

Assessment of Response of Kidney Tumors 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 antitumor activity for various types of cancer. Tuberous sclerosis (TSC) is a tumor syndrome caused by *TSC1* or *TSC2* mutations that lead to aberrant activation of mTOR and tumor 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 tumor 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 to track changes in tumor 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 tumor burden, while atorvastatin alone did not. Combined therapy with rapamycin and atorvastatin appeared to be more effective for treating renal tumors 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 tumors.

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Introduction

Atorvastatin, a synthetic inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA reductase, 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 antitumor activity for various types of cancer including renal carcinoma [2–5]. Tuberous sclerosis (TSC) is a tumor syndrome caused by *TSC1* or *TSC2* mutations and characterized by tumor 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 tumors exhibit aberrant activation of mTOR. Genetically engineered *Tsc1*^{+/-} or *Tsc2*^{+/-} mice spontaneously develop tumors 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 tumor 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 tumor number and size in the kidneys of a *Tsc1*^{+/-} mouse model and assess the efficacy of rapamycin and atorvastatin combination therapy.

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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*^{+/-} littermates were randomly allocated into four groups of six, 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 2 months and then sacrificed for assessment of tumor burden and analysis of protein expression and phosphorylation in tumors 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 anesthetized with 5% isoflurane in a 40% O₂/air mix at 1.2 l/min, injected with 2 × 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 anesthetic nose cone. Isoflurane was reduced to 1.5% to 2% for the maintenance of anesthesia during scanning. Body temperature, breathing rate, and heart rate were monitored throughout using the Model 1025 Monitoring and Gating System (SAI Inc., Stony Brook, NY). 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 × 4.0 cm, a matrix of 640 × 256, 64 × 0.5–mm coronal slices, a TE_{eff} of 26 milliseconds, a TR of 4100 milliseconds, a RARE factor of 4, and a BW of 100 kHz. The volumes of renal lesions were measured using the software Analyze 10.0 (Analyze Direct, Inc., Overland Park, KS). The measurement was conducted blindly in triplicate.

Histology

Mouse kidneys were fixed in 10% buffered formalin saline (Thermo Scientific, Runcorn, UK) for 24 hours. Fixed kidneys were processed and paraffin embedded according to standard procedures. A series of 5-μm coronal kidney sections was 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-8by4aUCFclNfAods3eg1w>).

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 Signaling Technology (Danvers, MA). Antibodies against Ki67 and phosphorylated RAF1 at S259 were supplied by Abcam (Cambridge, UK). SignalStain Boost Rabbit specific IHC Detection Reagent (Cell Signaling Technology, Danvers, MA) was used to stain antigens.

Western Blot

In addition to primary antibodies described above, primary antibody against β-actin and horseradish peroxidase-conjugated secondary antibody against rabbit were purchased from Cell Signaling

Technology (Danvers, MA) 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 micrograms of protein per sample was separated on NuPAGE 4% to 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 analyzed with ECL Select Western Detection Kit (GE Healthcare UK Ltd.), and signals were detected using Autochemi Imaging System (UVP, Upland, CA).

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 synthesize cDNA in a 20-μl solution using qScript cDNA SuperMix (Quanta BioSciences, Inc., Gaithersburg, MD). A PCR containing 1 μl cDNA, 2 μl 2.5 μM primer mix, 6.5 μl 2× Perfecta SYBR SuperMix (Quanta BioSciences, Inc., Gaithersburg, MD), 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 and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative quantification of target transcripts was obtained after normalization 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* < .05 was considered to be statistically significant.

Results and Discussion

Efficacy of Rapamycin, Atorvastatin, or Combination of Rapamycin and Atorvastatin on Renal Tumors in *Tsc1*^{+/-} Mice

Before starting treatment, *Tsc1*^{+/-} littermates were randomly allocated into 4 groups of 6, balanced for gender, and subjected to the first 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 (Supplementary 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 2 months. After treatment, *Tsc1*^{+/-} mice were subjected to the second MRI scanning to track changes in tumor number and size. First, we directly compared the total number and size of all renal lesions obtained from the second MRI scanning. As reported previously [12], atorvastatin alone did not reduce tumor burden in the kidneys of *Tsc1*^{+/-} mice (Figures 1 and 2; Supplementary Table 2). We found that combination of rapamycin and atorvastatin but not rapamycin alone reduced tumor number significantly, although both rapamycin alone and rapamycin combined with atorvastatin significantly reduced tumor volume (Figures 1 and 2; Supplementary Table 2). Fold changes in tumor volume over time have been widely used to assess changes in tumor

