and antiangiogenic activity. *Mol Cancer Ther*. 2007;6:2012-21.
6. Mendel DB, Laird AD, Xin X, Louie SG, Christensen JG, Li G, et al. In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship. *Clin Cancer Res*. 2003;9:327-37.
7. Vasudev NS, Goh V, Juttla JK, Thompson VL, Larkin JM, Gore M, et al. Changes in tumour vessel density upon treatment with anti-angiogenic agents: relationship with response and resistance to therapy. *Br J Cancer*. 2013;109:1230-42.
8. Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Rixe O, et al. Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. *The New England journal of medicine*. 2007;356:115-24.
9. Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Oudard S, et al. Overall survival and updated results for sunitinib compared with interferon alfa in patients with metastatic renal cell carcinoma. *J Clin Oncol*. 2009;27:3584-90.
10. Motzer RJ, Hutson TE, Cella D, Reeves J, Hawkins R, Guo J, et al. Pazopanib versus sunitinib in metastatic renal-cell carcinoma. *The New England journal of medicine*. 2013;369:722-31.
11. Sternberg CN, Davis ID, Mardiak J, Szczylik C, Lee E, Wagstaff J, et al. Pazopanib in locally advanced or metastatic renal cell carcinoma: results of a randomized phase III trial. *J Clin Oncol*. 2010;28:1061-8.
12. Rini BI, Atkins MB. Resistance to targeted therapy in renal-cell carcinoma. *Lancet Oncol*. 2009;10:992-1000.
13. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. *Nature reviews*. 2008;8:592-603.

14. Ebos JM, Lee CR, Kerbel RS. Tumor and host-mediated pathways of resistance and disease progression in response to antiangiogenic therapy. *Clin Cancer Res.* 2009;15:5020-5.
15. Shojaei F, Lee JH, Simmons BH, Wong A, Esparza CO, Plumlee PA, et al. HGF/c-Met acts as an alternative angiogenic pathway in sunitinib-resistant tumors. *Cancer research.* 2010;70:10090-100.
16. Welti JC, Gourlaouen M, Powles T, Kudahetti SC, Wilson P, Berney DM, et al. Fibroblast growth factor 2 regulates endothelial cell sensitivity to sunitinib. *Oncogene.* 2011;30:1183-93.
17. Casanovas O, Hicklin DJ, Bergers G, Hanahan D. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell.* 2005;8:299-309.
18. Huang D, Ding Y, Zhou M, Rini BI, Petillo D, Qian CN, et al. Interleukin-8 mediates resistance to antiangiogenic agent sunitinib in renal cell carcinoma. *Cancer research.* 2010;70:1063-71.
19. Horie S, Aruga S, Kawamata H, Okui N, Kakizoe T, Kitamura T. Biological role of HGF/MET pathway in renal cell carcinoma. *J Urol.* 1999;161:990-7.
20. Eguchi J, Nomata K, Kanda S, Igawa T, Taide M, Koga S, et al. Gene expression and immunohistochemical localization of basic fibroblast growth factor in renal cell carcinoma. *Biochem Biophys Res Commun.* 1992;183:937-44.
21. Nanus DM, Schmitz-Drager BJ, Motzer RJ, Lee AC, Vlamis V, Cordon-Cardo C, et al. Expression of basic fibroblast growth factor in primary human renal tumors: correlation with poor survival. *J Natl Cancer Inst.* 1993;85:1597-9.
22. Relf M, LeJeune S, Scott PA, Fox S, Smith K, Leek R, et al. Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. *Cancer research.* 1997;57:963-9.
23. Ciamporcero E, Miles KM, Adelaiye R, Ramakrishnan S, Shen L, Ku S, et al. Combination Strategy Targeting VEGF and HGF/c-met in Human Renal Cell Carcinoma Models. *Mol Cancer Ther.* 2015;14:101-10.
24. Alavi A, Hood JD, Frausto R, Stupack DG, Cheresh DA. Role of Raf in vascular protection from distinct apoptotic stimuli. *Science.* 2003;301:94-6.
25. Eliceiri BP, Klemke R, Stromblad S, Cheresh DA. Integrin alphavbeta3 requirement for sustained mitogen-activated protein kinase activity during angiogenesis. *The Journal of cell biology.* 1998;140:1255-63.

