Title:

Combination of everolimus with sorafenib for solid renal tumours in Tsc2+/− mice is superior to everolimus alone

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Tuberous sclerosis is an inherited tumour syndrome caused by mutations in \textit{TSC1} or \textit{TSC2} that lead to aberrant activation of mTOR and development of tumours in multiple organs including the kidneys. The mTOR inhibitors rapamycin and everolimus (rapalogs) have demonstrated clinical efficacy in treating TSC-associated tumours including renal angiomyolipomas. However, tumour responses are usually only partial and regrowth occurs after drug withdrawal. TSC-associated tumours are highly vascular and TSC patients with renal angiomyolipomas have elevated levels of circulating vascular endothelial growth factor A (VEGFA) and VEGFD. Sorafenib inhibits multiple kinases including VEGF receptors and has been used to treat metastatic epithelioid angiomyolipoma in one case but formal trials have not been undertaken. In this study, we investigated tumour angiogenesis and the therapeutic efficacy of everolimus in combination with sorafenib for renal tumours in \textit{Tsc2}\textsuperscript{+/−} mice. We found that these tumours exhibited remarkably variable angiogenesis despite consistent aberrant activation of mTOR and increased expression of HIF\textsubscript{1α} and VEGFA. Treatment of 11 month old \textit{Tsc2}\textsuperscript{+/−} mice for two months with a combination of everolimus and sorafenib significantly reduced the number and size of solid renal tumours, whereas everolimus or sorafenib alone did not. These results suggest that inhibition of mTOR and multiple kinases including VEGF receptors using combination therapy could hold promise for the treatment of TSC-associated tumours that have responded inadequately to a rapalog alone.

\textbf{Key words:} Tuberous sclerosis, renal tumours, mTOR, everolimus, sorafenib
INTRODUCTION

Tuberous sclerosis (TSC) is a tumour syndrome caused by mutations in \textit{TSC1} or \textit{TSC2} that lead to aberrant activation of mTOR and development of tumours in multiple organs. Over 80\% of TSC patients develop renal manifestations, usually multiple and bilateral angiomyolipomas (AML) that are the leading cause of adult deaths from the disease. Single and multiple renal cysts are also frequently observed and renal cell carcinoma (RCC) is found in around 2\% of TSC patients (1). Treatment with the mTOR inhibitor sirolimus (rapamycin) or its derivative everolimus significantly reduces the size of renal AML in TSC patients (2-4). Everolimus has also demonstrated clinical efficacy in TSC-associated renal carcinoma (5). However, AML and other TSC-associated tumour responses to mTOR inhibitors are partial and tumours that initially respond to treatment usually regrow after drug withdrawal. TSC-associated tumours are highly vascular (6) and TSC patients with renal AMLs have elevated levels of circulating vascular endothelial growth factor A (VEGFA) and VEGFD (7). The angiogenesis inhibitors sunitinib and sorafenib have been used to treat TSC-associated-RCC and epithelioid AML in a limited number of cases (5, 8, 9). Combination therapy using these multiple kinase inhibitors together with rapalogs may improve therapeutic efficacy for TSC-associated tumours.

Mouse models heterozygous for \textit{Tsc1} or \textit{Tsc2} have been described previously and develop lesions in multiple organs (10, 11). Renal lesions are prominent and include cysts, papillary adenomas, solid adenomas and carcinomas. These lesions are associated with somatic loss of function mutations of the corresponding second \textit{Tsc1} or \textit{Tsc2} allele and aberrant activation of the mTOR signalling pathway (12). Expression of HIF1 and VEGFA is increased in \textit{Tsc2}\textsuperscript{+\textendash} mouse embryo fibroblasts (MEF) but rapamycin only partially down-regulates VEGFA. Serum levels of VEGFA are increased and appear to be associated with the extent of tumour development in \textit{Tsc2}\textsuperscript{+\textendash} mice (13).
In this study, we investigated angiogenesis in renal lesions and the anti-tumour efficacy of everolimus in combination with sorafenib in the kidneys of \( \text{Tsc2}^{+/-} \) mice. We show that combination of everolimus with sorafenib is superior to everolimus alone for treating solid renal tumours in \( \text{Tsc2}^{+/-} \) mice.