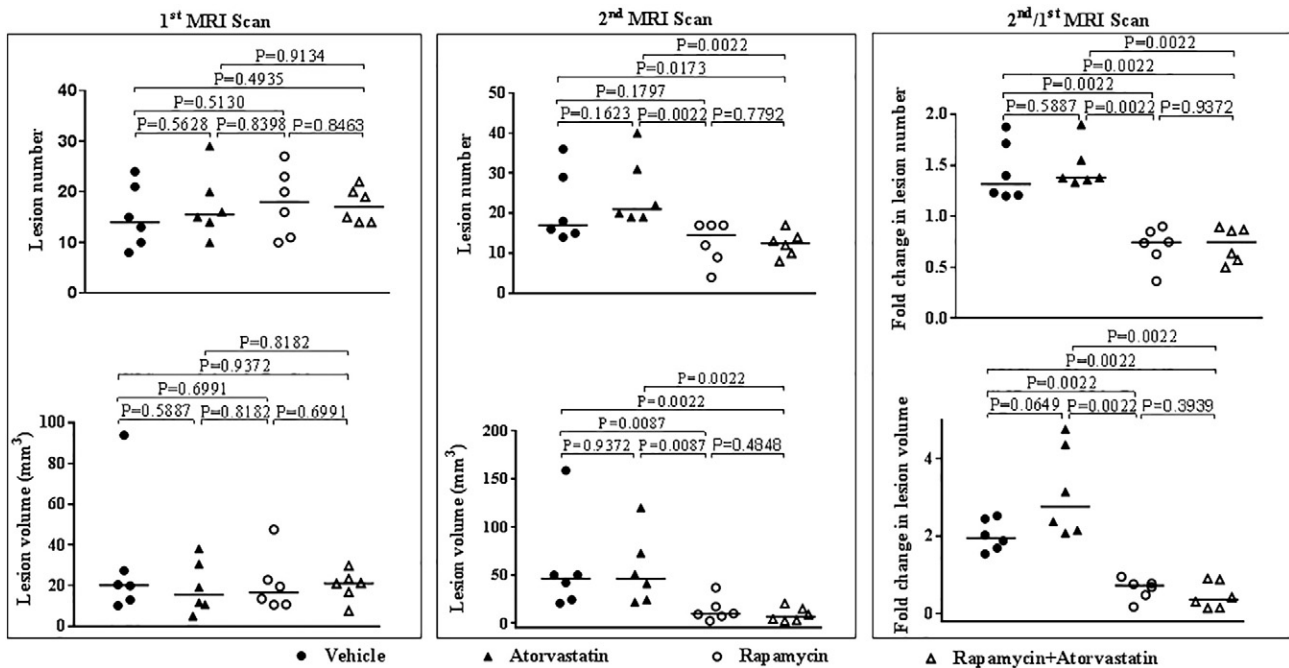


Figure 1. MRI assessment of treatment efficacy on renal tumors of *Tsc1*^{+/-} mice. *Tsc1*^{+/-} mice were subjected to MRI scanning before and after treatment, respectively, *in vivo*. Total number and volume of all renal lesions (cystic/papillary/solid) per mouse were obtained using Analyze 10.0. Left panel: Comparison of total number and volume of renal tumors between treatment groups before starting treatment at the age of 12 months (the first MRI scan). Middle panel: Comparison of total number and volume of renal tumors between treatment groups after treatment at the age of 14 months (the second 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 2 months before the second MRI scanning. Right panel: Comparison of fold change of total renal tumor number and volume between treatment groups (the second/first scan). See Supplementary Tables 1-3 for statistical details.

burden in preclinical xenograft models and clinical settings. We compared fold changes of total tumor number and volume (second/first scan). We confirmed that atorvastatin alone did not have any therapeutic efficacy for renal tumors of *Tsc1*^{+/-} mice and that rapamycin alone or combination of rapamycin and atorvastatin significantly reduced tumor burden in the kidneys of these mice (Figures 1 and 2; Supplementary Table 3). Combination of rapamycin and atorvastatin appeared to have greater therapeutic

efficacy for renal tumors, but the difference was not significant (Figure 1; Supplementary Table 3).

