Sirolimus and Autophagy Inhibition in Lymphangioleiomyomatosis

Results of a Phase I Clinical Trial

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BACKGROUND: Animal and cellular studies support the importance of autophagy inhibition in lymphangioleiomyomatosis (LAM). In a cohort of subjects with LAM, we tested the hypothesis that treatment with sirolimus and hydroxychloroquine (an autophagy inhibitor) at two different dose levels is safe and well tolerated. Secondary end points included changes in lung function.

METHODS: This 48-week, two-center phase I trial evaluated the safety of escalating oral hydroxychloroquine doses (100-200 mg) given twice a day in combination with sirolimus to eligible patients ≥ 18 years old with LAM. Subjects received combination therapy for 24 weeks followed by an observation phase without taking study drugs for an additional 24 weeks.

RESULTS: Fourteen patients provided written informed consent. Thirteen were treated in cohorts of three patients each with escalating hydroxychloroquine doses (200 and 400 mg) and an extension phase at the 400-mg dose. The most common adverse events were mucositis, headache, and diarrhea. No drug-related serious adverse events were reported. Secondary end points showed improvement in lung function at 24 weeks, with a decrease in lung function at the 48-week time point. When the higher dose of hydroxychloroquine was analyzed separately, FEV₁ and FVC remained stable at 48 weeks, but the 6-min walk distance showed a decrease toward baseline.

CONCLUSIONS: The combination of sirolimus and hydroxychloroquine is well tolerated, with no dose-limiting adverse events observed at 200 mg twice a day. Potential effects on lung function should be explored in larger trials.

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ABBREVIATIONS: 6-MWD = 6-min walk distance; AML = angio-myolipoma; DLCO = diffusion coefficient for carbon monoxide; DLT = dose-limiting toxicity; LAM = lymphangioleiomyomatosis; MTD = maximal tolerated dose; NCI = National Cancer Institute; SGRQ = St George's Respiratory Questionnaire; TSC = tuberous sclerosis complex; VEGF-D = vascular endothelial growth factor D

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Lymphangioleiomyomatosis (LAM), a multisystem disorder primarily affecting women, is characterized by cystic lung destruction, axial lymphatic abnormalities, and abdominal angiomyolipoma (AML).¹⁻⁷ LAM occurs sporadically in patients with no evidence of a germline genetic abnormality and in about one-third of women with tuberous sclerosis complex (TSC).⁸⁻¹⁰ As in TSC, sporadic LAM is thought to be caused by the aberrant proliferation of LAM cells with biallelic mutations in the *TSC1* or, more frequently, the *TSC2* gene.^{11,12}

In LAM cells, mutations in the *TSC1* or *TSC2* gene result in defective regulation and increased activity of mechanistic/mammalian target of rapamycin complex 1 (mTORC1). The Multicenter International LAM Efficacy of Sirolimus (MILES) trial¹³ showed that 1 year of sirolimus therapy was associated with stabilized lung function. Sirolimus is now approved by the US Food and Drug Administration and is recommended for the treatment of LAM.¹⁴ However, during the observation year of the MILES trial, when patients did not receive sirolimus therapy, changes in FEV₁ and FVC in the sirolimus and placebo groups did not differ significantly, which indicates that the beneficial effects of sirolimus are not permanent.¹³ Therefore, there is a need for additional therapy to maintain the benefits achieved with sirolimus.

Methods

Study Subjects

Subjects 18 years of age or older were eligible to enroll if they carried a definite diagnosis of LAM as defined by the European Respiratory Society¹⁹ or cystic lung disease with a VEGF-D level > 800 pg/mL and an FEV₁ < 80% predicted or diffusion coefficient for carbon monoxide (DLCO) < 70% predicted. Further details regarding inclusion and exclusion criteria are listed in e-Table 1.

