Statin therapy, alone or with rapamycin, does not reverse monocrotaline pulmonary arterial hypertension: the rapamycin-atorvastatin-simvastatin study

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Statin therapy, alone or with rapamycin, does not reverse monocrotaline pulmonary arterial hypertension: the rapamycin-atorvastatin-simvastatin study. Am J Physiol Lung Cell Mol Physiol 293: L933–L940, 2007. First published August 3, 2007; doi:10.1152/ajplung.00310.2006.—Pulmonary arterial hypertension (PAH) is characterized by excessive pulmonary artery smooth muscle cell proliferation and impaired apoptosis leading to obstruction of resistance pulmonary arteries. We hypothesized that antiproliferative (rapamycin) and proapoptotic (statins) agents, already used clinically for other indications, would decrease experimental PAH, facilitating translation to human therapies. Prior studies in the rat monocrotaline-PAH model have indicated that simvastatin regresses and rapamycin prevents, but cannot reverse, PAH. Two PAH prevention strategies (rapamycin monotherapy vs. rapamycin + atorvastatin) and one prevention strategy (simvastatin) were tested in a rat monocrotaline-PAH model. Adult male Sprague-Dawley rats were randomized to saline ($n = 6$) or monocrotaline (60 mg/kg ip, $n = 36$) treatment groups. Monocrotaline rats were randomized to gavage with vehicle, rapamycin (2.5 mg/kg 1-day$^{-1}$), or rapamycin + atorvastatin (10 mg/kg 1-day$^{-1}$) treatment groups, beginning 12 days post-monocrotaline. Echocardiographic and hemodynamic end points were assessed 2 wk later. Additional monocrotaline-PAH rats ($n = 20$) were randomized to vehicle or simvastatin (2 mg/kg 1-day$^{-1}$) treatment groups and followed echocardiographically for 4 wk. Monocrotaline-PAH increased lung p70 S6 kinase phosphorylation, and this was reversed by rapamycin, confirming the biological activity of rapamycin. Despite the use of high doses, neither rapamycin nor rapamycin + atorvastatin improved survival nor reduced PAH, vascular remodeling, and right ventricular hypertrophy. Although prophyllactic simvastatin slowed PAH progression, by 4 wk PAH severity and mortality were not different from placebo. Apart from the new finding of p70 S6 kinase phosphorylation in monocrotaline-PAH, this is a negative therapeutic trial (none of these promising therapies improved monocrotaline-PAH). These negative results should be considered as human trials with these agents are underway (simvastatin) or proposed (rapamycin).

p70 S6 kinase; mammalian target of rapamycin; publication bias

PULMONARY ARTERIAL HYPERTENSION (PAH) is a syndrome characterized by proliferative and obstructive remodeling of the resistance pulmonary arteries (PAs) (18) that, together with excessive vasoconstriction, increases pulmonary vascular resistance (PVR), leading to right ventricular (RV) hypertrophy (RVH). Ultimately, patients succumb to right heart failure and premature death (43). While drugs that are considered to be vasodilators have been the traditional mainstay of PAH therapy (9, 19), it is increasingly recognized that a cure for PAH will likely involve drugs, or drug combinations, that target the excess proliferation and disordered apoptosis that occurs within the resistance PAs in PAH (29, 41). Here, two promising PAH therapies, rapamycin (34), an antiproliferative immunosuppressor that arrests cells in the G1 phase of the cell cycle (4), and 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors (statins) (35), were examined in randomized, vehicle-controlled trials using a rodent model of severe PAH. PAH was induced by an injection of monocrotaline (MCT), an alkaloid derived from Crotalaria spectabilis. Because each medication is currently approved for clinical practice (for other indications), they offer the potential for rapid translation to human PAH therapies. Although these agents are rapidly moving toward clinical trials in human PAH, the number of supporting animal studies is relatively modest.