MATERIALS AND METHODS

Animal procedures

Animal procedures were performed in accordance with the UK Home Office guidelines and approved by the Ethical Review Group of Cardiff University. \( \text{Tsc2}^{+/-} \) balb/c mice were described previously (11). To test whether combination of everolimus with sorafenib could improve anti-tumour efficacy, we first determined the combined maximum tolerated dose (MTD) in the \( \text{Tsc2}^{+/-} \) mice in a two weeks pilot treatment study. \( \text{Tsc2}^{+/-} \) litter mates were randomly allocated into 4 groups of 10, balanced for gender and of the same age. Animals were treated from the age of 11 months with vehicle, everolimus (10 mg/kg), sorafenib (42 mg/kg), or everolimus (6 mg/kg) plus sorafenib (30 mg/kg). All mice were treated 5 times a week via gavage for two months and then sacrificed for assessment of tumour burden and analysis of protein expression and phosphorylation in the kidneys. Vehicle and everolimus were supplied by Novartis Pharma AG, Basel Switzerland. Sorafenib was supplied by Bayer Schering Pharma AG, Leverkusen, Germany.

Histology

Assessment of tumour burden in the kidneys of mice was performed as described previously (14). Mouse kidneys were fixed in 10% buffered formalin saline (Thermo Scientific, Runcorn, UK) for 24 h. Fixed kidneys were processed and paraffin embedded according to standard procedures. A series of 5 μm coronal kidney sections were prepared at 200 μm intervals from each kidney. Kidney sections were HE-stained and scanned to create virtual haematoxylin and eosin slides using an Aperio system (http://www.aperio.com/?gclid =
Virtual slides were used for lesion quantification. Maximum cross-sectional whole area and cellular area of each renal lesion were measured, respectively, using ImageJ (http://rsbweb.nih.gov/ij). Cellular areas of renal lesions were obtained from parenchyma and stroma. To estimate average size of tumour cells, cysts, papillary and solid tumours on HE-stained kidney sections were randomly chosen using ImageJ. Average size of tumour cells was determined from parenchyma (not stroma) through total area of all tumour cells divided by the number of tumour cells examined. Analysis was conducted blindly with respect to treatment status.

**Immunohistochemistry (IHC)**

Primary antibodies against VEGFR2, PDGFRα, PDGFRβ, ABCB1, and phosphorylated S6 ribosomal protein at S235/236, Akt at S473 and Erk1/2 at T202/Y204 were supplied by Cell Signalling Technology (Danvers, MA, USA). Antibodies against VEGFA, VEGFR1, CD34, HIF1α and Ki67, phosphorylated RAF1 at S259 were supplied by Abcam (Cambridge, UK). Antibody against ABCC1 was supplied by St John’s laboratory Ltd (London, UK). Antibody against ABCG2 was supplied by Santa Cruz Biotechnology, Inc (Dallas USA). Antibody against RALBP1 was supplied by Proteintech Europe (Manchester, UK). SignalStain Boost Rabbit specific IHC Detection Reagent (Cell Signalling Technology, Danvers, USA) was used to stain antigens.

**Western blot**

In addition to primary antibodies described above, primary antibody against β-actin and horseradish peroxidise-conjugated secondary antibody against rabbit were purchased from Cell Signalling Technology (Danvers, USA) for Western blot. Extracts of tumour samples were prepared using AllPrep DNA/RNA/Protein Mini Kit (QIAGEN Ltd-UK, Crawley, UK). Proteins were purified according to the kit supplier’s instruction. Twenty μg of protein per sample was separated on NuPAGE 4–12% Bis-Tris Gels (Fisher Scientific UK Ltd,
Loughborough, UK) and transferred onto Hybond ECL Membranes (GE Healthcare UK Ltd, Little Chalfont, UK). Blots were analysed with ECL Select Western Detection Kit (GE Healthcare UK Ltd) and signals were detected using Autochemi Imaging System (UVP, Upland, CA, USA).

