Chloroquine Prevents Progression of Experimental Pulmonary Hypertension via Inhibition of Autophagy and Lysosomal Bmpr-II Degradation

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ABSTRACT

**Rationale:** Pulmonary arterial hypertension (PAH) is characterised by excessive proliferation and apoptosis resistance in pulmonary artery smooth muscle cells (PASMCs).

**Objective:** We reasoned that chloroquine, based on its ability to inhibit autophagy and block lysosomal degradation of the bone morphogenetic protein type II receptor (BMPR-II), might exert beneficial effects in this disease.

**Methods and Results:** PAH was induced in male Sprague-Dawley rats by administration of monocrotaline (MCT). The induction of PAH was associated with changes in lung expression of LC3B-II, ATG5 and p62, consistent with increased autophagy, and decreased BMPR-II protein expression. Administration of chloroquine prevented the development of PAH, right ventricular hypertrophy and vascular remodelling following MCT, and prevented progression of established PAH in this model. Similar results were obtained with hydroxychloroquine. Chloroquine treatment increased whole lung and PASMC p62 protein levels consistent with inhibition of autophagy, and increased levels of BMPR-II protein. Chloroquine inhibited proliferation and increased apoptosis of PASMCs in vivo. In cultured rat PASMCs we confirmed that chloroquine both inhibited autophagy pathways and increased expression of BMPR-II protein via lysosomal inhibition. Consistent with the in vivo findings, chloroquine inhibited the proliferation and stimulated apoptosis of rat PASMCs in vitro, with no effect on endothelial cell proliferation or survival. Moreover, direct inhibition of autophagy pathways by ATG5 siRNA knockdown inhibited proliferation of rat PASMCs.

**Conclusions:** Chloroquine and hydroxychloroquine exert beneficial effects in experimental PAH. The mechanism of action includes inhibition of autophagy pathways and inhibition of lysosomal degradation of BMPR-II.

**Keywords:** Autophagy, pulmonary hypertension, bone morphogenetic protein Type-II receptor, smooth muscle cells, chloroquine

**Nonstandard Abbreviations:**
- BMPR-II: bone morphogenetic protein type II receptor
- CLQ: chloroquine
- HCQ: hydroxychloroquine
- LV+S: left ventricle plus septum
- MCT: monocrotaline
- PAH: pulmonary arterial hypertension
- PAECs: pulmonary artery endothelial cells
- PASMCs: pulmonary arterial smooth muscle cells
- qPCR: quantitative PCR
- RV: right ventricle
- RVH: right ventricular hypertrophy
- RV/LV+Sep: right ventricular hypertrophy
- RVSP: right ventricular systolic pressure
- TUNEL: terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive disease characterised by a marked elevation in pulmonary arterial pressure and right ventricular hypertrophy. The increase in pulmonary vascular resistance is due to adventitial, medial and intimal thickening of small pulmonary arteries,
resulting from fibroblast, smooth muscle and endothelial cell proliferation. Increased proliferation of pulmonary arterial smooth muscle cells (PASMCs) and resistance to apoptosis is a central feature of diverse forms of PAH. Without treatment, progression of pulmonary hypertension leads to right ventricular failure and death.

Recent studies have highlighted a key role for the transforming growth factor-β/bone morphogenetic protein superfamily in the pathobiology of PAH. Some 6-10% of cases of severe PAH have a further affected family member. Mutations in the bone morphogenetic protein type II receptor (BMPR-II) are now known to underlie at least 70% of cases of heritable PAH and are found in 10-40% of cases of apparently sporadic PAH. Loss of BMPR-II function due to haploinsufficiency or missense mutation reduces signalling via Smad1/5 proteins resulting in loss of expression of key BMP target genes such as the inhibitors of DNA binding transcription factors and failure of growth suppression in PASMCs. Evidence has accumulated that loss of BMPR-II expression or function is an important contributor to PAH pathobiology, even in the absence of BMPR-II mutation. Thus patients with idiopathic PAH, in whom BMPR-II mutation is excluded exhibit reduced lung BMPR-II protein expression. Further studies have confirmed the reduction in BMPR-II expression in PASMCs from patients with idiopathic PAH. Moreover, commonly used rat models of pulmonary hypertension, due to monocrotaline exposure or chronic hypoxia are associated with a marked reduction in lung BMPR-II protein expression. A causal role for loss of BMPR-II in these models is supported by studies showing prevention of pulmonary hypertension following targeted BMPR-II gene delivery to the pulmonary circulation. Recent studies from our lab have shown that cell surface BMPR-II is targeted for ubiquitination and degradation via the lysosome, raising the possibility that lysosomal inhibitors, such as chloroquine, may preserve BMPR-II protein expression. A further well-established action of chloroquine is the inhibition of autophagic protein degradation. By blocking the last step of the autophagy pathway, chloroquine treatment leads to the accumulation of ineffective autophagosomes. In cells reliant on autophagy for survival, for example tumour cells, inhibition of autophagy by chloroquine leads to cell death by apoptosis.

In the present study, we provide evidence for activation of autophagy pathways in the lungs of rats exposed to monocrotaline. Chloroquine inhibited the development and progression of pulmonary hypertension in monocrotaline-exposed rats and inhibited autophagy pathways. In addition chloroquine preserved the expression of lung BMPR-II protein in monocrotaline-exposed animals. In vitro, we confirmed that chloroquine inhibited proliferation and increased apoptosis of rat PASMCs, an effect associated with inhibition of autophagy and increased expression of BMPR-II protein. This study provides evidence that autophagy is involved in experimental PAH and that inhibition of the lysosome may be a novel approach in the treatment of PAH.