Rapamycin is used clinically in transplantation medicine, as an immunosuppressant (21) that prevents T lymphocyte proliferation (40), and in cardiovascular medicine, as an antiproliferative agent applied to coronary stents to reduce local restenosis (32). Rapamycin was originally isolated from the bacterium Streptomyces hygroscopicus (49). Rapamycin binds to a intracellular receptor, FK506 binding protein 12 (1), and this complex inhibits mammalian target of rapamycin (mTOR) (4, 15, 44), a ~280-kDa serine/threonine kinase (8). Rapamycin also inhibits mitogen-induced activation (phosphorylation) of a kinase that is important in cell proliferation, p70 S6 kinase (36, 48). Activated p70 S6 kinase phosphorylates the 40S ribosomal protein S6 (48) and thereby regulates protein synthesis. By inhibiting S6 kinase, rapamycin reduces the translation of mRNA encoding ribosomal proteins and elongation factors, decreasing protein synthesis (8). Relevant to this study, this pathway offers a simple marker to ensure that rapamycin dosing is adequate (namely, that it inhibits p70 S6 kinase phosphorylation).

Rapamycin may also have promise as an anticancer agent (37, 42). The similarities between PAH and cancer (2, 26, 50) further recommend testing of rapamycin as a therapeutic molecule in PAH, despite its toxicities (infections relating to immunocompromise, impaired wound healing, hyperlipidemia, and thrombocytopenia) (23). Rapamycin inhibits hypoxia-induced activation of S6 kinase in PA adventitial fibroblasts (10), suggesting a possibility for therapeutic benefit in PAH. Rapamycin also has been shown to attenuate experimental PAH and suppress neointimal proliferation in a pneumonectomy + MCT.

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Simvastatin has been shown to reverse established monocrotaline-PAH in rats by inducing apoptosis of neointimal smooth muscle cells (35). Simvastatin has also been shown to reverse PAH induced in rats by a combination of VEGF inhibition and chronic hypoxia by inducing endothelial cell apoptosis (47).

Although there are no randomized human trials of simvastatin in PAH, this drug is being used empirically at some centers, and a positive observational series has been published (22).

Furthermore, there is a clinical trial, based at Imperial College London (London, UK), that is currently randomizing patients with PAH to simvastatin or placebo (ClinicalTrials.gov Identifier: NCT00180713). In addition, a clinical trial in PAH studying simvastin and aspirin in a randomized, double-blind, placebo-controlled study using a 2 × 2 factorial design has recently been launched (NCT 00384865). There have been recent studies of other statins, notably pravastatin (14) and atorvastatin (39), in rat MCT-PAH. Pravastatin was demonstrated to be effective in preventing the development of MCT-PAH, whereas atorvastatin was not, despite very similar effects of both agents in enhancing endothelial NO synthase (eNOS) expression and reducing endothelial cell apoptosis. Atorvastatin is a more potent statin than simvastatin, in terms of reducing cholesterol and cardiovascular end points (12), and it may have more potent pleiotropic effects, such as enhancement of the bioavailability of endothelium-derived NO, suppression of inflammation, and inhibition of oxidative stress (24). We sought to confirm whether a therapeutic role exists for atorvastatin in reversing established PAH, alone or in concert with rapamycin, and whether any kind of therapeutic class effect exists for these statin agents in PAH.

We hypothesized that combination therapy with high-dose rapamycin (2.5 mg · kg⁻¹ · day⁻¹), alone or in combination with atorvastatin (10 mg · kg⁻¹ · day⁻¹), would reverse established MCT-PAH in rats. When these results proved negative, we attempted to replicate prior positive studies, using simvastatin to prevent PAH. However, we conclude that neither strategy is beneficial when the duration of observation is adequate.

**MATERIALS AND METHODS**

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agreed to the manuscript as written.

