Title:

Assessment of response of kidney tumours to rapamycin and atorvastatin in Tsc1+/− mice

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Abstract

Atorvastatin is widely used to lower blood cholesterol and to reduce risk of cardiovascular disease-associated complications. Epidemiological investigations and preclinical studies suggest that statins such as atorvastatin have anti-tumour activity for various types of cancer. Tuberous sclerosis (TSC) is a tumour syndrome caused by TSC1 or TSC2 mutations that lead to aberrant activation of mTOR and tumour formation in multiple organs. Previous studies have demonstrated that atorvastatin selectively suppressed growth and proliferation of mouse Tsc2 null embryonic fibroblasts through inhibition of mTOR. However, atorvastatin alone did not reduce tumour burden in the liver and kidneys of Tsc2+/− mice as assessed by histological analysis, and no combination therapy of rapamycin and atorvastatin has been tried. In this study, we used T2 weighted magnetic resonance imaging (MRI) to track changes in tumour number and size in the kidneys of a Tsc1+/− mouse model and to assess the efficacy of rapamycin and atorvastatin alone and as a combination therapy. We found that rapamycin alone or rapamycin combined with atorvastatin significantly reduced tumour burden while atorvastatin alone did not. Combined therapy with rapamycin and atorvastatin appeared to be more effective for treating renal tumours than rapamycin alone but the difference was not statistically significant. We conclude that combined therapy with rapamycin and atorvastatin is unlikely to provide additional benefit over rapamycin as a single agent in the treatment of Tsc-associated renal tumours.

Keywords

MRI, mTOR, renal tumour, tuberous sclerosis, rapamycin, atorvastatin

Abbreviations

TSC, tuberous sclerosis; mTOR, mechanistic target of rapamycin; mTORC1, mechanistic target of rapamycin complex 1; mTORC2, mechanistic target of rapamycin complex 2;
MAPK, mitogen-activated protein kinase; IHC, immunohistochemistry; HE, haematoxylin and eosin.

**Introduction**

Atorvastatin, a synthetic inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), also known as Lipitor, is widely used to lower blood cholesterol and to reduce risk of cardiovascular disease-associated complications [1]. Epidemiological investigations and preclinical studies suggest that statins such as atorvastatin have anti-tumour activity for various types of cancer including renal carcinoma [2-5]. Tuberous sclerosis (TSC) is a tumour syndrome caused by *TSC1* or *TSC2* mutations and characterised by tumour formation in multiple organs [6,7]. TSC2 is a GTPase activating protein towards Rheb and forms a functional complex with TSC1 that downregulates mTOR (mechanistic target of rapamycin) [8]. TSC-associated tumours exhibit aberrant activation of mTOR. Genetically engineered *Tsc1*+/− or *Tsc2*+/− mice spontaneously develop tumours in multiple organs including the liver and the kidneys [9,10]. Previous studies have demonstrated that atorvastatin selectively suppressed growth and proliferation of mouse *Tsc2* null embryonic fibroblasts through inhibition of mTOR [11]. Atorvastatin also inhibited mTOR in normal tissues of *Tsc2*+/− mice. However, atorvastatin alone did not reduce tumour burden in the liver and kidneys of these mice as assessed by histological analysis [12]. In this study, we used T2 weighted magnetic resonance imaging (MRI) to track changes of tumour number and size in the kidneys of a *Tsc1*+/− mouse model and assess the efficacy of rapamycin and atorvastatin combination therapy.

**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. $Tsc1^{+/−}$ balb/c mice were described previously [9]. $Tsc1^{+/−}$ litter mates were randomly allocated into 4 groups of 6, balanced for gender. Animals were treated from the age of 12 months with vehicle by i.p., rapamycin (5 mg/kg) by i.p., atorvastatin (100 mg/kg) by gavage, or rapamycin (5 mg/kg) plus atorvastatin (100 mg/kg) 5 times a week for two months and then sacrificed for assessment of tumour burden and analysis of protein expression and phosphorylation in tumours and normal tissues. Rapamycin was purchased from LC Laboratories, Woburn, MA, USA. Atorvastatin (Lipitor) was gift from University Hospital of Wales, Cardiff, UK.