MATERIALS AND METHODS

Monocrotaline rat model of pulmonary hypertension.
Male Sprague-Dawley rats (250-300g) were used throughout. All protocols and surgical procedures were approved by the local animal care committee. For the prevention study, animals received a single subcutaneous injection of monocrotaline (MCT) (60mg/kg) at day 0 to induce pulmonary hypertension. To determine the effect of chloroquine or hydroxychloroquine on the development of pulmonary hypertension, rats received chloroquine (20 or 50mg/kg), hydroxychloroquine (50mg/kg) or vehicle, by daily intraperitoneal injection from day 0 to day 20. Chloroquine (N,N'-[4-(7-Chloro-4-quinolinyI)-N,N'-dimethyl-1,4-pentanediameIne diphosphate salt) and hydroxychloroquine (7-Chloro-4-[4-(N-ethyl-N-b-hydroxyethylamino)-1-methylbutylamino]quinoline sulphate) were both purchased from Sigma-Aldrich, Poole, UK. Three weeks following monocrotaline injection, rats were anaesthetised for haemodynamic assessment and lung tissue harvesting. Immediately following haemodynamic assessment, rats were exsanguinated and the lungs were removed for further analysis. One lung was fixed in situ in the distended state by infusion of 10% buffered formalin into the pulmonary artery (at 25mmHg pressure) and trachea for 1 minute. Lungs were then placed in 4%
paraformaldehyde prior to embedding in paraffin. The remaining lung was immediately frozen in liquid nitrogen for protein and RNA isolation.

In further groups of MCT treated rats, the ability of chloroquine to prevent the progression of established pulmonary hypertension was tested. For these experiments animals were administered MCT 40mg/kg, since higher doses were associated with an unacceptably high mortality beyond 3-4 weeks. After 3 weeks animals received daily intraperitoneal injections of chloroquine (50mg/kg), or vehicle, for a further 10 days prior to measurement of haemodynamics and right ventricular hypertrophy.

**Haemodynamic evaluation and right ventricular hypertrophy.**

Animals were anesthetized, body weight was recorded, and a Millar 1.4 French pressure-volume microtip catheter was inserted via the right external jugular vein to record right ventricular pressures. To assess the extent of right ventricular hypertrophy (RVH), the heart was removed and the right ventricle (RV) free wall was dissected from the left ventricle plus septum (LV+S) and weighed separately. The degree of right ventricular hypertrophy was determined from the ratio RV/LV+S. In further groups of animals we assessed the effect of chloroquine on left ventricular function and cardiac output. A Millar 2.0 French pressure-volume microtip catheter was placed into the left ventricle through the right carotid artery. Hemodynamic parameters were collected and analyzed with the PVAN software (Millar Instrument) according to the manufacturer’s instructions. Systemic blood pressure was measured from aortic pressure traces.

**Pulmonary vascular morphometry.**

To determine the degree of muscularization of small pulmonary arteries lung tissue sections were stained with anti-smooth muscle α-actin. At least 20 arteries accompanying alveolar ducts were identified per tissue section. These arteries were scored according to whether they were completely muscular, partially muscular, or non-muscular, as previously described23. In addition, we measured wall thickness in all groups of animals in larger arteries associated with terminal bronchioles (>100µm in diameter), as previously described23.

**Western blotting.**

Frozen lung tissue was homogenised in lysis buffer (250 mM Tris-HCl, pH 6.8, 4% SDS, 20% v/v glycerol and 1x EDTA-free protease inhibitor cocktail - Roche, West Sussex, UK) and sonicated for approximately 1 minute and then centrifuged for 15 minutes at 15,000xg. Cultured rat smooth muscle cells were lysed in lysis buffer (50mM Tris-HCl, pH 8, 150mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS and 1x EDTA-free protease inhibitor cocktail). The protein concentration was determined using the Bio-Rad Lowry assay (Bio-Rad Laboratories, Hemel Hempstead, UK), using BSA as the standard. An equal amount of protein (70µg) from each sample was diluted with 5x sample loading buffer and boiled for 5 minutes. The protein suspensions were separated by SDS-PAGE and transferred to a nitrocellulose membrane and incubated with blocking buffer. Membranes were probe by BMPR-II (1:250, mouse monoclonal antibody, BD Transduction Laboratories, NJ, USA); ATG5 (1:1000, rabbit polyclonal antibody, Novus Biologicals, Littleton, USA); micottubule-associated protein-1 light chain 3 (LC3B) (1:1000, rabbit polyclonal antibody, Abcam, Cambridge, UK); p62 (1:1000, rabbit polyclonal antibody, Sigma-Aldrich, Poole, UK) was used. Additional antibodies for phospho-Smad1/5, Id1 and Id3 were used as previously described14. Blots were then incubated with an appropriate horseradish-peroxidase-conjugated antibody and enhanced chemiluminescence reagent (GE Bioscience, Little Chalfont, UK). To confirm equal loading blots were incubated with an anti-β-actin or α-tubulin antibody (Sigma-Aldrich, Poole, UK).

**Immunohistochemistry.**

Tissue sections were treated in a 0.4mol/L sodium citrate buffer at pH 6 and antigen retrieval performed using microwave (Surgipath, Peterborough, UK) followed by enzymatic digestion with Proteinase K (DakoCytomation, Ely, UK) for 10 minutes. Endogenous tissue peroxidase was quenched using hydrogen peroxidase (HP) blocking solution (DakoCytomation, Ely, UK). Polyclonal rabbit anti-LC3B (1:200, Abcam), polyclonal rabbit anti-Ki67 (1:400, Abcam) and rabbit polyclonal
anti-p62 (1:200, Sigma-Aldrich) were labelled using an affinity purified anti-rabbit streptavidin biotin complex (StreptABC) peroxidase (Vector Laboratories, Peterborough, UK), visualised using 3-3' diaminobenzidine hydrochloride (DAB) substrate (DakoCytomation, Ely, UK) and counterstained in Carrazi's haematoxylin (Bios, Skelmersdale, UK).

**Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) in tissue sections.**

Apoptotic cells were labelled using a TACS® 2 TdT-DAB *In Situ* Apoptosis Detection Kit (R&D Systems, Abingdon, UK). In brief, 10µm sections were deparaffinised, rehydrated and treated with 50µl Proteinase-K prior to *in situ* hybridisation and labelled following the manufacturer's protocol. Positive control material was generated using TACS-nuclease. Sections were counterstained with light green (TCS Biosciences, Buckingham, UK) and examined by light microscopy.

**Real time qPCR.**

Lung mRNA expression of BMPR-II, LC3B and ATG5 was evaluated using real-time quantitative PCR. Frozen lung tissue was homogenised and total RNA was extracted using TRI reagent (Sigma-Aldrich, Poole, UK) according to the manufacturer’s instruction. DNA from each sample was removed by TURBO DNA-free™ DNase treatment and removal reagents (Applied Biosystems, Warrington, UK). Reverse transcription was then performed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Warrington, UK). Synthesized complementary DNA was amplified by a standard PCR protocol using SYBR®-GreenJumpStart™ Taq ReadyMix™ (Sigma-Aldrich, Poole, UK) and rat-specific primers (Online Table I). Parallel amplifications with primers for β-actin (BACT) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed. Cycling conditions were: 3 minutes preincubation at 95°C, 30 seconds denaturation at 95°C, 30 seconds annealing at 58°C, and 30 seconds extension at 72°C for 50 cycles using iCycler (Bio-Rad Laboratories, Hemel Hempstead, UK). The fluorescent product was detected at the end of each cycle. Product specificity was confirmed by agarose gel electrophoresis and routinely by melting-curve analysis. Real-time PCR data were analysed by using iCycler software (Bio-Rad Laboratories, Hemel Hempstead, UK). The ratio of a specific gene to β-actin or GAPDH was calculated in each sample.

**Cell culture.**

Rat pulmonary artery smooth muscle cells (PASMCs) were isolated from small pulmonary arteries, as described previously. ATG5-/- MEFs were obtained as previously described. Human pulmonary artery endothelial cells (PAECs) were purchased from Lonza, Workingham, UK. Cells were maintained in complete endothelial cell growth medium-2 (EGM-2) and were used at passages 4–8. For proliferation studies, early passage PASMCs (passage 2 to 3) were plated in 24-well plates grown to subconfluence, then quiesced in serum-free medium for 24 hours before chloroquine (10µM) or hydroxychloroquine (10 or 30µM) treatment in 10% FCS for 24 hours. 3H-thymidine (0.5µCi) was added for the final 6 hours. For cell counting studies, cells were plated at 2 x 10⁴ cells per well in 24-well plates, adhered overnight in 10% serum, prior to the addition of chloroquine treatment. Medium was changed every 48 hours. Cell counts were performed on days 0, 2, 5, and 7. PAECs were plated into 24-well plates at 2 x 10⁵ cells per well. Cell media was changed every 48 hours. Cell counts were performed on days 0, 2, 5, and 7.

**Apoptosis assays.**

Apoptosis assays were performed in rat PASMCs in the presence and absence of chloroquine. Two methods were used: i) the morphological assessment of nuclear chromatin after Hoechst-33342 and propidium iodide (PI) staining and ii) AnnexinV-FITC apoptosis detection kit (BD Biosciences, NJ, USA). Cells were grown to subconfluence, then quiesced in serum-free medium for 24 hours before the addition of chloroquine (10µM) in SFM, or vehicle. Only AnnexinV-FITC positive and propidium iodide (PI) negative cells were counted as apoptotic cells. PAEC apoptosis was assessed using the AnnexinV-FITC apoptosis detection kit. Cells were plated 2 x 10⁵ cells and after 48 hours treated with chloroquine (10µM) overnight. To induce apoptosis PAECs were treated for 6 hours with
cycloheximide (20μg/ml) and TNFα (3ng/ml), or vehicle. Only AnnexinV-FITC positive and propidium iodide (PI) negative cells were counted as apoptotic cells.

**RNA interference.**

PASMCs were seeded in 6-well plates (7.5 x10⁴ cells/well) for RNA and protein extraction and in 24-well plates (1.5 x10⁴) for cell counting and ³H-thymidine incorporation the day before transfection. PASMCs were transfected with 10nM rat ATG5 siRNA (On-TARGETplus, Dharmacon, LOC365601) or non-targeting siRNA (siCP) (Perbio Science UK Ltd.) in complex with DharmaFECT2™ diluted in Opti-MEM I (Invitrogen, Paisley, UK). Cells were incubated with the complexes for 4 hours at 37°C, followed by incubation with 10% FCS in DMEM for 24 hours. To confirm the efficiency of siRNA knockdown parallel wells were transfected. Specific reduction of the relevant RNA was quantified using qPCR and specific reduction of the relevant protein level was also confirmed by Western blotting.

**Statistics.**

Data are presented as means ± standard error. Data between groups were compared using a two-tailed t-test or a one way analysis of variance followed by Tukey’s HSD test, whichever was appropriate. P<0.05 was considered statistically significant.