**Experimental protocols.** All experiments were conducted with ethical approval from the University of Alberta Animal Policy and Welfare Committee. Adult male Sprague-Dawley rats (200–300 g) were injected with MCT (60 mg/kg ip) or saline vehicle. Beginning on day 12, MCT-treated rats were randomized to placebo, rapamycin (2.5 mg · kg⁻¹ · day⁻¹), or rapamycin (2.5 mg · kg⁻¹ · day⁻¹) plus atorvastatin (10 mg · kg⁻¹ · day⁻¹) treatment groups (n = 12 rats/group) and followed for 12 days prior to invasive evaluation. The doses of rapamycin and atorvastatin were high, based on the literature (33, 34), a choice made to avoid concerns about underdosing in the eventuality of negative results. A second cohort of rats (n = 10 rats/group) were randomized to simvastatin (2 mg · kg⁻¹ · day⁻¹) or placebo treatment groups, beginning 1 day postinjection of MCT, consistent with a previous study (35) of simvastatin in rat MCT-PAH. Both active drugs and placebo were given by a single daily gavage, which ensured the medication was ingested. Rapamycin was purchased in oral liquid form (Wyeth Pharmaceuticals, ON, Canada). Atorvastatin (Pfizer, QC, Canada) and simvastatin (Merck Frosst, QC, Canada) tablets were crushed and suspended in simple syrup (2 parts sugar and 1 part distilled water) to facilitate gavage. Doses were freshly prepared each day. For experiments in the rapamycin plus atorvastatin protocol, echocardiography and invasive hemodynamic measurements (see below) were made on day 24 post-MCT, followed by animal euthanasia. Animals in the simvastatin/placebo protocol were examined serially using weekly echocardiography.

**Echocardiography and hemodynamics.** RV free wall thickness and PA Doppler signals were measured in the parasternal short-axis view at the level of the aortic valve using a Sonos 5500 echo machine with a 15-MHz probe (Phillips, ON, Canada). Rats were anesthetized with ketamine (60 mg/kg ip) and xylazine (20 mg/kg ip) and placed on a warmed surgical stage. Invasive left heart catheterization [carotid artery pressure and left ventricular (LV) pressure] and right heart catheterization [PA pressure (PAP)] were performed, as previously described, using a 1.4-Fr Millar catheter (Millar Instruments, Houston, TX) (30). Cardiac output was measured in triplicate using a thermodilutor probe by a validated thermodilution method (ADInstruments, Colorado Springs, CO) and 0.5-ml injections of iced saline (38). The PVR index was calculated as follows: (mean PA – LV end-diastolic pressure)/cardiac index.

**Measurement of RVH and morphometric analysis of RVs and PAs.** RVH was measured as the RV/(LV + septum) wet weight ratio measured after the animal was killed. Lungs were inflated with formalin, fixed overnight, and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin or anti-von Willebrand factor antibody. Five rats per group were studied, and from each rat, at least two separate lung sections were examined. Resistance PAs [external diameter (ED): 20–150 μm] were randomly chosen from low-power fields for analysis (~50 arteries/group, 2–3 slides/rat). Analysis was performed by two blinded investigators using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). ED and medial thickness (MT) were measured, and the percent MT was calculated as 2 × MT/100/ED.

**Immunoblots.** Protein expression in whole lungs was measured with immunoblot analysis using available antibodies, as previously described (31). The actin and phospho-p70 S6 kinase (Thr³⁸⁸) primary antibodies were rabbit and goat polyclonal antibodies, respectively (Santa Cruz Biotechnology, Santa Cruz, CA). The intensity of the bands was normalized to the intensity of a reporter protein (actin) using the Kodak Gel-doc system (Kodak, ON, Canada).

**Statistics.** Values are expressed as means ± SE. P < 0.05 was considered statistically significant. Sample sizes were based on previous studies that reported beneficial effects of rapamycin or simvas- tatin in experimental PAH using sample sizes of 5 and 12 per group, respectively. Assuming a treatment effect of a reduction of mean PAP of 10 mmHg, the estimated power for a sample size of 6 rats/group was 89%. The Kruskal-Wallis test or ANOVA was used as appropriate. Fisher’s probable least-significant difference test was used for post hoc analysis if the overall ANOVA indicated significance (StatView 4.02, SAS Institute).