Study Design

This was a two-site study (Brigham and Women's Hospital, Boston MA, and the National Heart, Lung, and Blood Institute [NHLBI], which was conducted at the National Institutes of Health Clinical Center, Bethesda, MD). The study was designed by the investigators, and the protocol (e-Appendix 1) was approved by the Department of Defense and the Institutional Review Boards at Brigham and Women's Hospital (Partners IRB, protocol No. 2012P000669) and the NHLBI (National Institutes of Health—NHLBI Protocol No. 14-H-0003). All participants provided written informed consent.

This dose-escalation study was designed to enroll two cohorts of patients, the first receiving 200 mg of hydroxychloroquine once a day in addition to sirolimus to keep levels between 5 and 15 ng/mL. Once safety was established at this dose, a second cohort received hydroxychloroquine 200 mg twice a day in addition to sirolimus. All subjects enrolled in this study were treatment naive

Autophagy ("self-eating") is an evolutionarily conserved mechanism through which cells maintain energy homeostasis and "recycle" proteins and organelles. Autophagy allows cells to produce energy and maintain essential molecules through the use of breakdown products.¹⁵ Increased autophagy can promote cell survival, especially in the setting of bioenergetic stress.¹⁵⁻¹⁷ mTORC1 is a known inhibitor of autophagy. We have previously shown that *TSC2*-deficient cells have low levels of autophagy,¹⁸ and blocking mTORC1 with sirolimus might result in increased autophagy and cell survival. We have also shown that the combination of sirolimus and an autophagy inhibitor (chloroquine) resulted in increased cell death in vitro and decreased tumor size in vivo compared with sirolimus alone.¹⁸

Based on these preclinical data we designed the Sirolimus and Autophagy Inhibition in LAM (SAIL) trial, a phase I safety and tolerability dose-escalation study of the combination of sirolimus (2 mg adjusted to keep trough levels between 5 and 15 ng/mL) and hydroxychloroquine (200 mg or 400 mg) taken orally daily in treatment-naïve patients with LAM. Secondary end points included the effectiveness of the combination therapy on lung function, 6MWD, quality of life and serum vascular endothelial growth factor (VEGF)-D levels.

and had not previously received either mTORC1 inhibitors or hydroxychloroquine.

Dose escalation was to be halted if the maximal tolerated dose (MTD) was reached; MTD was defined as one dose level below that at which a dose-limiting toxicity (DLT) was observed in one-third or more of the patients. If one of the three patients in a dose cohort experienced a DLT, three more patients were added to the cohort. If no further DLTs were observed in the group after 28 days, an additional cohort of three patients was enrolled at the next higher dose.

Toxicities were graded using the National Cancer Institute (NCI) Common Toxicity Criteria (version 3). If an NCI grade did not apply, the adverse event was graded as mild, moderate, or severe. DLT was defined as any drug-related adverse event qualifying as NCI grade 3 or 4.

The study design consisted of a screening visit and a 24-week treatment period with sirolimus and hydroxychloroquine, followed by a 24-week observation period with the subjects not receiving the study drugs. Subjects were enrolled consecutively at escalating dose levels.

Secondary End Points

Pulmonary function testing and 6MWD were performed according to established criteria.^{20,21} To assess quality of life, the St George's Respiratory Questionnaire (SGRQ)²² was administered at every visit. Subjects underwent MRI of the kidneys at the screening visit. If

AMLs were detected, subjects then had follow-up MRIs at the end of treatment (week 24) and the end of observation (week 48).

VEGF-D Serum Levels

Peripheral blood samples were obtained at each study visit. Serum samples were collected and stored at -80°F . Serum VEGF-D levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's instructions.

Results

Study Enrollment and Patient Characteristics

From November 2012 through October 2015, a total of 14 patients provided written informed consent to participate in the study. One patient provided informed consent but did not meet all inclusion and exclusion criteria. Thirteen patients were enrolled and received study drugs (Fig 1). Three subjects were enrolled at the first dose level (200 mg of hydroxychloroquine), and ten were enrolled at the second dose level (400 mg hydroxychloroquine). Baseline demographics and clinical characteristics are outlined in Table 1.