**RESULTS**

**Effect of chloroquine and hydroxychloroquine on MCT-induced pulmonary hypertension.**

Three weeks following exposure to MCT rats exhibited increased right ventricular systolic pressure (RVSP) and right ventricular hypertrophy (RV/LV+Sep). Chloroquine administered at both 20 mg/kg and 50mg/kg daily for 3 weeks prevented the elevation of RVSP in MCT exposed rats (Figure 1A and B). Representative right ventricular pressure traces are shown in Figure 1C. In control rats, daily chloroquine treatment (50mg/kg) for 3 weeks had no effect on systolic blood pressure (Figure 1D), heart rate, left ventricular end-diastolic pressure, cardiac output, or other hemodynamic parameters (Online Table II). In MCT-exposed rats, chloroquine significantly increased cardiac output and indices of cardiac contractility (Online Table II). These observations suggest that chloroquine lowered pulmonary vascular resistance without adversely affecting cardiac function. Consistent with this, chloroquine (50mg/kg) significantly inhibited the muscularization of small pulmonary arteries observed in MCT-exposed rats and prevented the increase in wall thickness in arteries >100µm diameter (Figure 1E and F). Administration of hydroxychloroquine (50mg/kg) significantly prevented both elevated RVSP and RV/LV+Sep in monocrotaline treated animals (Online Figure IA-C). Similar to chloroquine, hydroxychloroquine had no effect on systolic blood pressure (Online Figure ID).

In further experiments we evaluated the effect of chloroquine at 50mg/kg on established pulmonary hypertension. Daily treatment with chloroquine was started 3 weeks after MCT exposure and continued for 10 days. Rats developed pulmonary hypertension 3 weeks after MCT exposure. By day 31, vehicle treated rats exhibited a further rise in RVSP (Figure 2A). Chloroquine treated animals had a lower RVSP and RV/LV+Sep compared with the vehicle-treated animals (Figure 2A and B). At day 31 chloroquine treated animals had reduced body weight (BW) by an average of 31.2g (8.9%). Nevertheless, when RV weight was corrected for body weight we still observed a significant reduction in RV/LV+Sep in chloroquine treated rats compared to vehicle treated animals (Figure 2C). Muscularization of pulmonary arterioles was advanced by day 21 following MCT exposure and was not increased further by day 31. Treatment with chloroquine for 10 days did not significantly reverse the degree of distal muscularization (Figure 2D), but did reverse the medial thickening of larger arteries >100µm diameter (Figure 2E).
MCT-induced pulmonary hypertension is associated with increased autophagy, which is inhibited by chloroquine.

The expression of the autophagy markers, LC3B and p62, were determined by immunoblotting in lung samples from control rats and rats treated with MCT/saline, and MCT/chloroquine for 3 weeks. In normal lungs, LC3B-II was present at a low level but increased markedly in the lungs of MCT exposed rats (Figure 3A and B). As expected, chloroquine did not inhibit the expression of LC3B-II since chloroquine, as a lysosomal inhibitor, would favour accumulation of LC3B-II. A more direct indication of the status of autophagy pathways is the expression of p62. Lung p62 expression was reduced following MCT exposure, consistent with the activation of autophagy (Figure 3A and B). Chloroquine prevented the reduction in p62 expression, consistent with inhibition of autophagy (Figure 3A and B). In addition, immunohistochemistry for LC3B-II demonstrated increased staining in the walls of small pulmonary arteries in the lungs of rats following exposure to MCT, compared to saline treated animals (Online Figure II).

Chloroquine inhibits autophagy and proliferation of PASMCS in vivo and induces their apoptosis.

To determine whether the beneficial effects observed with chloroquine could be directly attributed to inhibition of autophagy in PASMCS in vivo, we stained lung sections for p62. In control animals, abundant p62 staining was observed in the media of small pulmonary arteries, as well as the surrounding parenchyma (Figure 3C). Three weeks after MCT exposure (50mg/kg), the thickened media of small pulmonary arteries were devoid of p62 immunostaining. In MCT-exposed animals treated with chloroquine there was partial restoration of p62 expression in medial PASMCS. Quantification of the expression of markers of proliferation (Ki67) and apoptosis (TUNEL) in lung sections (Figure 3D) further confirmed that chloroquine treatment was associated with a reduction in MCT-induced PASMC proliferation (Figure 3D and E, arrowheads) and the induction of PASMC apoptosis (Figure 3D and F, arrowheads) in MCT-exposed rats.

Chloroquine preserves lung expression of BMPR-II.

The expression of BMPR-II protein was reduced in the lungs of MCT-exposed rats, consistent with our previous report. Chloroquine prevented the reduction in BMPR-II protein levels in MCT-exposed rat lung (Figure 4A). MCT exposure led to a reduction in phospho-Smad1/5 activity and a reduction in the protein expression of Id1 and Id3, all of which were partly restored by chloroquine therapy (Figure 4A and B). The expression of BMPR-II mRNA was also reduced in MCT-exposed rat lung, as previously reported (Figure 4C). Chloroquine treatment did not significantly increase BMPR-II mRNA expression, despite the increase in levels of BMPR-II protein, consistent with the notion that the effects of chloroquine on BMPR-II are mainly at the post-transcriptional level (Figure 4C).

Chloroquine inhibits autophagy in rat pulmonary artery smooth muscle cells in vitro.

Having demonstrated the effect of chloroquine administration in vivo, we next sought to establish whether chloroquine inhibits autophagy pathways in isolated rat PASMCs. Serum deprivation of rat PASMCs led to increased expression of LC3B-II protein, as expected (Figure 5A). Chloroquine treatment further increased LC3B-II expression in these cells consistent with the in vivo data. Serum deprivation had a similar effect on other autophagy markers including ATG5 and Beclin-1. Chloroquine also increased expression of LC3B-II and ATG5 mRNA (Figure 5B and C). To determine the impact of chloroquine on protein degradation and lysosome function, we examined the effects of chloroquine on p62, a protein known to be uniquely degraded by autophagy pathways. Serum deprivation markedly reduced p62 levels, which were partly restored by chloroquine treatment (Figure 5A).
Chloroquine inhibits proliferation and induces apoptosis of PASMCs in vitro.