**RESULTS**

**Atorvastatin with or without rapamycin.** Rats injected with MCT developed PAH and RVH by day 10, which was severe...
by day 24. All rats survived to have echocardiography and invasive hemodynamic measurements. No significant reduction was observed in mean PAP with either rapamycin or rapamycin plus atorvastatin therapy (Figs. 1 and 2). Similarly, there were no differences in cardiac index or systemic blood pressure in the treatment groups versus placebo group. No significant decrease in the PVR index was observed with either therapy versus placebo, although there was a trend toward a small benefit in both rapamycin (80.6 ± 5.8 vs. 94.9 ± 5.8 mmHg·min⁻¹·kg⁻¹·ml, \( P = 0.07 \)) and rapamycin plus atorvastatin groups (80.3 ± 5.8 vs. 94.9 ± 5.8 mmHg·min⁻¹·kg⁻¹·ml, \( P = 0.07 \)) versus placebo (Fig. 2). No synergy was observed with the combination of rapamycin plus atorvastatin over rapamycin alone.

Echocardiographic measurements, in accordance with the invasive hemodynamics, showed no significant differences in the PA acceleration time [PAAT; a validated measure of mean PAP that shortens as mean PAP rises (20)] or RVH (Fig. 3). MCT increased the percent MT of intraparenchymal resistance PAs from 24.4 ± 1.4% to 40.1 ± 1.6% (Fig. 4). None of the therapies reduced this vascular remodeling.

Phosphorylation of p70 S6 kinase in homogenized lung tissue was induced in MCT-PAH, supporting the rationale for studying rapamycin as a treatment strategy. Moreover, rapamycin treatment largely eliminated phosphorylation of p70 S6 kinase, confirming the adequacy of rapamycin dosing (Fig. 5).

Simvastatin results. The results of simvastatin treatment are shown in Fig. 6. Unlike the atorvastatin treatment, simvastatin was given as a “prevention” strategy (1 day post-MCT), given the negative results with atorvastatin in our protocol with a relatively short duration of therapy. Unlike combination therapy with rapamycin and atorvastatin, or rapamycin monotherapy, simvastatin initially (for 2–4 wk) appeared to significantly improve pulmonary hemodynamics, as measured by echocardiography. RV thickness was also transiently less in the simvastatin group compared with placebo. Likewise, the PAAT was, for several weeks, significantly greater (nearer normal) in the simvastatin group compared with placebo. However, this improvement was not sustained, with all end points deteriorating to levels seen in placebo animals by week 4. Thus, simvastatin therapy delayed, but did not prevent, the development of fatal PAH.

**DISCUSSION**

This study carefully tested promising oral therapies for PAH, using drugs that are approved for human use (for other indications) and that have previously been reported to be beneficial in experimental PAH. In contrast to the hypothesized benefit of rapamycin alone and the proposed synergy from the combination of rapamycin and atorvastatin, neither significantly improved survival or any hemodynamic or morphological surrogate in rats with established MCT-PAH. On March 23, 2007, a PubMed search found reports of 91 studies of therapies for rodent MCT-pulmonary hypertension published since January 1, 2000. Of these, 88 studies demonstrated that the agent studied either prevented or reversed MCT-pulmonary
hypertension; only 3 “negative” studies were found (5, 28, 39). It is very possible that the literature is biased toward positive studies, with negative results being underreported. Although disappointing, these results are consistent with aspects of the study by Nishimura et al. (34), who found that, whereas rapamycin would prevent PAH if given at the same time as MCT, it was ineffective in reversing established MCT-PAH. It is unlikely that higher doses of rapamycin would be useful, as 2.5 mg·kg⁻¹·day⁻¹ is a very high dose compared with doses currently used in patients (maintenance doses of 2 mg/day for adults weighing over 40 kg). Higher doses are typically limited by toxicity. We chose not to test rapamycin as a prevention strategy, as prevention has limited relevance to PAH, a syndrome where presentation and diagnosis occurs almost inevitably relatively late in the course of the disease.