Serum Levels of Sirolimus and Adherence to Medication

Sirolimus levels were maintained between 5 and 15 ng/mL (e-Table 2), except for one patient in whom the drug was stopped temporarily for a dental procedure. Subjects were provided with a medication diary, and pill counts confirmed that all subjects were taking hydroxychloroquine as prescribed.

Statistical Analysis

Baseline characteristics and adverse events were summarized descriptively. Repeated-measure analysis of variance was used to assess a change of the secondary outcome variables for the treatment and observation phases separately. VEGF-D values were log-transformed to meet statistical assumptions. All statistical analysis was performed using SAS, version 9.4 (SAS Institute).

Analysis of the Primary End Point: Safety and Adverse Events

No dose-limiting toxicities were encountered at any dose level. A total of 223 nonserious adverse events were reported for 13 patients. One serious adverse event was also reported; left flank pain requiring hospitalization was found to be related to a herniated disk and not related to study drugs. The majority of nonserious adverse events (96.4% [215 of 223]) were rated as moderate (Common Terminology Criteria for Adverse Events grade 2) or lower (Table 2). The majority of adverse events happened during the treatment phase and were deemed to be related to the study drug (78.13% possibly, probably, or definitely related). The most commonly reported nonserious adverse events were rash/acne (15 events in eight subjects), oral mucositis (15 events in eight subjects), and diarrhea (13 events in eight subjects). A complete list of adverse events is provided in Table 3.

Five subjects had their study participation terminated early. One subject who received the lower dose of

Figure 1 – Flow chart of study enrollment: A total of 14 subjects were enrolled at two dose levels of hydroxychloroquine. HCQ = hydroxychloroquine.

TABLE 1] Baseline Demographics of Subjects Who Received Study Drugs

Characteristic	Frequency (%) or Median (Range) or Mean $\pm$ SD
Sex	
Female	13 (100)
Ethnicity	
Not Hispanic or Latino	11 (85)
Hispanic or Latino	2 (15)
Race	
White	12 (92)
Asian	1 (8)
Age, y	49 (40-65)
Postmenopausal women	6 (46)
Pneumothorax	9 (69)
Angiomyolipoma	2 (15)
TSC-LAM	None
Biopsy sample	8 (62)
Chylothorax	1 (7.6)
Pulmonary function test	
FEV ₁ (L)	1.6 $\pm$ 0.7
FEV ₁ (%)	59 $\pm$ 21
FVC (L)	2.8 $\pm$ 0.7
FVC (%)	81 $\pm$ 16
Dlco mL/min/mm Hg	9.8 $\pm$ 3.1
Dlco (%)	43 $\pm$ 15
6MWD, m	442 $\pm$ 103
SGRQ	43.8 $\pm$ 19.2
Log VEGF-D	3.11 $\pm$ 0.33

6MWD = 6-min walk distance; Dlco = diffusion coefficient for carbon monoxide; SGRQ = St George's Respiratory Questionnaire; TSC-LAM = tuberous sclerosis complex-lymphangiomyomatosis; VEGF-D = vascular endothelial growth factor D.

hydroxychloroquine had a marked decline in FEV₁ in the observation phase and was withdrawn from the study by the study team; one subject withdrew because of intractable diarrhea attributable to sirolimus and one subject withdrew because of a skin rash attributable to hydroxychloroquine; and two subjects withdrew to take sirolimus alone. Of note, two subjects experienced QT

prolongation at the end of the treatment phase (one in each dose level). The QT interval returned to baseline after medications were stopped.

Prespecified Secondary End Points

Pulmonary function testing: A secondary objective of the SAIL trial was to determine the effects of the combination therapy on lung function during 24 weeks of combination therapy, followed by the 24-week observation phase. The least square mean of postbronchodilator FVC values (mL) during the treatment and observation phases were not significantly different ($P = .1381$ and $P = .7122$, respectively). However, postbronchodilator FEV₁ (mL) increased significantly during the treatment phase ($P = .0323$) and subsequently declined at the end of observation compared with the end of treatment ($P = .0303$). DLCO did not significantly change throughout the study period (Fig 2, Table 4).