**Statistical analysis**

Wilcoxon rank-sum test was performed for comparisons of treatment efficacy on mouse renal lesions. Fisher’s exact test was used to assess massive cell death in mouse solid tumours. P<0.05 was considered to be statistically significant. Analyses were performed using STATA 13.

**RESULTS**

**Angiogenesis of renal lesions in Tsc2+/− mice is variable**

We investigated the expression of the drivers of tumour angiogenesis HIF1α and VEGFA in kidney sections by immunohistochemistry (IHC). Consistent with activation of mTORC1, these proteins were highly expressed in these lesions as reported previously (Figure 1) (15). RAF1 and Erk1/2 are positive regulators of tumour angiogenesis. As observed previously (12), RAF1 and Erk1/2 were highly phosphorylated in kidney lesions (Figure 2). We also investigated the expression of several receptor kinases that contribute to tumour angiogenesis using IHC. More VEGFR1 and PDGFRα but less VEGFR2 and PDGFRβ were detected in the lesions than in adjacent normal tissues (Figure 2). We used CD34 to assess angiogenesis and found that tumour angiogenesis in the kidneys of the Tsc2+/− mice is remarkably variable and showed no apparent association with lesion size or type (Figure 3).

**Combination of everolimus with sorafenib is more effective than everolimus alone for solid renal tumours in Tsc2+/− mice**
To test whether combination of everolimus with sorafenib could improve anti-tumour efficacy, Tsc2\(+/-\) mice were randomly allocated to 4 groups of 10 mice each: vehicle, everolimus, sorafenib, and everolimus plus sorafenib (Supplemental Table 1). We treated animals for two months from the age of 11 months. Three mice from the everolimus plus sorafenib group were euthanized due to significant loss of body weight within the first month of treatment and were excluded from further analysis in this study. We also noticed that 2 animals treated with everolimus plus sorafenib moved slowly and suffered from diarrhoea with sore eyes in the last two weeks of treatment. Drug doses were then reduced from 6 mg/kg to 3 mg/kg for everolimus and from 30 mg/kg to 15 mg/kg for sorafenib to treat these 2 mice until the end of the experiment. When all renal lesions were analysed, everolimus alone and everolimus plus sorafenib both significantly reduced lesion number, size and cellular area (P< or =0.002). The reductions were slightly greater for the combination of everolimus with sorafenib than everolimus alone but the difference was not statistically significant (Figure 4; Supplemental Table 2). Sorafenib alone appeared to reduce lesion number, size and cellular area but not significantly when all lesions were analysed (Figure 4; Supplemental Tables 2). Similar results were obtained when cystic/papillary lesions were analysed except that sorafenib alone slightly reduced lesion size (P=0.046; Figure 4, Supplemental Table 3). For solid lesions, however, everolimus in combination with sorafenib significantly reduced total number, size and cellular area (P< or =0.004), whereas everolimus or sorafenib alone did not (Figure 4; Supplemental table 4).

**Everolimus or sorafenib alone and in combination inhibit growth and proliferation of renal tumour cells from Tsc2\(+/-\) mice**

To assess effect of treatment on growth of tumour cells, average cell size was determined using HE stained kidney sections. We also used IHC-stained Ki67 to determine the effect of treatment on proliferation of tumour cells. To determine average cell size and cell
proliferation, 25 lesions were randomly chosen from each treatment group including 15 cysts and 10 papillary/solid lesions. At least 1000 cells from an area randomly chosen from each lesion or all cells from a lesion that had no more than 1000 cells were examined. We found that everolimus and everolimus plus sorafenib reduced the median cross sectional area of tumour cells from $184.15 \, \mu m^2$ to $77.84 \, \mu m^2$ and $66.55 \, \mu m^2$ respectively ($P=0.0001$) and also reduced median percentage of Ki67-positive tumour cells from $18.78\%$ to $0.33\%$ and $0.17\%$ respectively ($P=0.0001$) (Figures 5 and 6; Supplemental Table 5). Sorafenib alone also reduced the median cross sectional area of tumour cells ($P=0.0305$) and median percentage of Ki67-positive tumour cells ($P=0.0407$) but to a much smaller extent (Figures 5 and 6; Supplemental Table 5). These results were consistent with the decreased phosphorylation of RAF1 and Erk1/2 in tumours treated by everolimus, sorafenib or both but decreased phosphorylation of S6 ribosomal protein only in tumours treated with everolimus or everolimus plus sorafenib as detected by Western blot analysis (Figure 7). No significant increase or decrease of phosphorylation of Akt was observed in everolimus or sorafenib or everolimus plus sorafenib treated tumours (Figure 7).