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

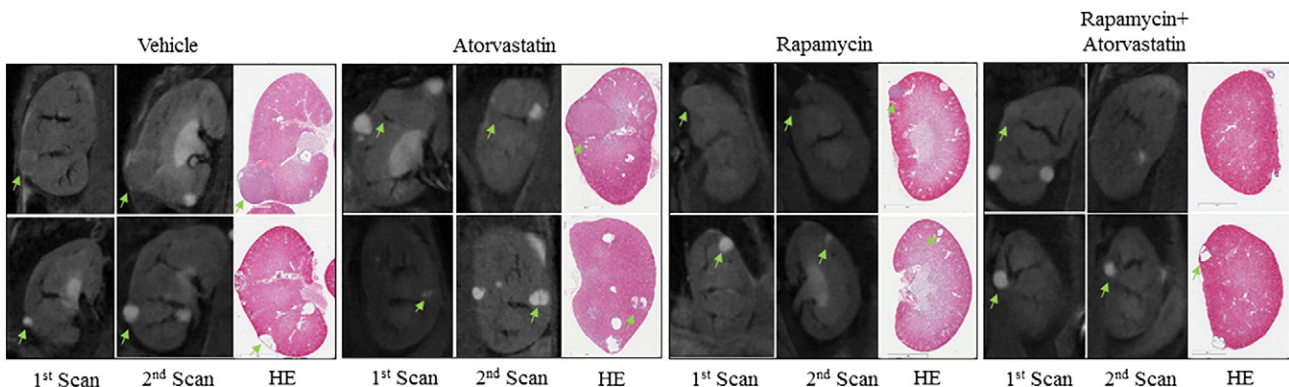


Figure 2. Changes in renal tumor size of *Tsc1*^{+/-} mice detected by MRI and histology. *Tsc1*^{+/-} mice were scanned using MRI *in vivo* as in Figure 1 and sacrificed after the second scan for kidney section preparation. Kidney sections were HE-stained. Representative MRI images and HE sections were presented to show size of different tumor types at specified treatment groups.

Effect of Rapamycin, Atorvastatin, or Combination of Rapamycin and Atorvastatin on Cell Proliferation and Oncogenic Signaling in Renal Tumors of *Tsc1*^{+/-} Mice

Atorvastatin was reported to selectively suppress growth and proliferation of mouse *Tsc2* null embryonic fibroblasts *in vitro* [11]. In the current study, rapamycin alone or combination of rapamycin and atorvastatin inhibited proliferation of tumor cells as indicated by reduced number of Ki67-positive tumor 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 tumors (Figure 5). Rapamycin alone or combination of rapamycin and atorvastatin potentially inhibited mTORC1 in renal tumors (Figure 5). In a previous study, atorvastatin was found to effectively delay tumor 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

