Renal and liver tumors in $Tsc2^{+/−}$ mice, a model of tuberous sclerosis complex, do not respond to treatment with atorvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor

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Abstract
Inactivating mutations of the tumor suppressor gene TSC2 are associated with tumorigenesis in tuberous sclerosis complex (TSC) and lymphangioleiomyomatosis. Statins, as 3-hydroxy-3-methylglutaryl CoA reductase inhibitors, have the potential to limit the growth of these tumors by limiting the isoprenylation of activated GTPases in Tsc2-null cells. We tested atorvastatin as a therapy for (a) ethylnitrosourea (ENU)-enhanced renal cystadenoma and (b) spontaneous liver hemangioma in 129Sv/Jae $Tsc2^{+/−}$ mice. ENU-treated $Tsc2^{+/−}$ mice were given atorvastatin chow (0.1%, w/w) for 1 or 3 months before sacrifice at 6 months; 129Sv/Jae $Tsc2^{+/−}$ mice were given atorvastatin chow (0.1%, w/w) for 6 months before sacrifice at 12 months. All treatment groups were compared with mice of identical genotype and strain background that were fed control chow. Pathologic analyses revealed a predominance of renal cystadenoma or spontaneous liver hemangioma as compared with the untreated groups. In conclusion, although the marked reduction in cholesterol levels indicates that atorvastatin was effective as a 3-hydroxy-3-methylglutaryl CoA reductase inhibitor, it did not inhibit the growth of tumors that develop in these $Tsc2^{+/−}$ models, suggesting that it is unlikely to have benefit as a single-agent therapy for TSC-associated tumors. [Mol Cancer Ther 2009;8(7):1799–807]

Introduction
Mutations in either of two genes, TSCI and TSC2, are causally linked to the development of tuberous sclerosis complex (TSC), a tumor suppressor gene syndrome characterized by multiple tumors of the brain, kidney, heart, and skin (1, 2). Renal involvement by angiomylipomas and lung involvement by lymphangioleiomyomatosis (LAM) are both common in TSC, are a significant source of morbidity and mortality, and seem to be due to complete loss of TSCI/TSC2 complex function. Pulmonary LAM is characterized by smooth muscle cell proliferation in nodular lesions with cyst formation. It can be rapidly progressive and fatal, but can also have a slow natural history that spans decades. LAM occurs both in TSC and in sporadic patients without features of TSC. A decade of research has led to improved understanding of the tumor suppressor functions of hamartin and tuberin, the protein products of TSCI and TSC2, respectively. The hamartin-tuberin complex suppresses the activation of the small GTPase Rheb (ras homologue enriched in brain) through a GTPase-activating domain near the COOH terminus of tuberin (3, 4). Rheb is a major regulator of mTORC1 (mammalian target of rapamycin complex 1). Thus, in cells lacking functional hamartin or tuberin, elevated levels of active Rheb (GTP-Rheb) lead to constitutive activation of mTORC1, resulting in phosphorylation of p70 S6 kinase (S6K), S6, and eukaryotic translation initiation factor 4E–binding protein 1, to regulate protein translation and cell growth (5, 6).

Therapeutic options for TSC, including LAM, have recently focused on targeting mTORC1 (7, 8). Rapamycin, a mTORC1 inhibitor, has a well-established role as an immunosuppressant to limit transplant rejection (9, 10) and as a smooth muscle cell growth inhibitor in vascular stents (11, 12). In addition, Bissler et al. (13) have recently shown in a phase 1-2 trial that rapamycin has promising activity in decreasing the size of TSC-associated renal angiomylipomas to an average of 53% of their starting volume in 20 patients who stayed on drug for a year, suggesting possible improvement in pulmonary function in patients...
with LAM. However, benefits seemed to be short-lived, with regrowth of renal tumors to 86% of starting tumor volume on average, during a follow-up period of 1 year off drug, suggesting that continued rapamycin treatment may be necessary for sustained response. Because rapamycin has many potential significant side effects, including immunosuppression and infection risk (14, 15), and 25% of patients came off treatment in this trial due to side effects (13), there is a continuing need for alternative treatment approaches.