Chloroquine (10µM) profoundly inhibited serum-induced proliferation of rat PASMCs over 7 days in culture (Figure 6A), and inhibited ³H-thymidine incorporation over 24 hours (Online Figure IIIA). Furthermore, hydroxychloroquine (10 and 30µM) administered to rat PASMCs significantly inhibited serum-induced proliferation and ³H-thymidine incorporation (Online Figure IVA and B). Proliferation of human pulmonary endothelial cells (PAECs) was unaffected by chloroquine at 10µM (Figure 6B). Chloroquine also markedly stimulated apoptosis of PASMCs as determined by AnnexinV-FITC analysis and nuclear morphology after 24 hours treatment in serum free media (Figure 6C and Online Figure IIIB). Treatment with chloroquine had no effect on PAEC apoptosis, either under basal conditions, or under conditions where PAEC apoptosis was mediated by TNFα and cycloheximide stimulation (Figure 6D).

Inhibition of autophagy inhibits proliferation of PASMCs.

To confirm the effect of autophagy inhibition on the proliferation of rat PASMCs we employed siRNA silencing of ATG5-12. Knockdown of ATG5 protein was confirmed by immunoblotting and led to a simultaneous reduction in expression of the downstream LC3B-II protein (Figure 7A). Inhibition of ATG5-12 expression by siRNA led to a significant inhibition of serum-stimulated proliferation and a reduction in serum-stimulated ³H-thymidine incorporation (Figure 7B).

Inhibition of autophagy preserves BMPR-II protein expression.

To determine whether the effect of chloroquine on BMPR-II expression observed in vivo could be directly linked to inhibition of autophagy we employed mouse embryonic fibroblasts (MEFs) deficient in ATG5. Compared with wild type MEFs, ATG5−/− MEFs demonstrated increased expression of p62, consistent with inhibition of autophagy in these cells, and increased BMPR-II protein expression (Figure 7C). Furthermore, inhibition of lysosomal function with either chloroquine (10µM) or another lysosomal inhibitor, concanamycin A (50nM), increased the expression of BMPR-II protein in rat PASMCs (Online Figure V).

DISCUSSION

In this study we provide the first evidence that the widely used anti-malarial and anti-rheumatoid drug, chloroquine, prevents the development of MCT-induced pulmonary hypertension and inhibits the progression of established disease. Recent studies have demonstrated that chloroquine and hydroxychloroquine are potent inhibitors of autophagy in cancer and can increase tumour cell death either alone, or can enhance tumour killing in combination with chemotherapeutic agents. We reasoned that chloroquine, via inhibition of autophagy might also demonstrate beneficial therapeutic effects on the course of pulmonary hypertension. Pulmonary hypertension, similar to cancer, is characterised by increased cell proliferation and resistance to apoptosis. Here we show that the development of pulmonary hypertension is associated with increased lung autophagy as evidenced by increased expression of LC3B-II and reduced expression of p62 in the lungs of rats exposed to MCT. In addition, immunohistochemistry revealed increased expression of LC3B-II and reduced expression of p62 in muscularized small pulmonary arteries following MCT exposure, accompanied by increased medial thickness and increased proliferation of PASMCs in vivo. Increased expression of LC3B-II has been reported previously in the lungs of mice exposed to chronic hypoxia and in the lungs of patients with pulmonary hypertension of various aetiologies. This is the first report of activation of these pathways in the MCT rat model, a widely used model for pulmonary hypertension.

Treatment with chloroquine inhibited autophagy in the lungs of MCT-exposed rats, as evidenced by the partial restoration of p62 expression in the lung tissue of these animals. Furthermore, we confirmed that chloroquine treatment restored p62 levels in the media of small pulmonary arteries.
Chloroquine treatment was also associated with inhibition of proliferation and induction of apoptosis in PASMCs in small pulmonary arteries.

We have recently shown that BMPR-II is constitutively degraded by the lysosome in HeLa cells. Chloroquine is known to inhibit lysosomal acidification, accounting for its blockade of autophagy, where the lysosome is the final destination of autophagic vesicles. Inhibition of lysosomal degradation of BMPR-II is another novel mechanism by which chloroquine might exert beneficial effects in pulmonary hypertension. Consistent with this hypothesis chloroquine treatment increased lung protein expression of BMPR-II in MCT rats, with minimal impact on lung BMPR-II mRNA expression. Furthermore, exposure of rat PASMCs to chloroquine, or the specific and potent inhibitor of vacuolar ATPase, concanamycin A, increased BMPR-II protein expression. Moreover, we provide evidence for a direct link between autophagy and BMPR-II expression since ATG5-/- MEFs, which are unable to initiate autophagy, exhibited increased expression of p62 and BMPR-II. A diagram summarising these mechanisms of action of chloroquine in the setting of PASMC proliferation and apoptosis resistance is shown in Figure 8.

Only one previous study has assessed the role of autophagy in rat models of pulmonary hypertension. In that report the authors employed mice deficient in LC3B and showed that lack of LC3B led to increased susceptibility to hypoxia-induced pulmonary hypertension. In addition, LC3B knockdown using siRNA in vitro in PASMCs increased reactive oxygen species production, hypoxia-inducible factor-1α stabilization, and hypoxic cell proliferation. The protective effect of autophagy appeared specific to LC3B in the hypoxic model, since Beclin +/- mice did not demonstrate an exaggerated pulmonary hypertensive response to hypoxia. In supplementary data the same authors reported that chloroquine had no impact on the development of pulmonary hypertension in the monocrotaline rat model or in the hypoxic mouse at a dose of 20mg/kg per day. The authors did not present data to confirm whether chloroquine at that dose impacted on autophagy pathways and the animals were studied only 2 weeks, rather than 3 weeks, following MCT exposure, when minimal pulmonary hypertension had developed. In the present study we clearly demonstrate the impact of chloroquine on autophagy pathways in vivo. Moreover we confirmed that at 3 weeks following MCT exposure chloroquine reduced RVSP in rats at doses of 20mg/kg and 50mg/kg. In addition, we confirmed inhibition of autophagy pathways in vitro by knockdown of ATG5 expression, a key mediator of autophagy, led to inhibition of rat PASMC proliferation and increased PASMC apoptosis, similar to the effect seen with chloroquine.