**Massive cell death induced by sorafenib in large solid renal tumours is not correlated with RALBP1 expression in Tsc2+/− mice**

In some large solid tumours of the kidneys in $Tsc2^{+/−}$ mice treated by sorafenib, massive cell death was observed (Figure 8, Supplemental Figure 1). Everolimus or everolimus plus sorafenib also caused massive cell death but to a much smaller extent (Figure 8). The dying or dead cells exhibited features of anucleate ghost cells characteristic of necrosis (16). Greatest cell death was found for the sorafenib only group (Table 1) ($P=0.002$). However, the solid tumour count was low in the combination therapy group and it is unsurprising that although their results were comparable to those of the sorafenib only group, they did not reach statistical significance.
To explore the possible mechanisms of massive cell death caused by sorafenib, we examined by IHC the expression of some ABC transporters, and RALBP1 that was reported to be involved in sorafenib resistance in renal cancer (17). We found that expression of RALBP1 was variable while the ABC transporters were consistently suppressed in all untreated tumours examined (Supplemental Figure 2). We then investigated the protein level of RALBP1 in sorafenib treated tumours. We did not find a consistent reduction of RALBP1 in sorafenib treated tumours with massive cell death (Supplemental Figure 2).

DISCUSSION
We investigated angiogenesis in renal lesions of Tsc2+/− mice and tested the anti-tumour efficacy of everolimus in combination with sorafenib on these lesions. We found that tumour angiogenesis is very variable although HIF1 and VEGFA are consistently highly expressed in all lesions. Renal lesions, mainly, AMLs, in TSC patients are also very variable in vascularity. This variability may affect the response to therapy with angiogenesis inhibitors. We have shown that the combination therapy of everolimus with sorafenib represents an effective strategy to treat solid tumours whereas everolimus alone is effective on cystic/papillary lesions in Tsc2+/− mice. This suggests that advanced lesions may be more dependent on tumour angiogenesis than early ones. In clinical settings, rapalogs alone have been used to treat renal AML in TSC patients (2-4). Angiogenesis inhibitors and rapalogs have occasionally been used sequentially to treat TSC-associated-RCC and epithelioid AML (5, 9). No combined therapy has been reported in the treatment of TSC associated renal lesions in patients. This study suggests that rapalogs in combination with multiple kinase inhibitors may improve therapeutic efficacy for TSC-associated solid tumours. The challenge of combination therapy is the increased toxicity as observed in this study and in sporadic RCC patients treated with both rapalogs and angiogenesis inhibitors together (18). Lenvatinib
is a kinase inhibitor targeting multiple kinases including VEGFR1, VEGFR2 and VEGFR3, and was first approved by the U. S. Food and Drug Administration (USFDA) in 2015 for the treatment of locally recurrent or metastatic thyroid cancer (19). Recently, combination therapy of lenvatinib with everolimus has been reported to significantly prolong progression-free survival in RCC with manageable toxicity and approved for the treatment of advanced RCC by USFDA (20, 21). Lenvatinib plus everolimus may also be a promising combination therapy for TSC patients with renal tumours that do not respond adequately to rapalogs, and possibly for people with sporadic RCC containing TSC1 or TSC2 mutations.