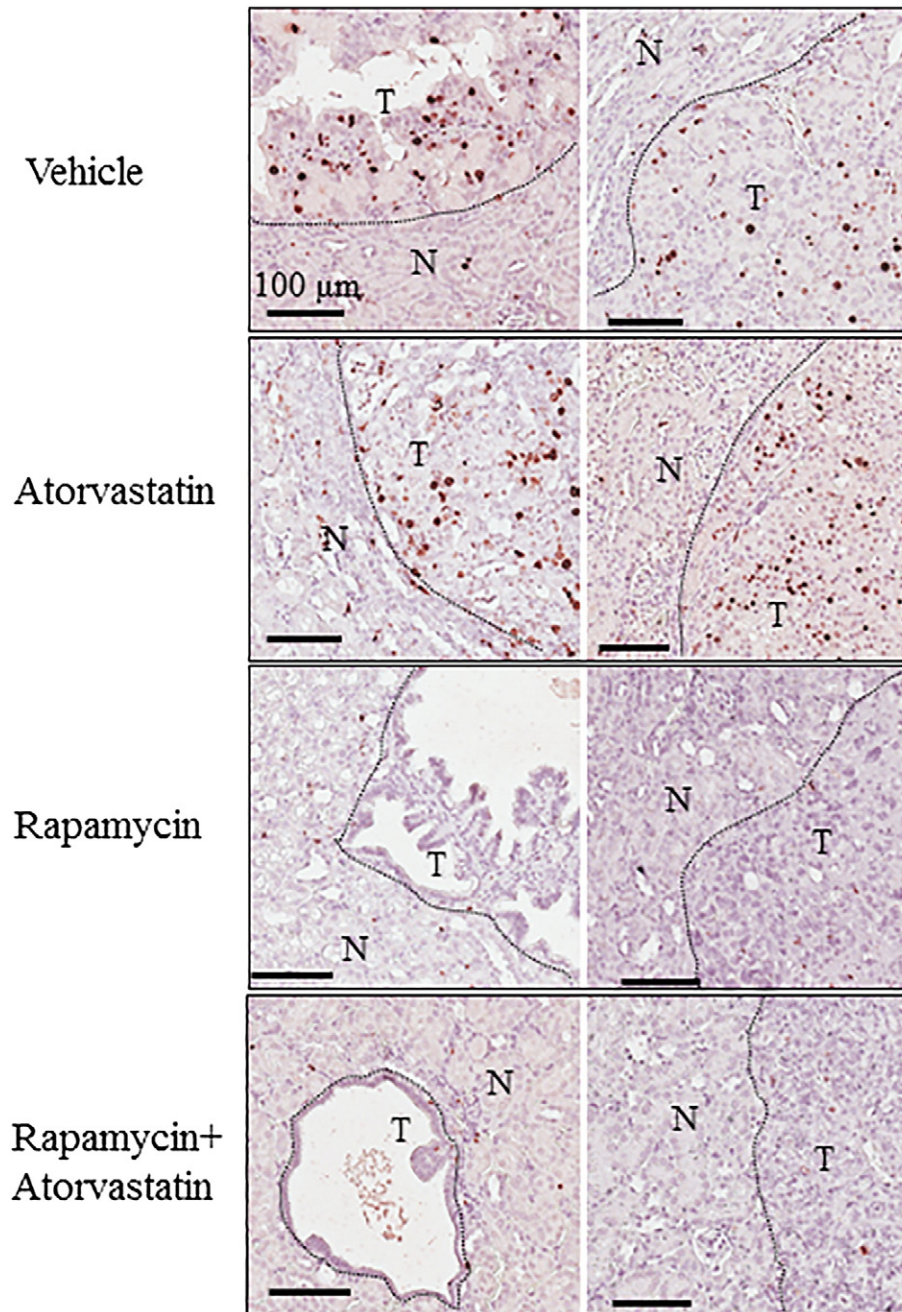


Figure 3. Treatment effect on proliferation of renal tumor cells in *Tsc1*^{+/-} mice. Kidney sections, prepared from 14-month-old *Tsc1*^{+/-} mice after the second MRI scan, were stained with antibody against Ki67 to assess proliferation of tumor cells. Representative sections were presented to show expression of Ki67 in tumor cells of specified treatment groups.

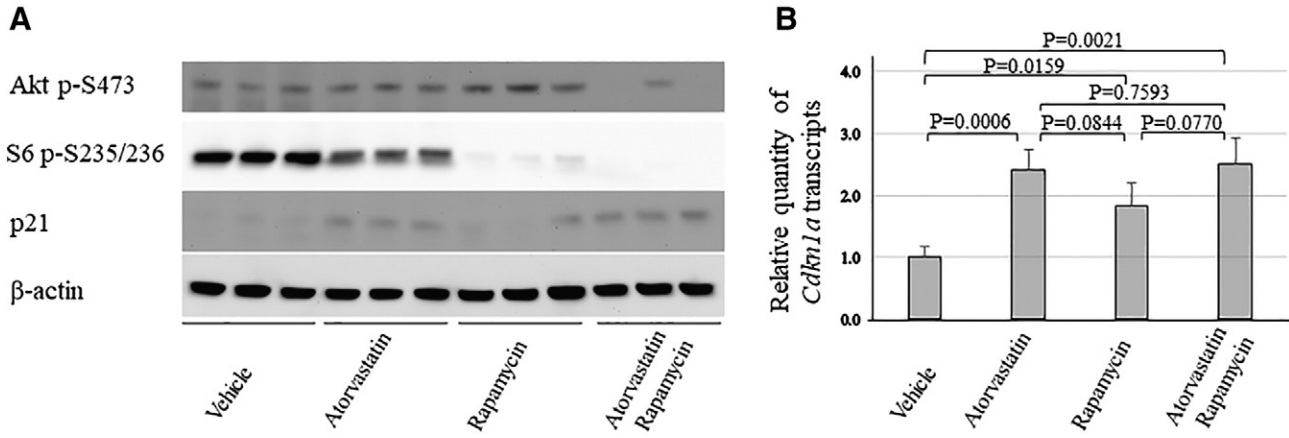


Figure 4. Treatment effect on mTOR signaling and expression of p21 in the liver of *Tsc1*^{+/-} mice. (A) Western analysis. Proteins were prepared from liver tissues dissected from *Tsc1*^{+/-} mice treated for 2 months with vehicle, atorvastatin, rapamycin, or atorvastatin/rapamycin combination. β-Actin 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 2 months with vehicle, atorvastatin, rapamycin, or atorvastatin/rapamycin combination (*n* = 5 each group). RNA was used to synthesize cDNA, and real time q-PCR was performed to estimate relative quantity of *Cdkn1a* transcripts.