The role of autophagy in cell survival and death is complex, as is widely recognised in the cancer literature. Autophagy has been shown to be associated with increased susceptibility to tumorigenesis, but also with resistance to anticancer therapies. The type of autophagy depends on the stimulus and a distinction has been made between stress- and starvation-induced autophagy, utilising different components of the autophagy pathway. For example, the cargo protein p62 plays a major role in stress or substrate-induced autophagy. Therefore, it is not altogether surprising that inhibition of autophagy may generate different outcomes in different models of pulmonary hypertension, in particular when considering the use of knockout mice with a background deficiency of autophagy components (e.g. LC3B), compared with the inhibition of autophagy in the adult animal following the induction of pulmonary hypertension, as employed in the present study. The outcome might be very different depending on whether the animal was unable to initiate autophagy, as in the case of the LC3B knockout mouse, compared with the pharmacological inhibition of autophagy in the smooth muscle cells of an animal that had activated autophagy pathways. We would submit that the in vivo model employed in the present study is closer to the potential therapeutic use of such agents in human PAH.

Chloroquine and hydroxychloroquine have been widely used as malarial prophylaxis for over 60 years. They have acceptable toxicity with monitoring and are inexpensive. One of the well-recognized side effects is retinopathy, which occurs in 0.5–1% of patients on long term therapy, though this can be avoided or reversed with screening. More recently these 4-aminoquinolones have emerged as anti-inflammatory agents and are used in the treatment of rheumatoid arthritis, lupus...
erythematous and sarcoidosis and a number of dermatological conditions. They exert a number of potentially beneficial effects that would be favourable in patients with pulmonary hypertension. Inflammation and altered immunity is well recognised in patients with pulmonary hypertension, and predicts a poor prognosis in these patients. Chloroquine and hydroxychloroquine decrease the production of cytokines from T-lymphocytes and monocytes and reduce proinflammatory cytokine levels, including IL-6, in patients with systemic lupus erythematosus. In addition, these drugs are known to improve insulin resistance and the metabolic syndrome, which have been implicated in the pathobiology of pulmonary hypertension. Furthermore, hydroxychloroquine improves vascular function in patients with lupus. There are case reports of aminoquinolones causing cardiomyopathy and arrhythmia following acute administration of high doses, but in the chronic treatment of autoimmune disease this is not observed. Our measurements from animals exposed to 50mg/kg of chloroquine for up to 3 weeks did not reveal any signs of cardiac dysfunction. Indeed, in MCT-exposed animals, chloroquine improved cardiac output and other indices of cardiac function.

In the present study animals were treated with 20 or 50mg/kg daily of chloroquine, comparable to the doses used in murine cancer studies. Hydroxychloroquine is considered to be more potent and better tolerated than chloroquine and is the drug of choice for non-malarial indications. Importantly, both drugs accumulate in tissues over a period of weeks with tissue concentrations being several hundred times that found in plasma. Hydroxychloroquine at a dose of 600mg per day achieves concentrations sufficient for inhibition of autophagy in humans. The promising therapeutic profile of the 4-aminoquinolones, taken together with the present findings, supports the further evaluation of the efficacy and safety of these agents in patients with pulmonary arterial hypertension.

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DISCLOSURES
None.

REFERENCES


FIGURE LEGENDS

Figure 1. Chloroquine attenuates monocrotaline-induced pulmonary hypertension. Bar charts showing mean RV systolic pressure (RVSP) measurements (A), indices of RV weight (RV/LV+Sep) (B), and right ventricle pressure traces (C). Bar chart represents mean systolic blood pressure (D). Bar chart representing the percentage of muscularized pulmonary arterioles at the level of the alveolar ducts plus, example photomicrographs of serial sections of peripheral rat lung containing small arteries from control animals or monocrotaline (MCT) treated rats with saline vehicle or chloroquine (CLQ) for 3 weeks. Sections were immunostained for anti-smooth muscle α-actin (E). All at 100x magnification. Bar = 50μm. Bar chart of the percentage of medial thickening of larger arteries (F). RV/(LV+Sep) indicates ratio of RV free wall to left ventricle plus septum. *P<0.05, ***P<0.01 compared with control; **P<0.05, #P<0.01 compared with monocrotaline- and saline-treated rats.

Figure 2. Chloroquine partially inhibits the progression of established pulmonary hypertension in the MCT model. Bar charts showing mean RV systolic pressure (RVSP) measurements (A), indices of RV weight (RV/LV+Sep) (B), RV/BW (C), the percentage of muscularized pulmonary arterioles at the level of the alveolar ducts (D), and the percentage of medial thickening in larger arteries (E). Rats were exposed to monocrotaline (MCT) for 21 days or 31 days. Animals exposed to MCT for 31 days were treated with chloroquine or saline for 10 days after 3 weeks MCT exposure. *P<0.01 compared with MCT 21 days; **P<0.05 compared with MCT 31 days; ***P<0.01 compared with control.

Figure 3. Autophagy markers are increased in MCT-exposed rat lung. Immunoblots of lung LC3B/II and p62 expression in control animals or monocrotaline (MCT) treated rats with saline vehicle or chloroquine (CLQ) for 3 weeks (A). Densitometry of immunoblots showing quantification of changes in LC3BII and p62 expression (B). Photomicrographs of serial sections of peripheral rat lung containing small arteries from control animals or rats exposed to monocrotaline with saline vehicle or chloroquine (CLQ) for 3 weeks (C and D). Sections were immunostained for the anti-autophagy marker p62 (C), for proliferation using Ki67 and apoptosis using TUNEL (D). All at 100x magnification. Bar = 50μm. Bar chart of the number of Ki67 positive nuclei per vessel from control animals or monocrotaline with saline vehicle or chloroquine (CLQ) (E). Bar chart of the number of TUNEL positive nuclei per vessel (F). *P<0.05, ***P<0.01 compared with control; **P<0.05 compared with monocrotaline- and saline-treated rats 21 days.