**Magnetic resonance imaging (MRI)**

As described previously [13], MRI scans were carried out using a high-field (9.4 T) small bore (20 cm) Bruker Biospec 94/20 magnetic resonance spectroscopy (MRI/MRS) spectrometer. Mice were anaesthetised with 5% isoflurane in a 40% O2/Air mix at 1.2 L/min, injected with 2x 0.2 ml of 4% glucose/0.18% saline solution subcutaneously and transferred to specialist MRI bed model #T10532 (Bruker, Ettlingen, Germany) with integrated circulating heated water and an anaesthetic nose cone. Isoflurane was reduced to 1.5–2% for the maintenance of anaesthesia during scanning. Body temperature, breathing rate and heart rate were monitored throughout using the Model 1025 Monitoring and Gating System (SAI Inc., Stony Brook, New York). Body temperature was maintained during recovery in a warm air V1200 recovery chamber (Peco Services Ltd.) set at 27°C. T2 weighted, respiratory gated, fat suppressed RARE scans were performed with an FOV of 10.0 x 4.0 cm, a matrix of 640 x 256, and 64 x 0.5 mm coronal slices, a TEeff of 26 ms, a TR of 4100 ms, a RARE factor of 4, a BW of 100 kHz. The volumes of renal lesions were measured using the software Analyze 10.0 (Analyze Direct, Inc., Overland Park, USA). The measurement was conducted blindly in triplicate.

**Histology**

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 HE slides using an Aperio system (http://www.aperio.com/?gclid = CNXN-8by4a UCFcINfAods3egIw).

**Immunohistochemistry (IHC)**

Primary antibodies against 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 Ki67 and phosphorylated RAF1 at S259 were supplied by Abcam (Cambridge, 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. Primary antibody against p21 was supplied by Abcam (Cambridge, UK). Extracts of liver tissues 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 Amersham Protran Premium 0.45 μm nitrocellulose blotting 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).

**Quantitative real time PCR (q-PCR)**

Total RNA was isolated from mouse liver tissues using AllPrep DNA/RNA/Protein Mini Kit and TissueRuptor (QIAGEN Ltd-UK, Crawley, UK). One microgram RNA was used to synthesise cDNA in a 20 μl solution using qScript cDNA SuperMix (Quanta BioSciences, Inc., Gaithersburg, USA). A PCR reaction containing 1 μl cDNA, 2 μl 2.5 μM primer mix, 6.5 μl 2xPerfecta SYBR SuperMix (Quanta BioSciences, Inc., Gaithersburg, USA) and 3.5 μl
water was performed in a 7500 Real Time PCR System (**Life Technologies Ltd**, Paisley, UK). PCR was cycled as 95°C for 3 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative quantification of target transcripts was obtained after normalisation to Gapdh. Primer sequences of Cdkn1a for real time q-PCR were CCTGGTGATGTCCGACCTG and CCATGAGCGCATCGCAATC.

**Statistical analysis**

Wilcoxon rank-sum test was performed for comparisons of treatment efficacy on mouse renal lesions. Analyses were performed using GraphPad Prism 7.01. Two tailed Student’s t-Test was used for comparison of q-PCR results. P<0.05 was considered to be statistically significant.