Six-minute walk test: The walk distance increased significantly at the end of the treatment phase compared with the screening visit ($P = .0003$). During the observation phase, walk distance decreased toward baseline and was significantly different than at the end of treatment ($P = .0391$).

Patient-reported outcomes: There was no change in the SGRQ over the treatment or observation phase ($P = .4183$ and $P = .5899$, respectively).

VEGF-D serum levels: Mean serum VEGF-D levels were elevated at baseline (Table 4). Nine of 13 subjects had VEGF-D levels > 800 pg/mL. During the treatment phase, logVEGF-D levels decreased significantly ($P < .0001$), and during the observation phase, logVEGF-D levels increased significantly ($P < .0001$) (Table 4), demonstrating that levels returned to near baseline once treatment was stopped.

Angiomyolipoma Size

Two subjects enrolled at the higher dose level were found to have kidney AML at the screening visit. One subject was withdrawn from the study 2 months after

TABLE 2] Nonserious Adverse Events by Severity for Each Hydroxychloroquine Dose

Severity	Hydroxychloroquine 200 mg	Hydroxychloroquine 400 mg	Total
Mild	39 (88.6%)	134 (74.9%)	173 (77.6%)
Moderate	5 (11.4%)	37 (20.7%)	42 (18.8%)
Severe	0 (0.0%)	8 (4.4%)	8 (3.6%)
Total	44	179	223

TABLE 3] Adverse Events by Body System for Each Dose

Body System	Dose		
	200 mg No. (%)	400 mg No. (%)	Total No. (%)
Cardiac disorders	1 (2.27)	2 (1.11)	3 (1.34)
Eye disorders	0 (0)	2 (1.11)	2 (0.89)
GI disorders	8 (18.18)	40 (22.22)	48 (21.43)
General disorders and administration site conditions	8 (18.18)	12 (6.67)	20 (8.93)
Immune system disorders	0 (0)	1 (0.56)	1 (0.45)
Infections and infestations	7 (15.91)	15 (8.33)	22 (9.82)
Injury, poisoning, and procedural complications	0 (0)	1 (0.56)	1 (0.45)
Investigations	3 (6.82)	36 (20)	39 (17.41)
Metabolism and nutrition disorders	0 (0)	6 (3.33)	6 (2.68)
Musculoskeletal and connective tissue disorders	0 (0)	8 (4.44)	8 (3.57)
Nervous system disorders	2 (4.55)	9 (5.00)	11 (4.91)
Psychiatric disorders	0 (0)	1 (0.56)	1 (0.45)
Renal and urinary disorders	0 (0)	14 (7.78)	14 (6.25)
Reproductive system and breast disorders	3 (6.82)	2 (1.11)	5 (2.23)
Respiratory, thoracic, and mediastinal disorders	1 (2.27)	17 (9.44)	18 (8.04)
Skin and subcutaneous tissue disorders	11 (25.00)	11 (6.11)	22 (9.82)
Vascular disorders	0 (0)	3 (1.67)	3 (1.34)
Total	44 (100)	180 (100)	224 (100)

No. = No. of events; % = percent of adverse events at each dose level.

the screening visit because of skin rash attributed to hydroxychloroquine. The end-of-study MRI did not show any significant change from baseline. The other subject had multiple kidney AMLs. The total AML area was 23.5 cm² at baseline, which decreased to 16.9 cm² at the end of 24 weeks of therapy and increased to 24.4 cm² at the end of 48 weeks of study enrollment.

Subgroup Analysis of Subjects Receiving Sirolimus and 400 mg Hydroxychloroquine

Additional analysis of the group of subjects who received 400 mg of hydroxychloroquine in combination with sirolimus showed that there was no change in postbronchodilator FVC or FEV₁ in both the treatment and observation phases (Fig 2, Table 5).