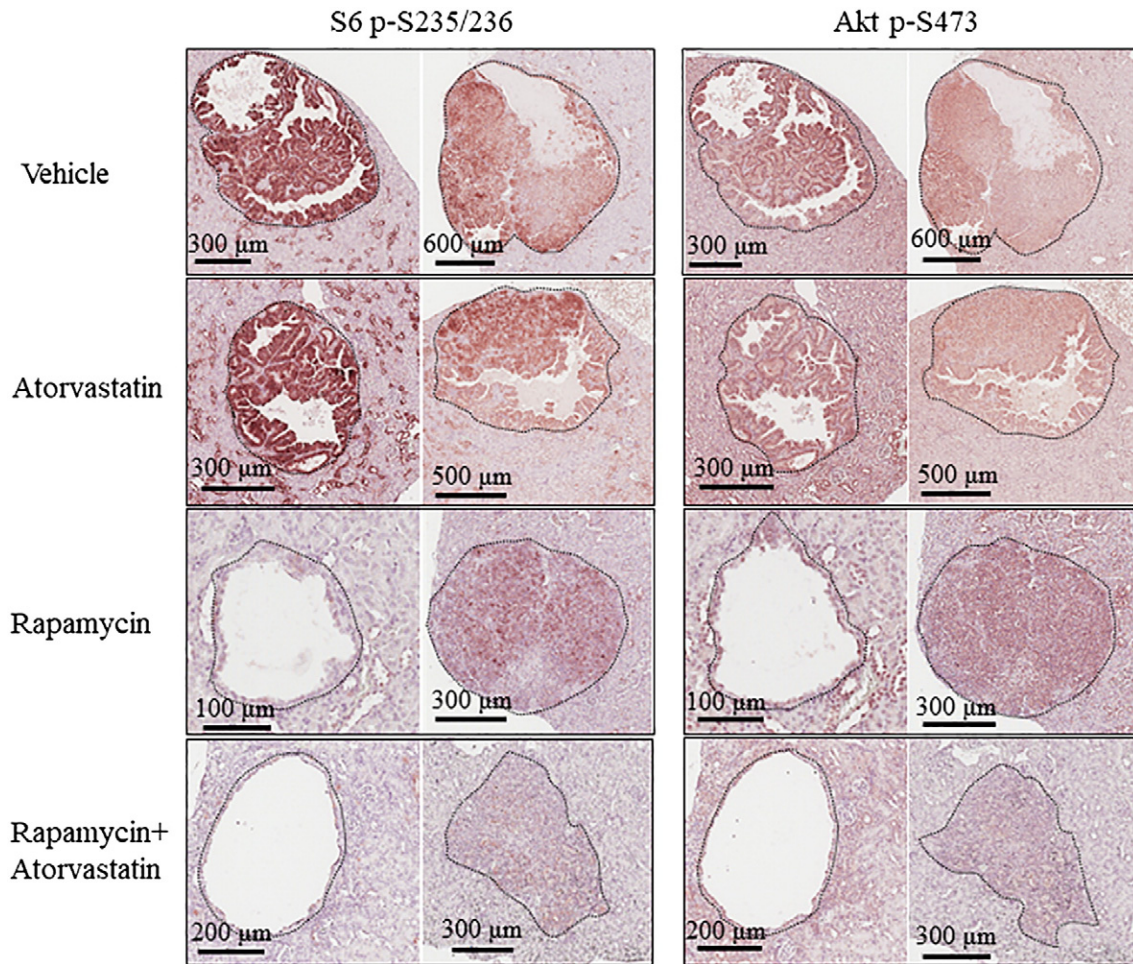


Figure 5. Treatment effect on mTOR signaling of renal tumors in *Tsc1*^{+/-} mice. Kidney sections, prepared from 14-month-old *Tsc1*^{+/-} mice after the second MRI scan, were used for IHC analysis. Representative IHC-stained sections were presented to show phosphorylation of S6 at S235/236 and Akt at S473 in renal tumors. Black lines are scale bars.