Statins are lipid-lowering agents, with an impressive safety record, that have pleiotropic effects in addition to their cholesterol lowering effect (16). Such pleiotropic benefits range from coronary artery plaque stabilization to antiproliferative effects on vascular smooth muscle and cancer cells (17). Statins are specific inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase; ref. 18). As a result of HMG-CoA reductase inhibition, statins inhibit not only the biosynthesis of cholesterol but also the synthesis of downstream isoprenoid lipid moieties. The process of isoprenylation (farnesylation and/or geranylgeranylation) is necessary for full functional activity of many small GTPase family members by enabling them to associate with relevant membranes for downstream signaling (19, 20). Rheb, which is constitutively activated in tuberin-null states, belongs to the Ras family of GTPases and is exclusively farnesylated (21). Moreover, the farnesylation of Rheb is important for the regulation of mTORC1-kinase activity toward S6K (22–24). In contrast, RhoA is typically geranylgeranylated, and although constitutively activated in tuberin-null states, its activation remains unaffected by inhibiting mTORC1 kinase (25). We have previously shown that atorvastatin blocks Rheb isoprenylation and downstream phosphorylation of mTOR-S6K-S6, in addition to blocking RhoA activity in tuberin-null cells (26). Atorvastatin resulted in significant and selective reduction of tuberin-null cell growth, making it a potential therapeutic candidate for diseases where tuberin function is dysregulated or lost.

Atorvastatin (also known as Lipitor) is a third-generation synthetic statin that is widely used clinically and very well tolerated. In this article, we describe the in vivo effects of atorvastatin as a single-agent therapy for renal cystadenoma and liver hemangioma, the two tumors that develop spontaneously at high frequency in Tsc2+/− mice.

Materials and Methods

Animals and Tissue Collection

All procedures were approved by the Children’s Hospital Animal Care and Use Committee. Atorvastatin (0.1%, w/w) was prepared in murine chow (Purina); controls were fed standard chow. The dose, 0.1% (w/w; ~100 mg/kg/d), was calculated based on the average amount of chow ingested by mice on a daily basis and is the maximum recommended dose of atorvastatin (Pfizer, Inc.; refs. 27–29).

Ethynitrosourea-Treated Cohort (Ethynitrosourea-Associated Tumors). Tsc2+−/− mice develop kidney tumors (cystadenoma, solid adenoma) and liver hemangiomas at a variable rate that is dependent on strain (30, 31). To study kidney tumors in these mice, we used mice from a defined strain background (50% C57BL/6, 50% 120Sv/Jae) and treated the mice at P9 with a single dose of N-ethyl-N-nitrosourea (ENU) at 60 μg/g i.p. to accelerate renal tumorigenesis.3 Tumors were allowed to develop, and animals were treated with atorvastatin for either 1 mo (n = 4) or 3 mo (n = 10) before sacrifice at 6 mo of age. Control mice (n = 7) also received ENU at the same age but were fed standard chow for 6 mo.

Non-ENU-Treated Cohort (Spontaneous Tumors). To study the effects of atorvastatin on spontaneous renal cystadenoma and liver hemangioma development, we used male and female Tsc2+−/− mice in the 129/SvJae strain, in which liver hemangiomas are common and severe (30, 31). Mice were treated with atorvastatin for 6 mo (n = 8) before sacrifice at 12 mo of age, or received a control chow diet (n = 12).

Mice from all cohorts were sacrificed, and blood, lungs, livers, and kidneys were harvested for tumor or biochemical analysis.

Standard Histology and Tumor Assessment

Standard histology sections were prepared from mouse kidneys after 10% formalin fixation and cutting into five 1- to 2-mm sections. After H&E staining, slides were viewed on a Nikon Eclipse E400 microscope, and images were captured using Spot software v4.0.5. Both gross and microscopic kidney pathologies were read by a blinded observer (K.P.) and scored according to a modification of a formula used previously (30, 31). The kidney tumor score for kidney cystadenoma was determined as a summed score for all lesions in a kidney, by scoring each individual tumor grossly as follows: 1 for tumors <1 mm; 2 for 1 to 1.5 mm; 5 for 1.5 to 2 mm; and 10 for > 2 mm. Microscopic kidney tumor scores were determined similarly, except that the score for each lesion was multiplied by 2 if the tumor had a papillary component, and by 4 if it was a solid adenoma. The percent cellularity of cystadenoma was determined as the percent of the tumor that contained proliferating cells as opposed to cyst cavity; pure cysts had a score of 0% cellularity whereas solid adenomas had a score of 100% cellularity.

For mouse liver hemangioma, livers were sectioned throughout at 1 mm, and sections were then read to determine the percent involvement by hemangioma on each section by a blinded observer (D.K.). The percent liver involvement by hemangioma was determined as the weighted average involvement over all sections.