**Results and Discussion**

**Efficacy of rapamycin, atorvastatin or combination of rapamycin and atorvastatin on renal tumours in Tsc1+/− mice**

Before starting treatment, Tsc1+/− litter mates were randomly allocated into 4 groups of 6, balanced for gender, and subjected to the 1st MRI scanning at the age of 12 months. Total number and volume of all renal lesions (cystic/papillary/solid) per mouse were determined using Analyze 10.0. As shown in the left panel of Figure 1, total number and volume of all renal lesions varied greatly from mouse to mouse but no significant difference was detected between any two groups before treatment (Supplemental Table 1). Tsc1+/− mice were then treated with vehicle (i.p.), atorvastatin (100 mg/kg, gavage), rapamycin (5 mg/kg, i.p.) or combination of atorvastatin and rapamycin 5 times a week for two months. After treatment, Tsc1+/− mice were subjected to the 2nd MRI scanning to track changes in tumour number and size. First, we directly compared the total number and size of all renal lesions obtained from
the 2\textsuperscript{nd} MRI scanning. As reported previously [12], atorvastatin alone did not reduce tumour burden in the kidneys of \( Tsc1^{+/−} \) mice (Figures 1 and 2; Supplemental Table 2). We found that combination of rapamycin and atorvastatin but not rapamycin alone reduced tumour number significantly although both rapamycin alone and rapamycin combined with atorvastatin significantly reduced tumour volume (Figures 1 and 2; Supplemental Table 2). Fold changes in tumour volume over time has been widely used to assess changes in tumour burden in preclinical xenograft models and clinical settings. We compared fold changes of total tumour number and volume (2\textsuperscript{nd}/1\textsuperscript{st} scan). We confirmed that atorvastatin alone did not have any therapeutic efficacy for renal tumours of \( Tsc1^{+/−} \) mice and that rapamycin alone or combination of rapamycin and atorvastatin significantly reduced tumour burden in the kidneys of these mice (Figures 1 and 2; Supplemental Table 3). Combination of rapamycin and atorvastatin appeared to have greater therapeutic efficacy for renal tumours but the difference was not significant (Figure 1; Supplemental Table 3).

A major advantage of using MRI instead of traditional histology to assess tumour burden in mice is the use of fewer animals to achieve statistical power. By analysing changes over time, MRI is particularly useful in genetically engineered mouse models such as \( Tsc1^{+/−} \) or \( Tsc2^{+/−} \) mice that show great variability in tumour number and size [9,10].

**Effect of rapamycin, atorvastatin or combination of rapamycin and atorvastatin on cell proliferation and oncogenic signalling in renal tumours of \( Tsc1^{+/−} \) mice**

Atorvastatin was reported to selectively suppress growth and proliferation of mouse \( Tsc2 \) null embryonic fibroblasts \textit{in vitro} [11]. In the current study, rapamycin alone or combination of rapamycin and atorvastatin inhibited proliferation of tumour cells as indicated by reduced
number of Ki67 positive tumour cells, but atorvastatin alone did not (Figure 3). Atorvastatin was previously shown to significantly inhibit mTOR complex 1 (mTORC1) in normal tissues [11]. We found that atorvastatin slightly inhibited mTORC1 in the liver whereas rapamycin alone or combination of rapamycin and atorvastatin greatly inhibited mTORC1 as indicated by marked reduction of S6 phosphorylation (Figure 4). In the liver, combination of rapamycin and atorvastatin appeared to reduce phosphorylation of Akt at S473, a marker of mTORC2 activation, but rapamycin or atorvastatin alone did not (Figure 4). As reported previously [12], atorvastatin alone did not inhibit mTORC1 in renal tumours (Figure 5). Rapamycin alone or combination of rapamycin and atorvastatin potently inhibited mTORC1 in renal tumours (Figure 5). In a previous study, atorvastatin was found to effectively delay tumour progression by inhibiting phosphorylation of Akt and Erk proteins in a mouse model of pancreatic cancer [4]. In the current study, we did not observe any effect of atorvastatin on phosphorylation of Akt and Erk1/2 in renal tumours by IHC analysis (Figures 5 and 6). In contrast, rapamycin alone or combination of rapamycin and atorvastatin inhibited phosphorylation of Akt at S473 and Erk1/2 in most solid renal lesions and some renal cysts (Figures 5 and 6) [14] although combination of rapamycin and atorvastatin appeared to have stronger inhibitory effect on phosphorylation at these sites. Previous studies suggest that atorvastatin upregulates expression of p21[4]. We investigated the expression of p21 by Western and q-PCR analysis to demonstrate the effective delivery of atorvastatin. We found that the levels of p21 protein and Cdkn1a transcripts were significantly increased in the liver treated with atorvastatin (Figure 4). These results suggest that the lack of efficacy of atorvastatin for renal tumours in Tsc mouse models is likely to be associated with insufficient inhibition of mTOR and Erk signalling in the tumours.
In conclusion, combined therapy of rapamycin and atorvastatin at doses used here is unlikely to provide additional benefit over rapamycin alone in the treatment of TSC-associated renal tumours. Further investigation is needed to test whether higher doses of atorvastatin in combination with rapamycin could bring any additional benefit without causing significant adverse effect for treating renal lesions in these models.