26. Mavria G, Vercoulen Y, Yeo M, Paterson H, Karasarides M, Marais R, et al. ERK-MAPK signaling opposes Rho-kinase to promote endothelial cell survival and sprouting during angiogenesis. *Cancer Cell*. 2006;9:33-44.
27. Gourlaouen M, Welti JC, Vasudev NS, Reynolds AR. Essential role for endocytosis in the growth factor-stimulated activation of ERK1/2 in endothelial cells. *J Biol Chem*. 2013;288:7467-80.
28. Samatar AA, Poulidakos PI. Targeting RAS-ERK signalling in cancer: promises and challenges. *Nat Rev Drug Discov*. 2014;13:928-42.
29. Long GV, Stroyakovskiy D, Gogas H, Levchenko E, de Braud F, Larkin J, et al. Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma. *The New England journal of medicine*. 2014;371:1877-88.
30. Long GV, Stroyakovskiy D, Gogas H, Levchenko E, de Braud F, Larkin J, et al. Dabrafenib and trametinib versus dabrafenib and placebo for Val600 BRAF-mutant melanoma: a multicentre, double-blind, phase 3 randomised controlled trial. *Lancet*. 2015.
31. Robert C, Karaszewska B, Schachter J, Rutkowski P, Mackiewicz A, Stroiakovski D, et al. Improved overall survival in melanoma with combined dabrafenib and trametinib. *The New England journal of medicine*. 2015;372:30-9.
32. Welti JC, Powles T, Foo S, Gourlaouen M, Preece N, Foster J, et al. Contrasting effects of sunitinib within in vivo models of metastasis. *Angiogenesis*. 2012;15:623-41.
33. Gilmartin AG, Bleam MR, Groy A, Moss KG, Minthorn EA, Kulkarni SG, et al. GSK1120212 (JTP-74057) is an inhibitor of MEK activity and activation with favorable pharmacokinetic properties for sustained in vivo pathway inhibition. *Clin Cancer Res*. 2011;17:989-1000.
34. Huang D, Ding Y, Li Y, Luo WM, Zhang ZF, Snider J, et al. Sunitinib acts primarily on tumor endothelium rather than tumor cells to inhibit the growth of renal cell carcinoma. *Cancer research*. 2010;70:1053-62.
35. Yeh TC, Marsh V, Bernat BA, Ballard J, Colwell H, Evans RJ, et al. Biological characterization of ARRY-142886 (AZD6244), a potent, highly selective mitogen-activated protein kinase kinase 1/2 inhibitor. *Clin Cancer Res*. 2007;13:1576-83.
36. Sebolt-Leopold JS, Dudley DT, Herrera R, Van Becelaere K, Wiland A, Gowan RC, et al. Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. *Nature medicine*. 1999;5:810-6.
37. Nakatsu MN, Hughes CC. An optimized three-dimensional in vitro model for the analysis of angiogenesis. *Methods in enzymology*. 2008;443:65-82.
38. <http://cancer.sanger.ac.uk/cosmic>.

39. Moreno Garcia V, Basu B, Molife LR, Kaye SB. Combining antiangiogenics to overcome resistance: rationale and clinical experience. *Clin Cancer Res.* 2012;18:3750-61.
40. Phan VT, Wu X, Cheng JH, Sheng RX, Chung AS, Zhuang G, et al. Oncogenic RAS pathway activation promotes resistance to anti-VEGF therapy through G-CSF-induced neutrophil recruitment. *Proc Natl Acad Sci U S A.* 2013;110:6079-84.
41. Sakai I, Miyake H, Fujisawa M. Acquired resistance to sunitinib in human renal cell carcinoma cells is mediated by constitutive activation of signal transduction pathways associated with tumour cell proliferation. *BJU international.* 2013;112:E211-20.
42. Harada K, Miyake H, Kusuda Y, Fujisawa M. Characterization of mechanism involved in acquired resistance to sorafenib in a mouse renal cell cancer RenCa model. *Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico.* 2014;16:801-6.
43. Azad N, Ball D, Sherman S, Rudek M, Falchook G, Nelkin B, et al. Abstract B279: A phase I study determining the safety and tolerability of combination therapy with Pazopanib (P), a VEGFR/PDGFR/Raf inhibitor, and GSK1120212 (Trametinib: T), a MEK inhibitor, in advanced solid tumors with expansion cohorts in advanced differentiated thyroid cancer (DTC), cholangiocarcinoma (ChCA), and soft tissue sarcoma (STS). *Molecular Cancer Therapeutics.* 2013;12:B279.

Table 1 Activity of MEKIs and TKIs for inhibition of ERK1/2 activation in endothelial cells and inhibition of endothelial cell tubule formation

Drug	Inhibition of ERK activation determined from western blots (IC₅₀ ± SEM)	Inhibition of endothelial cell tubule formation (IC₅₀ ± SEM)
Trametinib	1.33 ± 0.05 nM	0.09 ± 0.02 nM
PD184352	20.35 ± 13.21 nM	6.34 ± 3.73 nM
Selumetinib	6.60 ± 2.88 nM	7.33 ± 0.76 nM
Sunitinib	3174.33 ± 1564.04 nM	39.60 ± 8.35 nM
Pazopanib	640.75 ± 329.27 nM	2.75 ± 0.25 nM
Sorafenib	not determined	199.80 ± 11.40 nM

Figure legends

Figure 1 Heterogeneous response to sunitinib in a preclinical model of renal cell carcinoma

A,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 tumours included in the study are shown in Supplementary Figure 1.

C. Tumor vessel density in the control, sensitive, early resistance and late resistance categories. Graph shows the density of endomucin-positive tumor vessels per $\text{mm}^2 \pm \text{SEM}$ ($n = 6$ tumors from each category). Example staining of endomucin is shown.