Immunohistochemistry

Immunohistochemistry for phospho-S6 (pS6) was done on paraffin-embedded sections as previously described (32). Antibodies used for immunohistochemistry were pS6 (Ser235/236) and pS6 (Ser240/244; Cell Signaling Technology). In brief, slides were deparaffinized and antigen retrieval was done using Dako Target Retrieval Solution pH 6 (DAKO). Sections were stained by the immunoperoxidase technique using AEC (HRP-AEC Cell & Tissue Staining

3 Pollizzi and Kwiatkowski, unpublished observations.
Kit, R&D Systems, Inc.) with counterstaining done using hematoxylin (DAKO). After staining, slides were viewed on a Nikon Eclipse E400 microscope, and images captured using Spot software v4.0.5. Slides were read by a blinded observer (L.M.) and scored as follows: 0, no detectible tumor-specific stain; 1, light stain, similar to that seen in nearby renal parenchyma; 2, moderately intense stain, higher than that seen in nearby renal parenchyma; 3, strong stain, much higher than surrounding normal renal parenchyma.  

**Immunoblot Analysis**

Lung and liver extracts were prepared by homogenization in lysis buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L MgCl2, 1% Triton-X, 10% glycerol] using a dounce homogenizer, beginning with one lobe of the lungs or a small portion of liver. Equal amounts of protein were loaded and analyzed for pS6 levels by immunoblotting as previously described (33). Primary antibodies used were against pS6 (Ser235/236), S6, phospho-S6K (pS6K, Thr421/424; Cell Signaling Technology), RhoA, and Rheb (Santa Cruz). Secondary horseradish peroxidase–conjugated antibody (Cell Signaling Technology) was used with chemiluminescent signal detection by ECL (Cell Signaling Technology). Semiquantitative densitometric analysis was done by ImageQuant software (Molecular Dynamics). Data are expressed as fold activation relative to control. Images of immunoblots were obtained and cropped using Adobe Photoshop 6.0 (Adobe systems, Inc.).

**GTP-RhoA Pull-down Assay**

Whole-cell lysate GTP-RhoA was affinity purified using glutathione S-transferase-rhotekin Rho binding domain agarose beads (Upstate/Millipore) as previously described (26).

**Measurement of Cholesterol Levels**

Total and free serum cholesterol levels were measured using a standard ELISA kit (Biovision Cholesterol/Cholesteryl Ester Quatitation Kit). Approximately 1 mL of whole blood was removed at the time of sacrifice. Blood was centrifuged at 10,000 × g for 5 min. Serum was removed and stored at −80°C until analysis. All samples were analyzed together and in duplicate. Cholesterol reaction mix with and without cholesterol esterase was added to each sample and incubated at 37°C for 1 h. Fluorescence was measured at excitation/emission wavelengths of 540/595 nm (Tecan Spectrafluor PLUS). Readings were corrected for background and read off of a standard curve.

**Statistical Analysis.** Results are expressed as mean ± SD. Statistical analysis using a nonparametric Kruskal-Wallis ANOVA with Dunn’s multiple comparison test for follow-up analysis was done using GraphPad Prism 3.0 software.

**Results**

ENU-Treated *Tsc2*+/− Mice (Accelerated Renal Cystadenoma)

**Survival and Tumor Type.** All mice (with the exception of one) survived to the age of 6 months. The kidney tumors that were observed in ENU-treated *Tsc2*+/− mice were histologically similar to those previously reported in non-ENU-treated mice of age 12 to 18 months (30, 31). Liver hemangiomas were not observed in this cohort of mice. Histologic analysis of ENU-associated kidney tumors in both control and atorvastatin treatment groups revealed multiple cystadenomas (Fig. 1) that, on gross inspection, were not appreciably different.

**Cholesterol Levels.** Chow weight was measured on a weekly basis, and no significant difference in chow consumption was observed between control and atorvastatin treatment groups, 1 month or 3 months. Figure 2A shows that there was a major, significant reduction in serum total cholesterol levels (65 ± 2% decrease, 1 month; 66 ± 2% decrease, 3 months; mean ± SD; *P* < 0.001) and in free cholesterol levels (81 ± 3% decrease, 1 month; 79 ± 2% decrease, 3 months; mean ± SD; *P* < 0.001) in the atorvastatin-treated mice. Total and free cholesterol levels were similar in the 3-month and 1-month atorvastatin treatment cohorts.

**Gross and Microscopic Tumor Scores.** Both gross and microscopic kidney tumor scores were similar among the control, atorvastatin 1 month, and atorvastatin 3 months treatment groups (Fig. 2B, *P* = 0.52 for gross, *P* = 0.84 for microscopic; control, 12.3 ± 9.1; atorvastatin 1 month, 15.9 ± 12.5; atorvastatin 3 months, 13.7 ± 7.1; mean ± SD for gross scores). In addition, the percent cellularity did not differ according to treatment (Fig. 2B; *P* = 0.36), suggesting that there was no effect on the rate of proliferation of kidney tumors in mice treated with atorvastatin.