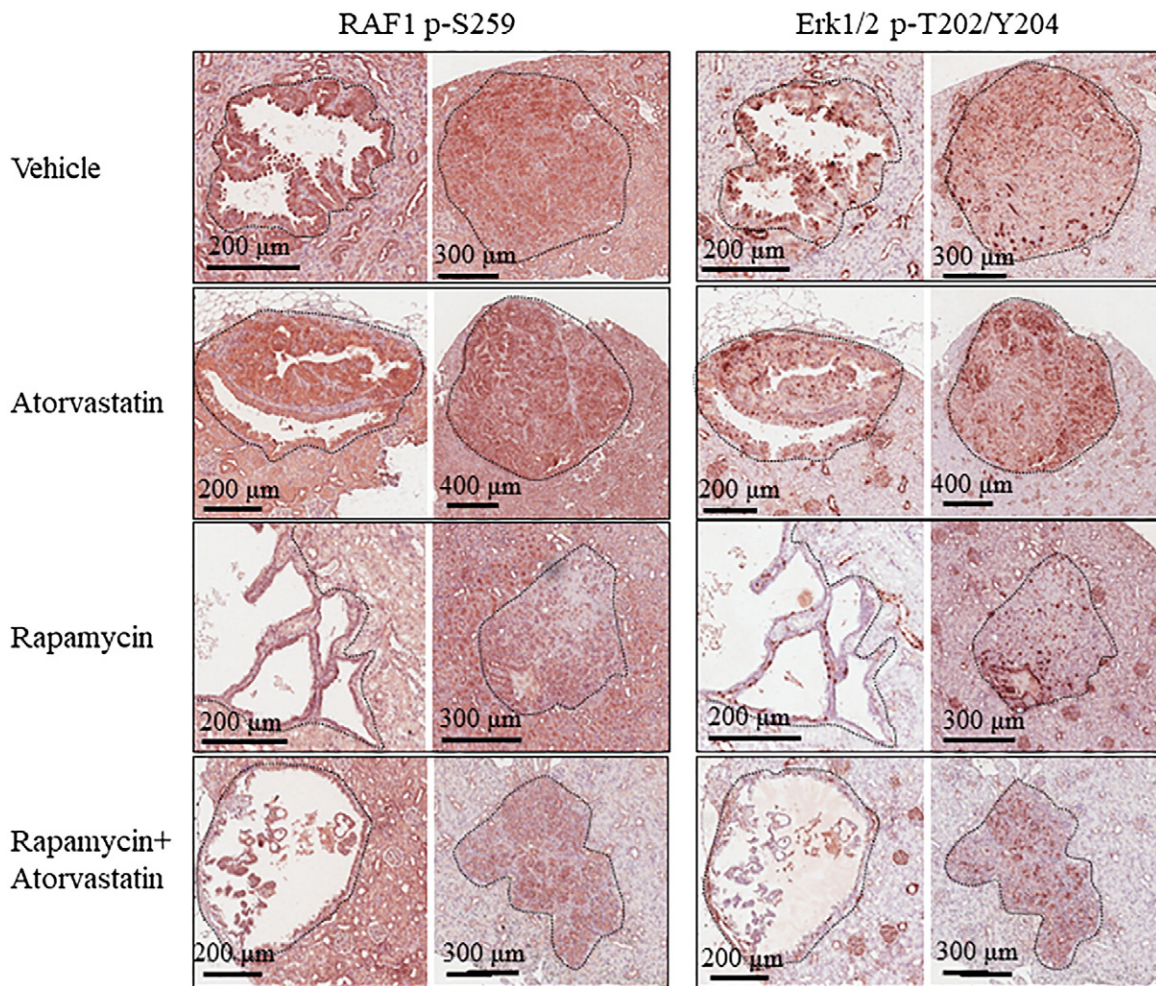


Figure 6. Treatment effect on MAPK signaling of renal tumors in *Tsc1*^{+/-} mice. Kidney sections, prepared from 14-month-old *Tsc1*^{+/-} mice after the second MRI scan, were used for IHC analysis. Representative IHC-stained sections were presented to show phosphorylation of RAF1 at S259 and Erk1/2 at T202/Y204 in renal tumors. Black lines are scale bars.

Erk1/2 in renal tumors 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 tumors in Tsc mouse models is likely to be associated with insufficient inhibition of mTOR and Erk signaling in the tumors.

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 tumors. 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.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.tranon.2017.07.009>.

Conflict of Interest Statement

None declared.

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