D. Tumor vessel proliferation in the control, sensitive, early resistant and late resistant categories. Graph shows the percentage of endothelial cells with Ki67-positive nuclei per field $\pm \text{SEM}$ (15 high power fields were analyzed from across 3 tumors in each category). Example staining of a Ki67-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 $\pm \text{SEM}$ (15 high power fields were analyzed from across 3 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,E**).

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. Cell lysates were then probed for phosphorylated ERK1/2 or total ERK.

Figure 3 Anti-angiogenic activity of MEKIs and TKIs within an *in vitro* angiogenesis assay

A. Inhibition of new tubule formation by drugs. HUVEC-coated beads were embedded in a fibrinogen gel and stimulated with VEGF and FGF2 for 7 days to induce tubule formation. Assays were performed to assess the activity of six drugs (trametinib, selumetinib, PD184352, sunitinib, pazopanib or sorafenib) or vehicle alone. Graph shows tubule formation at the indicated drug concentration relative to the vehicle control \pm SEM (n = 3 independent experiments).

B. Retraction of pre-existing tubules induced by drugs. Tubules were allowed to form for 7 days in the absence of drug and were then treated with vehicle or drug for 48 hours. Change in tubule length that occurred during the drug incubation period was quantified. Graph shows the percentage change in tubule length at the indicated drug concentration \pm SEM (n = 3 independent experiments).

Figure 4 Combination of trametinib with sunitinib is more effective than either drug alone *in vivo*

A-C. Mice with established subcutaneous 786-0-R tumors were treated with vehicle alone, trametinib, sunitinib or a combination of sunitinib and trametinib. Graph in **A** shows tumor volume measured at the indicated timepoints \pm SEM. Graph in **B** shows tumor vessel density at endpoint (number of endomucin-positive vessels per $\text{mm}^2 \pm$ SEM is shown). Representative fields of endomucin staining from the indicated experimental groups are shown in **C**. $n = 7$ mice per experimental group.

D,E. Mice with established subcutaneous 786-0-R tumors were treated for 2 weeks with vehicle alone, trametinib, sunitinib or a combination of sunitinib and trametinib. Graph in **D** shows phosphorylated ERK1/2 (pERK1/2) quantified in tumor vessels \pm SEM. Graph in **E** shows phosphorylated pERK1/2 quantified in cancer cells \pm SEM (20 high power fields were analyzed from across 4 tumors in each experimental group).

nsd = no significant difference, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

Figure 5 Efficacy of trametinib in a discontinuous schedule or when added after progression or when combined with pazopanib

A. Efficacy of trametinib in a discontinuous schedule. Mice with established subcutaneous 786-0-R tumors were treated with sunitinib on a two week on / one week off schedule (n = 10 mice) or a combination of sunitinib plus trametinib on a two week on / one week off schedule (n = 10 mice) or they were treated with vehicle alone (n = 5 mice). Graph shows tumor volume measured at the indicated timepoints \pm SEM. Bars below the x-axis indicate periods when drugs were administered.

B. Efficacy of trametinib when added after progression. Mice with established subcutaneous 786-0-R tumors were treated with sunitinib for 28 days (n = 10 mice). On day 29, mice were randomized to either continue on sunitinib for 14 days (n = 5 mice) or switch to treatment with sunitinib plus trametinib for 14 days (n = 5 mice). For the purpose of controls, mice with established subcutaneous 786-0-R tumors were treated with vehicle alone (n = 5 mice) or sunitinib plus trametinib for the duration of the experiment (n = 10 mice). Note: the vehicle group for this experiment was shared with the experiment show in panel A. Main graph shows tumor volume measured at the indicated timepoints \pm SEM. The inset graph shows percentage change in tumor volume between day 28 and day 42 for the mice that remained on sunitinib versus the mice that switched to sunitinib plus trametinib.

C. Efficacy of trametinib when combined with pazopanib. Mice with established subcutaneous 786-0-R tumors were treated with vehicle alone, trametinib, pazopanib or a combination of pazopanib plus trametinib. Graph shows tumor volume measured at the indicated timepoints \pm SEM. n = 10 mice per group.

** $P < 0.01$, *** $P < 0.0001$.

Figure 6 Efficacy of the combination regimen in a PDX model of RCC

A. Tissue from four different regions (R1 to R4) of the primary tumor were grafted subcutaneously into recipient mice (1st generation). A tumor formed in the mouse grafted with region 4 was then passaged into a further five recipient mice (2nd generation). Tumors formed in these mice were then passaged into a further twenty recipient mice (3rd generation).

B-E. Histology of the patient's primary renal cancer (**B**) and the 1st (**C**), 2nd (**D**) and 3rd (**E**) generation of the PDX.

F. Mice with established subcutaneous tumors from the 3rd generation of the PDX were treated with vehicle alone, trametinib, sunitinib or a combination of sunitinib plus trametinib. Graph shows tumor volume measured at the indicated timepoints \pm SEM.

Scale bar, 30 μ M. * $P < 0.05$.