**Figure 1.** Histologic appearance of ENU-induced renal cystadenoma in *Tsc2*+/− mice. H&E-stained sections show the two major tumor types in the kidneys of *Tsc2*+/− mice: solid adenoma (A) and cystadenoma (B). Magnification, ×20.
pS6 and GTP-RhoA Levels. Immunoblot analysis of healthy lung and liver homogenates in mice treated with atorvastatin for 1 month (Fig. 3A) showed significant and consistent reduction in pS6K (Thr421/424) and pS6 (Ser235/236) levels (P < 0.001 for all comparisons). Similar significant reductions in GTP-RhoA levels were also observed in atorvastatin-treated mice (Fig. 3A; P < 0.001). Immunoblot analysis of lung and liver homogenates in
the atorvastatin 3 months cohort (Fig. 3B) showed similar major reductions in pS6K (Thr421/424), pS6 (Ser235/236), and GTP-RhoA levels (Fig. 3B; $P < 0.01$ for all comparisons).

**Non–ENU-Treated 129/SvJae Tsc2$^{+/−}$ Mice (Spontaneous Liver Hemangioma)**

**Survival and Tumor Type.** The Tsc2$^{+/−}$ mice were evaluated at 12 months of age; in this strain (129/SvJae), liver hemangioma predominate (Fig. 4A) and kidney cystadenoma are less common. There were no deaths observed during the 12 months of this trial.

**Cholesterol Levels.** No difference in consumption of chow was observed between control and treatment groups (data not shown). A major reduction in serum total (58 ± 6% decrease; mean ± SD; $P < 0.01$) and free (71 ± 4% decrease; mean ± SD; $P < 0.01$) cholesterol levels was seen in the atorvastatin-treated mice (Fig. 4B).

**Gross and Microscopic Tumor Scores.** Both gross and microscopic kidney tumor scores were similar in the control and atorvastatin treatment groups (Fig. 4C, $P = 0.86$ for gross, $P = 0.83$ for microscopic; control males, 5.1 ± 5.2; atorvastatin males, 6.4 ± 7.2; control females, 6.8 ± 10.2; atorvastatin females, 3.9 ± 4.9; mean ± SD for gross scores). Although the gross kidney tumor scores were somewhat lower in atorvastatin-treated females compared with controls, this did not achieve statistical significance and showed the opposite trend in the microscopic kidney tumor scores.

Similar to findings in Tsc1$^{+/−}$ mice, liver hemangiomas were somewhat more severe in female than in male mice.

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**Figure 3.** pS6 and GTP-RhoA levels in healthy tissues of atorvastatin and control Tsc2$^{+/−}$ mice. Immunoblots of whole tissue lysates of the lungs and livers of a representative cohort of mice treated with atorvastatin for 1 mo (A) and 3 mo (B). pS6K(T421/424; pS6K), pS6(S235/236; pS6), total S6, GTP-RhoA, and total RhoA were assessed in control and atorvastatin-treated mice. Intensity of bands was measured by densitometry and expressed as fold difference of levels in atorvastatin-treated (atorv) mice compared with controls (ctrl). $\ast$, $P < 0.05$. These images are cropped; full-length blots shown in A and B can be viewed in Supplementary Figs. S1 and S2, respectively.
However, there was no evidence of a treatment response to atorvastatin based on quantitative assessment of percent liver hemangioma (Fig. 4C, \( P = 0.98 \); control males, 4.7 ± 1.2; atorvastatin males, 4.7 ± 3.1; control females, 6.8 ± 9.1; atorvastatin females, 6.0 ± 5.5; mean ± SD).

**pS6 and GTP-RhoA Levels.** Similar to the ENU-treated cohort, immunoblot analysis of lung homogenates (Fig. 5) showed significant reduction in pS6K (Thr421/424; 2.39-fold reduction ± 0.22; mean ± SD; \( P < 0.05 \)), pS6 (Ser235/236; 1.88-fold reduction ± 0.12; mean ± SD; \( P < 0.05 \)), and GTP-RhoA (2.14-fold reduction ± 0.20; mean ± SD; \( P < 0.05 \)) levels in atorvastatin-treated mice.

We also performed histologic analysis of kidney tumors in both control and atorvastatin treatment groups to examine the potential effects of drug on pS6 expression by the cystadenomas. Cystadenomas expressed variable levels of...
pS6, which did not differ significantly according to treatment group (Fig. 6). To confirm the effects of atorvastatin treatment on Rheb and RhoA, we examined organ extracts by immunoblotting. Rheb could not be detected in liver extracts, in contrast to cultured cell lysates. However, we observed increased levels of RhoA in atorvastatin-treated mouse liver extracts (Fig. 6C), consistent with depletion of the isoprenoid pool and altered distribution of RhoA, as reported previously (34).