Figure 4. Chloroquine restores defective BMP signalling in MCT-exposed rat lung. Immunoblots of lung BMPR-II, phospho-Smad1/5, Id1 and Id3 in control animals or monocrotaline (MCT) treated rats with saline vehicle or chloroquine (CLQ) for 3 weeks (A and B). Densitometry of immunoblots showing quantification of changes in BMPR-II, phospho-Smad1/5, Id1 and Id3 expression (A and B). Lung BMPR2 mRNA expression determined by real-time qPCR in control animals or monocrotaline (MCT) treated rats with saline vehicle or chloroquine (CLQ) for 3 weeks (C). *P<0.05, ***P<0.01 compared with control; ** P<0.01 compared with monocrotaline- and saline-treated rats 21 days.

Figure 5. Chloroquine induces autophagy marker expression in rat pulmonary microvascular smooth muscle cells. Representative immunoblots of LC3BII/II, ATG5-12, Beclin 1, and p62 from rat pulmonary artery smooth muscle cells (PASMC) in growth medium (10% FCS), serum-free medium or serum-free medium with 10μM chloroquine (CLQ) for 24 hrs (A). LC3B (B) and ATG5-12 (C) mRNA expression determined by real-time qPCR in rat pulmonary artery smooth muscle cells (PASMC) in growth medium (10%FCS), serum-free medium or serum-free medium with 10μM chloroquine for 24 hrs. *P<0.05 compared with 10%FCS; #P<0.05 compared with serum-free medium. n=3. C, growth medium (10%FCS); SFM, serum-free medium; CLQ: serum-free medium + CLQ.

Figure 6. Chloroquine inhibits cell proliferation and induces apoptosis in rat pulmonary artery smooth muscle cells. Cell growth curves of rat pulmonary artery smooth muscle cells and human
pulmonary endothelial cells treated with 10μM chloroquine (A and B, respectively). Apoptosis was measured in rat pulmonary artery smooth muscle cells with serum free medium or serum free medium with 10μM chloroquine (CLQ) for 24hrs. Representative flow cytometry scatter plots of propidium iodide (PI) (y-axis) versus AnnexinV-FITC (x-axis) (C; left panel). Bar chart showing average of 3 independent experiments (C; right panel). Apoptosis was measured in human pulmonary endothelial cells after 16 hour 10μM chloroquine treatment in 2% FCS and then 6 hours treatment with cycloheximide (20μg/ml) and TNF (3ng/ml). Representative flow cytometry scatter plots of propidium iodide (PI) (y-axis) versus AnnexinV-FITC (x-axis) (D; left panel). Bar chart showing average of 3 independent experiments (D; right panel). **P<0.05 compared with 10%FCS; ***P<0.001 compared with serum free medium. SFM: serum free medium.

**Figure 7.** Knock-down of ATG-5 inhibited pulmonary artery smooth muscle cells proliferation. Immunoblots of ATG5-12 and LC3BI/II were performed 24 hours post ATG-5 knockdown (A). Cell numbers were counted on days 2, 4, and 7 after knockdown (top panel). 3H-thymidine incorporation was measured 48hrs post ATG-5 knockdown (bottom panel) (B). Representative immunoblots of ATG5-12, BMPR-II and p62 expression in wild type and ATG-/ MEFs (C). *P<0.05 compared with DH, or siCP (siRNA control). SD1/SD2/SD3, pulmonary artery smooth muscle cells from 3 different rats; DH, transfection reagent only; siCP, non-targeting siRNA; siATG5, ATG-5 siRNA.

**Figure 8.** A schematic representation of proposed mechanism for the role of chloroquine in monocrotaline induced pulmonary hypertension. CLQ acts on the PASMC to prevent acidification of the lysosome, thereby preventing correct processing of the autophagosome and preventing degradation of BMPR-II. The resulting restoration of BMPR-II signalling and inhibition of autophagy contributes to a pro-apoptotic, anti-proliferative phenotype in PASMCs.
Novelty and Significance

What Is Known?

- Genetic and non-genetic forms of pulmonary arterial hypertension (PAH) are associated with reduced expression of the bone morphogenetic protein type II receptor (BMPR-II) protein in the lung vasculature.

- Cell surface BMPR-II is susceptible to lysosomal degradation.

- Chloroquine is an inhibitor of autophagy and inhibits autolysosomal degradation pathways.

What New Information Does This Article Contribute?

- Activation of autophagy pathways and loss of BMPR-II protein in the pulmonary hypertensive lung is prevented by chloroquine.

- Chloroquine and hydroxychloroquine can prevent the development and progression of pulmonary hypertension induced by monocrotaline in rats.

- Chloroquine therapy, via inhibition of autophagy, increases apoptosis and inhibits proliferation of pulmonary artery smooth muscle cells (PASMCs) in pulmonary hypertensive arteries.