Interestingly, the mitogen-activated kinase p70 S6 kinase was activated in MCT-PAH (Fig. 5), providing a strong rationale for rapamycin therapy. p70 S6 kinase has previously been reported to occur in pulmonary fibroblasts in response to another pulmonary hypertensive stimulus (chronic hypoxia).
(10) and in cancer cells (17, 36) but has not been reported in MCT-PAH. This observation is consistent with the growing literature showing that cancer and PAH share many disordered signaling pathways (50) including activation of the mTOR pathway and p70 S6 kinase (17, 36, 37, 40), activation of hypoxia-inducible factor and decreased expression of voltage-gated potassium channels (3), and mitochondrial hyperpolarization with a glycolytic shift: the “Warburg effect” (2, 26).

It is unlikely that the atorvastatin was underdosed, as the simvastatin dose used in the previous study was 2 mg·kg⁻¹·day⁻¹, and in the present study, atorvastatin, a more potent statin, was dosed at 10 mg·kg⁻¹·day⁻¹. A shorter duration of atorvastatin therapy could theoretically have contributed to the negative results, as the reports of benefit with simvastatin therapy involved administration of the statin from 2 to 6 wk compared with 12 days in our study. There may also be differences in the two models used. Although the MCT dose was 60 mg/kg ip for each study, Nishimura et al. used pneumonectomized rats at sea level versus our nonpneumonectomized rats at 668 m above sea level. Nishimura et al. achieved a mean PAP of ~40 mmHg in vehicle-treated controls versus our model, which achieved a mean PAP of only 34 mmHg. Higher PA initial pressures might offer a greater chance for demonstrating benefit. Additionally, the theoretical possibility exists that rapamycin somehow masked a potential beneficial effect of atorvastatin. This seems unlikely, given the known mechanisms of each drug and their frequent concomitant use in transplant patients and the negative results of the simvastatin experiments.

Because of the unexpected negative results with atorvastatin, which differed from a positive study (35) of similar sample size using simvastatin, we repeated the simvastatin prevention protocols. The goal was to distinguish whether atorvastatin’s failure related to lack of some missing pleiotropic property or discrepancies in the experimental design (use of the statin for regression or the combination with rapamycin). The simvastatin monotherapy experiment tested whether the drug would replicate existing work from other groups and prevent PAH. A more prolonged period of followup than previously published was initially planned to avoid prematurely concluding success in a notoriously progressive PAH model; however, disease progression and spontaneous mortality in both groups precluded adequate sample sizes to measure hemodynamics later.

Fig. 4. PAH was associated with increased resistance PA remodeling (%medial thickness; A), but this was not reduced by either rapamycin or rapamycin + atorvastatin therapy. B: representative images of resistance PAs from the control and treatment groups.

Fig. 5. Rapamycin treatment reduced phosphorylation of p70 S6 kinase (A) in homogenized lung tissue, consistent with adequate rapamycin dosing. MCT increased the phosphorylation of p70 S6 kinase, confirming the rationale for the study. B: intensity of the bands normalized to the intensity of a reporter protein (actin).
than 4 wk [the duration of followup of the Nishimura et al. study (35)]. The duration of followup is a critical factor as MCT-PAH continues to worsen for 4–8 wk, usually leading to death, and thus an intervention might appear beneficial if the followup period was too short.

Our results are in stark contrast to the improvements in pulmonary hemodynamics seen with a previous simvastatin study (35) of MCT-PAH, where mean PAP reductions were seen from 42 to 36 mmHg at 2 wk and to almost normal levels (24 mmHg) at 6 wk, or a study (47) of simvastatin in a PAH model induced by VEGF inhibition and chronic hypoxia, where PAP was reduced from 68 to 49 mmHg. In our model, treatment with simvastatin initially appeared to reduce mortality, but by 2 mo of therapy, it was clear it only reduced death by ~1 wk, far less impressive than the 100% survival rate previously observed (35). Echocardiographic parameters of PAH, including PAAT and RV thickness, were improved by simvastatin therapy at 1 and 2 wk post-MCT, but this effect was not sustained, and, ultimately, rats treated with simvastatin developed severe PAH and succumbed (Fig. 6). This suggests that simvastatin retards but does not reverse MCT-PAH and clearly tempers the enthusiasm for statin therapy in human PAH.