**Discussion**

The statins, as pharmacologic inhibitors of HMG-CoA reductase, have a wide array of biological effects including antiproliferative and anticancer properties (17, 35–37). Our results indicate that atorvastatin, given at near-maximal dosage with major effects on both total and free serum cholesterol, had little or no effect on the size of ENU-accelerated or spontaneous tumors in Tsc2−/− mice. This study was prompted by in vivo observations that atorvastatin was highly selective and effective at inhibiting the growth of cells lacking Tsc2 (26). Although no murine model perfectly replicates the tumor spectrum seen in TSC and LAM patients, we chose to test this therapy in a Tsc2−/− model where tumors develop de novo to more closely parallel human TSC and LAM. LAM cells in humans and tumors that develop in both human TSC and Tsc2−/− mice have all been shown to bear markers of Rheb-mTORC1-S6K activation (31, 38, 39), and growth of LAM lesions and TSC-associated tumors is relatively insensitive in the treatment arms, suggesting that the dose was not too high. It could be postulated that administration of drug by injection could have led to higher bioavailability. However, p.o. administration is the norm for statin use in human patients. Furthermore, the dose used in this study is similar to that reported to cause successful outcomes in other murine cancer models using oral statins (29, 42, 43). Last, the duration of atorvastatin treatment ranged from 4 weeks to 6 months with the consistent observation that tumor size and morphology remained unchanged. These data suggest that failure of atorvastatin to affect tumor size did not relate to dose, lack of systemic levels, ineffective HMG-CoA reductase blockade, treatment duration, or method of administration.

Murine models of melanoma, mammary, prostate, and colon cancers have all been responsive to a variety of oral or intraperitoneal statins including pravastatin, lovastatin, and atorvastatin (43–46). However, in the present study, tumor volume as well as cellularity and pS6 expression showed no response to atorvastatin. We consider two possible explanations for this lack of sensitivity to atorvastatin. First, it is possible that levels of drug within tumors were reduced in comparison with other cell types and tissues. However, this seems unlikely because the drug was effective in the treatment of other tumor types, although this may relate to effects on pathways other than mTOR signaling. Second, it is possible that there was incomplete in vivo inhibition of G protein function, specifically Rheb and RhoA, within tumor cells, in contrast to what was seen in normal tissues. Consistent with this possibility, it is known that whereas atorvastatin has significant effect as an inhibitor of Rheb and RhoA prenylation, levels of the active GTP-bound G proteins are not completely eliminated by micromolar concentrations of atorvastatin (26). In addition, residual farnesylated Rheb can be detected even after prolonged treatment with atorvastatin. Similarly, we and others

![Figure 5. Effects of atorvastatin on pS6 and GTP-RhoA levels in healthy tissues of Tsc2−/− mice. Immunoblots of whole tissue lysates from lung are shown. Levels of phospho-T421/4-S6 kinase (p-S6K), phospho-S235/236-S6 (p-S6), total S6, GTP-RhoA, and total RhoA were compared in vehicle control mice and those treated with atorvastatin for 6 mo. Densitometric evaluation of the indicated proteins was calculated and expressed as fold difference of levels in atorvastatin-treated (atorv) mice compared with vehicle controls (ctrl). *, P > 0.05. These images are cropped; full-length blots shown in this figure can be viewed in Supplementary Fig. S3.](https://mct.aacrjournals.org/article-fig-v5)
have shown that when the ability of Rheb to be farnesylated is altered, either by statins or by the expression of farnesylation mutant Rheb, inhibition of S6 phosphorylation, although significant, does not return to baseline (4, 26). Thus, it is possible that in Tsc2-null tumors in vivo, in which there is no GTPase-activating protein activity for Rheb and markedly elevated Rheb-GTP levels, the inhibition of farnesylation by atorvastatin is not sufficient to block mTORC1 activation.

In conclusion, several large-scale human population studies have indicated that statins may reduce the risk of development of several different cancer types (47–49). In this preclinical study, however, atorvastatin was not effective at reducing the frequency or severity of renal cystadenoma and liver hemangioma in Tsc2+/- mice. Nonetheless, an effective dose was delivered, as assessed by mTOR pathway effects in normal tissues, marked reduction in serum cholesterol, and effects on RhoA. Based on these findings, caution is appropriate in considering statins as potential monotherapy for patients with either TSC-associated tumors or LAM. However, combination therapy or other novel approaches involving statins may prove to have benefit.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


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