We demonstrated that everolimus or everolimus plus sorafenib reduced tumour burden by dramatically shrinking tumour cell size and by preventing cell proliferation through inhibiting mTORC1 and the mitogen-activated protein kinase (MAPK) pathway. In contrast, sorafenib suppressed tumour cell growth and proliferation although to a smaller extent through inhibiting the MAPK pathway but not mTORC1. TSC-associated tumours are characterised by the presence of “giant” or grossly enlarged cells (22). Our observations on the huge effect of mTOR inhibition on tumour cell size and proliferation suggest that much of the tumour response to mTOR inhibitors observed in the clinical setting, and the rapid regrowth of tumours on drug withdrawal, may be attributable to changes in tumour cell size as well as through effects on cell proliferation. We did not consistently see reduction or elevation of Akt phosphorylation by either everolimus or sorafenib or both. In some xenograft models of malignancy, everolimus plus sorafenib treatment reduces phosphorylation of Akt (23, 24). This discrepancy may reflect the difference in tumour cell types and other factors such as tumour microenvironments.
We have found that sorafenib causes massive cell death with typical “ghost” cells in some large solid tumours (16). Everolimus or everolimus plus sorafenib also causes massive cell death but to a much smaller extent. Necrosis and apoptosis in tumours caused by sorafenib have been documented in preclinical studies (25) but the mechanisms of sorafenib-induced massive tumour cell death are not fully understood. To better understand mechanisms of massive cell death caused by sorafenib, we examined the expression of drug transporters including ABCB1, ABCC1, ABCG2 and RALBP1. Protein levels of these transporters were remarkably reduced in renal tumours although RALBP1 expression was variable. RALBP1 is suggested to be an efficient transporter of sorafenib and its expression levels are negatively correlated with drug resistance in patients with renal carcinoma (17). However, in the current study, there was no obvious inverse correlation between expression of RALBP1 and massive cell death. Different levels of angiogenesis in these renal lesions may contribute to differences in massive cell death associated with sorafenib treatment. AMLs with large aneurysmal vessels cause most problems in TSC patients. Inhibition of mTORC1 reduces size of TSC-associated AMLs and prevents haemorrhage but tumours regrow after drug withdrawal. Angiogenesis inhibitors or combination of rapalogs with angiogenesis inhibitors might prevent tumour regrowth by causing similar massive cell death in highly vascularised tumours and this approach warrants further investigations in both preclinical and clinical settings.

We conclude that everolimus in combination with sorafenib is superior to everolimus alone for treating solid tumours in the kidneys of $Tsc2^{+/−}$ mice and that new agents for combination therapy with less toxicity may significantly improve therapy for TSC-associated solid tumours.
CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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REFERENCES


Figure Legends

Figure 1 mTORC1 activation and angiogenesis signalling in renal lesions of Tsc2<sup>+/−</sup> mice

Kidney sections prepared from 13 months old Tsc2<sup>+/−</sup> mice were used for IHC analysis. Kidney sections were stained with antibodies against S6 p-S235/236, HIF1α and VEGFA. Representative sections were shown demonstrating increased phosphorylation of S6 at S235/236 and increased expression of HIF1α and VEGFA in renal lesions including cysts (C), papillary adenomas (P) and solid tumours (S). Scale bars are 100.1 μm.

Figure 2 MAPK signalling and expression of angiogenesis-contributing receptor kinases in renal lesions of Tsc2<sup>+/−</sup> mice

Kidney sections prepared from 13 months old Tsc2<sup>+/−</sup> mice were used for IHC analysis. Kidney sections were stained with antibodies against RAF1 p-S259 and Erk1/2 p-T202/Y204, VEGFR1, VEGFR2, PDGFRα and PDGFRβ. Representative sections were shown demonstrating increased phosphorylation of RAF1 at S259 and Erk1/2 at T202/Y204, increased expression of VEGFR1 and PDGFRα, and diminished expression of VEGFR2 and PDGFRβ in renal lesions. Scale bars are 100.1 μm.