For this same group, 6MWD increased significantly in the treatment phase ($P < .0001$). In the observational phase of the study, the 6MWD decreased significantly

($P = .0021$) (Table 5). There was, however, no change in the SGRQ throughout the study visits.

Discussion

We report the results of a safety and tolerability study of the combination of an mTOR inhibitor (sirolimus) and an inhibitor of autophagy (hydroxychloroquine) in a treatment-naïve cohort of patients with LAM. The combination was well tolerated at usual doses of sirolimus (levels between 5 and 15 ng/mL) and hydroxychloroquine at two dose levels (200 mg or 400 mg orally daily). The protocol-defined MTD was not achieved, and no dose-limiting adverse event was recorded. The vast majority of adverse events were mild to moderate, with only 3.6% recorded as severe adverse events. The most frequently reported adverse events were GI incidents (diarrhea) and oral mucositis, which were likely due to mTOR inhibition. These adverse

Figure 2 – Changes in lung function over the study period. Shown are mean postbronchodilator FEV₁ (bars represent standard error) at baseline and follow-up visit in the entire cohort (blue line) and the cohort of patients receiving 400 mg hydroxychloroquine (red line). The number of patients for each cohort and each time point for whom FEV₁ data were available is also shown. Visits 1, 2, 3, 4, 5, 6, and 7 correspond to weeks 3, 8, 16, 24 (end of treatment), 36, and 48 (end of observation), respectively.

events were similar to those reported in the MILES trial¹³ and in the study of everolimus in LAM.²³ Of critical importance, no visual adverse events that could be attributed to hydroxychloroquine were noted.

Efficacy was evaluated in secondary end points during a treatment phase over 24 weeks followed by an observation phase for 24 weeks. Results showed that the combination therapy resulted in stabilization or improvement in lung function, similar to that seen with rapamycin alone, as demonstrated in the MILES trial and additional long-term follow-up of patients with

LAM.²⁴ In the observation phase, FEV₁ decreased to baseline levels.

Intriguingly, subjects who received the higher dose of hydroxychloroquine (400 mg) showed no statistically significant difference in lung function (FEV₁, FVC, DLCO values) at the end of observation compared with while receiving treatment. Whether this is related to the efficacy of the combination at the higher dose in achieving a cytotoxic response or to decreased power of the study to detect these differences merits further investigation. At the higher dose level, there was a

TABLE 4] Effects of Sirolimus and Hydroxychloroquine on Outcome Measures During Treatment and Observation Phases

Variable	Baseline ^a	Value at 24 Wk ^a	P Value ^b	Value at 48 Wk ^a	P Value ^c
FEV ₁ , ^d mL	1,772 ± 183	1,892 ± 184	.0323	1,755 ± 186	.0303
FVC, ^d mL	2,933 ± 174	3,091 ± 178	.13	3,047 ± 185	.7122
DLCO (mL/mm Hg/min)	9.79 ± 0.93	10.2 ± .94	.0989	9.83 ± .95	.1766
6MWD	442.5 ± 27.0	484.7 ± 27.3	.0003	460.9 ± 27.7	.0391
SGRQ	43.81 ± 5.41	41.92 ± 5.44	.4183	43.36 ± 5.58	.5899
LogVEGF-D	3.12 ± 0.09	2.91 ± 0.09	< .0001	3.08 ± 0.09	< .0001

See Table 1 legend for expansion of abbreviations.

^aLeast square means ± SEM.

^bBetween baseline and end of treatment.

^cBetween end of treatment and end of observation.

^dAfter bronchodilators.