Conflict of Interest Statement
None declared.

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References


**Figure Legends**

**Figure 1 MRI assessment of treatment efficacy on renal tumours of Tsc1+/- mice**

*Tsc1+/-* mice were subjected to MRI scanning before and after treatment respectively *in vivo*. Total number and volume of all renal lesions (cystic/papillary/solid) per mouse were obtained using Analyze 10.0. **Left panel:** Comparison of total number and volume of renal tumours between treatment groups before starting treatment at the age of 12 months (the 1st MRI scan). **Middle panel:** Comparison of total number and volume of renal tumours between treatment groups after treatment at the age of 14 months (the 2nd MRI scan). *Tsc1+/-* mice
were treated with vehicle, atorvastatin (100 mg/kg), rapamycin (5 mg/kg) or combination of rapamycin (5 mg/kg) and atorvastatin (100 mg/kg) for two months before the 2nd MRI scanning. **Right panel:** Comparison of fold change of total renal tumour number and volume between treatment groups (the 2nd/1st scan).

See supplemental Tables 1-3 for statistical details.

**Figure 2 Changes in renal tumour size of Tsc1+/− mice detected by MRI and histology**

*Tsc1+/−* mice were scanned using MRI in vivo as in Figure 1 and sacrificed after the 2nd scan for kidney section preparation. Kidney sections were HE-stained. Representative MRI-images and HE sections were presented to show size of different tumour types at specified treatment groups.

**Figure 3 Treatment effect on proliferation of renal tumour cells in Tsc1+/− mice**

Kidney sections, prepared from 14 months old *Tsc1+/−* mice after the 2nd MRI scan, were stained with antibody against Ki67 to assess proliferation of tumour cells. Representative sections were presented to show expression of Ki67 in tumour cells of specified treatment groups.

**Figure 4 Treatment effect on mTOR signalling and expression of p21 in the liver of Tsc1+/− mice**

A. Western analysis. Proteins were prepared from liver tissues dissected from *Tsc1+/−* mice treated for two months with vehicle, atorvastatin, rapamycin or atorvastatin/rapamycin combination. Beta-actin was used as a loading control. Representative Western blots were presented to show phosphorylation of Akt at S473 and S6 at S235/236, and expression of p21.
B. Q-PCR analysis. Total RNA was isolated from liver tissues dissected from Tsc1+/− mice treated for two months with vehicle, atorvastatin, rapamycin or atorvastatin/rapamycin combination (n=5 each group). RNA was used to synthesise cDNA and real time q-PCR was performed to estimate relative quantity of Cdkn1a transcripts.

**Figure 5 Treatment effect on mTOR signalling of renal tumours in Tsc1+/− mice**

Kidney sections, prepared from 14 months old Tsc1+/− mice after the 2nd MRI scan, were used for IHC analysis. Representative IHC-stained sections were presented to show phosphorylation of S6 at S235/236 and Akt at S473 in renal tumours. Black lines are scale bars.

**Figure 6 Treatment effect on MAPK signalling of renal tumours in Tsc1+/− mice**

Kidney sections, prepared from 14 months old Tsc1+/− mice after the 2nd MRI scan, were used for IHC analysis. Representative IHC-stained sections were presented to show phosphorylation of RAF1 at S259 and Erk1/2 at T202/Y204 in renal tumours. Black lines are scale bars.