Figure 3 Angiogenesis in renal lesions of Tsc2<sup>+/−</sup> mice

Kidney sections prepared from 13 months old Tsc2<sup>+/−</sup> mice were used for IHC analysis. Kidney sections were stained with antibody against CD34, an angiogenesis marker. Representative sections were shown demonstrating remarkable variation in angiogenesis in renal lesions. Scale bars are 100.1 μm.

Figure 4 Treatment efficacy on renal lesions of Tsc2<sup>+/−</sup> mice
Tsc2+/− mice were treated from 11 months old for 2 months (n=10 each group). Mice were sacrificed for tumour burden assessment at the age of 13 months. Three mice from the everolimus plus sorafenib group were euthanized due to significant loss of body weight within the first month of treatment and excluded from further analysis in this study. Dosages are described in methods. Kidney sections were prepared for histological assessment of treatment efficacy. Left panel: comparison of total number and size (area) as well as cellular area of all lesions (cystic, papillary and solid). Middle panel: comparison of total number and size (area) as well as cellular area of cystic/papillary lesions. Right panel: comparison of total number and size (area) as well as cellular area of solid lesions. Horizontal bars indicate a median. Anucleate ghost cells (dead cells) were not included for calculating cellular area of any lesions. For detailed statistical analysis see Supplemental Tables 2, 3 and 4.

**Figure 5 Effect of treatment on size of renal tumour cells in Tsc2+/− mice**

Kidney sections were prepared from 13 months old Tsc2+/− mice after treatment and HE stained for estimation of average size of tumour cells. (a) Tumour (T) and adjacent normal (N) cells. Representative sections were presented to show size of tumour cells after two months treatment with vehicle, everolimus, sorafenib and everolimus plus sorafenib. Scale bars are 100.1 µm. (b) Average size of tumour cells. Twenty five lesions randomly chosen from each group after two months treatment were used to estimate average size of tumour cells using ImageJ. Everolimus alone or everolimus plus sorafenib significantly reduced average size of tumour cells. Sorafenib alone significantly reduced average size of tumour cells but to a smaller extent.

**Figure 6 Effect of treatment on proliferation of renal tumour cells in Tsc2+/− mice**

Kidney sections were prepared from 13 months old Tsc2+/− mice after treatment and stained with antibody against Ki67 by IHC to assess proliferation of tumour cells. (a) Expression of
Ki67 in renal tumours. Representative sections were presented to show expression of Ki67 in tumour cells after two months treatment with vehicle, everolimus, sorafenib and everolimus plus sorafenib. Scale bars are 100.1 µm. (b) Proliferation of tumour cells. Twenty five lesions randomly chosen from each group after two months treatment were used to detect Ki67 positive tumour cells using ImageJ. Everolimus alone or everolimus plus sorafenib significantly inhibited proliferation of tumour cells. Sorafenib alone significantly inhibited proliferation of tumour cells but to a smaller extent.

**Figure 7 Effect of treatment on mTOR/MAPK signalling in renal lesions of Tsc2+/− mice**

Western blot was used to analyse mTOR/MAPK signalling in renal tumours. Proteins were prepared from solid renal tumours of Tsc2+/− mice treated for one month with vehicle, everolimus, sorafenib and everolimus plus sorafenib respectively. Beta-actin was used as a loading control. Representative Western blots were presented to show that sorafenib, everolimus alone or both attenuated phosphorylation of RAF1 and Erk1/2, and everolimus alone or everolimus plus sorafenib decreased phosphorylation of S6 but sorafenib alone did not in solid renal tumours.

**Figure 8 Massive cell death in solid renal tumours of Tsc2+/− mice**

Kidney sections were prepared from 13 months old Tsc2+/− mice after treatment and HE stained for examination of massive cell death in solid tumours. Left panel: representative solid tumours treated with vehicle, everolimus, sorafenib and everolimus plus sorafenib. Green arrows indicate massive cell death. Scale bars are 100.1 µm. Right panel: solid tumours with or without massive cell death. All solid tumours detected were presented. Relatively large solid tumours treated with sorafenib showed massive cell death.