Why would a drug that prevents development of experimental PAH (based on reports in the literature) not cause regression of PAH once the disease is established? Although there are examples in the literature of antiproliferative strategies reversing established MCT-PAH, such as dicholoracetate (27) or the PDGF antagonist STI-571 (45), there is also evidence to suggest that the timing of administration of antiproliferative strategies may be critical. In a study of [3H]thymidine uptake after MCT injection in rats, muscularization of small PAs begins at 3 days and PA smooth muscle cells show a peak uptake at day 5, with less proliferation thereafter, suggesting that the proliferation is most intense early in the syndrome. Perhaps there is a limited window of time, early in PAH, when an antiproliferative strategy is beneficial.

What is the mechanism of benefit for simvastatin, even if small and not durable, in MCT-PAH? The Nishimura et al. study (35), which demonstrated impressive hemodynamic responses to simvastatin therapy, also suggested simvastatin promotes apoptosis of smooth muscle cells in resistance PAs. Accompanying data demonstrated the downregulation of the inflammatory genes fos, jun, and TNF-α and upregulation of the cell cycle inhibitor p27Kip1, eNOS, and bone morpho- genetic protein receptor type 1a. These data implicate several molecular mechanisms. Our experiments demonstrate a significant downregulation of eNOS expression in simvastatin-treated animals (immunoblots not shown), suggesting that any increase in NO by simvastatin would relate to increased bioavailability, not enzyme expression. Other investigators have implicated simvastatin in a RhoA- and RhoB-dependent transcriptional inhibition of preproendothelin-1 (16), which is a precursor to endothelin-1, a potent pulmonary vasoconstrictor.

It is a challenge to reconcile the negative results of simvastatin in our model with the results of simvastatin in other models (35, 47). It may be that the length of followup is important. In our study, we followed rats out to 8 wk of therapy and found, ultimately, no survival benefit, despite results at earlier time points showing improved hemodynamics. Studies of the pneumonectomy and MCT model and the VEGF inhibition and chronic hypoxia model studied shorter durations of therapy: 6 and 4 wk, respectively. There may also be a role for chance and/or differences in molecular pathobiology among models in explaining the differences observed between these relatively small, brief rodent studies. Further work is necessary to explore the mechanism of simvastatin in MCT-PAH and to reconcile our results with those of others.

Even in the present study, simvastatin appears to have more potent beneficial effects on the pulmonary circulation in PAH than does atorvastatin. Simvastatin and atorvastatin have different chemistries, which, in turn, cause different pharmacokinetics and pharmacodynamics and thus different potencies and...
pleiotropic effects (6, 24). Although atorvastatin is generally believed to be more potent than simvastatin in terms of LDL reduction and certain pleiotropic effects, including anti-inflammatory and antioxidant properties, it is possible, although in our opinion unlikely, that simvastatin has more potent pleiotropic effects that are most relevant in MCT-PAH. For example, it has been suggested that simvastatin is a more potent coronary artery vasodilator than atorvastatin, perhaps due to the presence of a lactone ring (13). It might be that simvastatin behaves as a potent pulmonary vasodilator in MCT-PAH instead of an antiproliferative therapy.

Interestingly, there was a trend \( (P = 0.08) \) toward a reduction of RVH with the combination therapy of rapamycin and atorvastatin compared with placebo-treated rats. This was not observed in the rapamycin group, which suggests a possible beneficial effect of atorvastatin in the reduction of RVH. This is consistent with published reports of reductions of LV hypertrophy by atorvastatin in hypertensive rats (51) and a transgenic rabbit model of human hypertrophic cardiomyopathy (46). Whether a reduction in RVH without improvement in pulmonary hemodynamics would translate to a clinical benefit, such as improved mortality, improved functional capacity, or symptom reduction, is unknown.

We conclude that rapamycin does not significantly reverse established MCT-PAH and, therefore, may be of limited benefit in human PAH. Similarly, neither a simvastatin prevention strategy nor a rapamycin and atorvastatin regression strategy are beneficial in MCT-PAH. Perhaps further study of statins in MCT-PAH instead of an antiproliferative therapy.

### REFERENCES


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