TABLE 5] Effects of Sirolimus and Hydroxychloroquine (400 mg) on Outcome Measures During Treatment and Observation Phases

Variable	Baseline	Value at 24 Wk ^a	P Value ^b	Value at 48 Wk ^a	P Value ^c
FEV ₁ , ^d mL	1,783 ± 222	1,866 ± 224	.3160	1,771 ± 226	.9400
FVC, ^d mL	2,975 ± 220	3,069 ± 226	.4760	3,094 ± 234	.8653
D _L CO (mL/mm Hg/min)	10.01 ± 1.09	10.50 ± 1.09	.1168	10.25 ± 1.10	.4492
6MWD	429.4 ± 33.9	482.2 ± 34.2	< .0001	442.4 ± 34.5	.0021
SGRQ	47.05 ± 6.18	43.20 ± 6.22	.1283	45.71 ± 6.32	.3668
Log VEGF-D	3.11 ± 0.11	2.91 ± 0.11	< .0001	3.05 ± 0.11	.0003

See Table 1 legend for expansion of abbreviations.

^aLeast square means ± SEM.

^bBetween baseline and end of treatment.

^cBetween end of treatment and end of observation.

^dAfter bronchodilators.

decline (approximately 40 m) in the 6MWD at the end of observation compared with the end of therapy. The minimal clinically important difference in the 6MWD in LAM is unknown. However, studies in COPD and in idiopathic pulmonary fibrosis demonstrate that the minimal clinically important difference is 30 and 24 to 45 m, respectively.^{25,26} The observed change could therefore be clinically significant, although it did not correlate with changes in self-reported quality of life.

The SGRQ has been shown to be a valid tool for assessing quality of life in patients with LAM.²⁷

Surprisingly, our data did not show a significant change in the SGRQ during the treatment or observation phase. Whether this could be related to the short duration of treatment of 24 weeks vs 48 weeks in the MILES trial warrants further investigation.

Serum VEGF-D levels have previously been shown to correlate with disease severity and response to mTOR inhibition.²⁸ In our studies, VEGF-D levels decreased with combination therapy but increased when treatment was stopped, suggesting that combination therapy even at higher doses did not result in a durable suppression of VEGF-D production.

Overall, the observed stability at the higher dose could be due to the lack of power to detect a difference, since the trial was designed as a phase I safety study. Further, the observed changes in VEGF-D and 6MWD after therapy was stopped could be due to inadequate dosing or treatment duration.

Inhibition of autophagy in conjunction with cancer therapy has been shown to be effective in multiple preclinical cancer models.²⁹ There are currently more than 20 clinical trials using hydroxychloroquine to block autophagy in a variety of cancers, and in some

of these studies, hydroxychloroquine is used in conjunction with an mTOR inhibitor. In a study of hydroxychloroquine and sirolimus in combination therapy to treat melanoma, the MTD of hydroxychloroquine was not reached with doses up to 600 mg twice a day, which was also the only dose shown to inhibit autophagy in peripheral blood mononuclear cells.³⁰ In contrast, a combination of proteasome inhibition with autophagy inhibition identified hydroxychloroquine 400 mg daily as a dose sufficient to induce autophagic vacuole formation in bone marrow plasma cells.³¹ In that same study, autophagy inhibition in bone marrow plasma cells and peripheral blood mononuclear cells had very poor correlation, suggesting that different cell types might have different autophagic responses to hydroxychloroquine dosing. In the absence of a lung biopsy, it is a challenge in our study to understand if the higher dose of hydroxychloroquine was sufficient to induce autophagy in LAM cells.

Multiple clinical trials have evaluated the effectiveness of autophagy inhibition in solid malignancies.³⁰⁻³⁸ This is the first report, to our knowledge, of autophagy inhibition in lung disease. Our findings could have important application to other lung diseases such as COPD and idiopathic pulmonary fibrosis in which autophagy has been shown to be altered, and modulating autophagy could have therapeutic benefits.

In summary, the SAIL trial has established the safety of a combination of an mTOR inhibitor with hydroxychloroquine to block autophagy in patients with LAM. The higher dose level showed promising results in durable effects after study drugs were stopped. Larger phase II/III trials are needed to establish the long-term effectiveness of the combination.

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Additional information: The e-Appendix and e-Tables can be found in the Supplemental Materials section of the online article.

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