Despite existing therapies, pulmonary arterial hypertension carries a significant mortality. Mutations in the gene encoding BMPR-II are the most common heritable form of PAH and loss of BMPR-II expression is a feature of non-genetic forms of PAH in humans and animal models of the disease. It has been previously shown that BMPR-II is degraded in lysosomes and that activation of the lysosomal autophagy pathway is a feature of pulmonary hypertensive arteries. We reasoned that inhibition of lysosomal acidification using the antimalarial drug, chloroquine, might provide therapeutic benefit in PAH. In agreement with this hypothesis, we found that chloroquine or hydroxychloroquine prevent the development and halt the progression of pulmonary hypertension in monocrotaline-treated rats. Neither drug adversely affected cardiac function during chronic administration. The pulmonary vascular autophagy pathway was inhibited by chloroquine and this prevented the associated reduction in lung BMPR-II protein. Also, chloroquine markedly inhibited the proliferation and induced apoptosis of PASMCs \textit{in vitro} and \textit{in vivo} via inhibition of autophagy. These findings suggest that antimalarial drugs may represent an effective novel therapy for the treatment of PAH. In addition to its known anti-inflammatory actions, the benefit of this therapy include inhibition of autophagy and restoration of BMPR-II protein levels.
Chloroquine Prevents Progression of Experimental Pulmonary Hypertension via Inhibition of Autophagy and Lysosomal Bmpr-II Degradation

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SUPPLEMENTAL MATERIAL

Online Table I. Primer sequences used for real-time qPCR.

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<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>BMPR2</td>
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<td>5'-GCAGAAACGACGCAACTATCA-3'</td>
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<td>LC3B</td>
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<td>BACT</td>
<td>5'-TGTCACCAAATTGGAGACGATA-3'</td>
<td>5'-ACCCTCATAGATGGGCACAG-3'</td>
</tr>
</tbody>
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Online Table II. Left ventricular haemodynamics

| Group (n) | Heart rate (bpm) | End-systolic Volume (µL) | End-diastolic Volume (µL) | Maximum Pressure (mmHg) | Minimum Pressure (mmHg) | End-systolic Pressure (mmHg) | End-diastolic Pressure (mmHg) | Stroke Volume (µL) | Ejection Fraction (%) | Cardiac Output (µL/min) | dP/dt max (mmHg/s) | Tau_w (ms) | Tau_g (ms) |
|-----------|------------------|--------------------------|--------------------------|--------------------------|--------------------------|---------------------------|--------------------------|------------------|----------------------|------------------|------------------|-----------|
| Control (n=6) |                  |                          |                          |                          |                          |                           |                           |                  |                      |                  |                  |           |
| MEAN      | 478.3            | 69.8                     | 77.9                     | 103.3                    | 3.0                      | 96.9                      | 4.1                      | 18.9             | 23.6                 | 8997.3           | 11758.3         | 9.0        | 12.1       |
| SD        | 27.4             | 8.0                      | 2.3                      | 8.0                      | 0.7                      | 6.9                       | 0.8                      | 3.3              | 4.1                  | 1533.5           | 3146.9           | 1.8        | 1.6        |
| CLQ (n=8) |                  |                          |                          |                          |                          |                           |                           |                  |                      |                  |                  |           |
| MEAN      | 441.4            | 64.0                     | 81.3                     | 105.5                    | 2.9                      | 93.9                      | 5.3                      | 25.3             | 29.2                 | 11492.4          | 10211.5         | 10.4       | 15.1       |
| SD        | 37.4             | 10.4                     | 16.1                     | 28.6                     | 1.2                      | 25.4                      | 1.5                      | 11.7             | 8.5                  | 5923.4           | 4987.3           | 2.8        | 4.1        |
| MCT (n=10) |                  |                          |                          |                          |                          |                           |                           |                  |                      |                  |                  |           |
| MEAN      | 455.0            | 66.0                     | 74.6                     | 93.4                     | 2.3                      | 88.3                      | 4.0                      | 16.2             | 19.4                 | 7467.3           | 8673.4           | 10.4       | 13.5       |
| SD        | 35.2             | 5.9                      | 11.4                     | 10.2                     | 1.1                      | 11.5                      | 1.3                      | 9.2              | 8.7                  | 4462.7           | 2452.6           | 2.8        | 4.3        |
| MCT+CLQ (n=8) |                |                          |                          |                          |                          |                           |                           |                  |                      |                  |                  |           |
| MEAN      | 443.8            | 72.8                     | 96.5#                    | 112.4                    | 8.5                      | 98.2                      | 4.9                      | 30.4#            | 30.8#                | 13541.4#         | 11864.0#        | 11.4      | 15.5       |
| SD        | 27.0             | 6.2                      | 4.2                      | 9.1                      | 9.6                      | 7.8                       | 0.2                      | 3.7              | 3.0                  | 2387.4           | 1759.3           | 2.9        | 3.6        |

* p<0.05 compared to MCT
Online Figure I. Hydroxychloroquine attenuates monocrotaline-induced pulmonary hypertension. Bar charts showing mean RV systolic pressure (RVSP) measurements (A), indices of RV weight (RV/LV+Sep) (B), RV/BW (C) and mean systolic blood pressure (D). RV/(LV+Sep) indicates ratio of RV free wall to left ventricle plus septum. **P<0.01 compared with control; *P<0.05 compared with monocrotaline- and saline-treated rats.
Online Figure II. The autophagy marker, LC3B is increased in MCT-exposed rat lung. Photomicrographs of serial sections of peripheral rat lung containing small arteries from control animals or rats exposed to monocrotaline for 3 weeks. Sections were immunostained for autophagy marker LC3B. All at 100x magnification. Bar = 50μm. *P<0.05, ***P<0.01 compared with control; **P<0.05 compared with monocrotaline- and saline-treated rats 21 days. H&E: haematoxylin and eosin. LC3B: LC3B staining.
Online Figure III. Chloroquine inhibits cell proliferation and induces apoptosis in rat pulmonary artery smooth muscle cells. Bar chart representing ³H-thymidine incorporation; cells were treated with 10μM chloroquine (CLQ) for 24 hours (A). Apoptosis assessed by nuclear morphology changes following H-33342 staining (white arrows indicate apoptotic cells, top panel). Bar chart showing average of 3 independent experiment results (bottom panel) (B). *P<0.001 compared with serum free medium. SFM: serum free medium; CLQ, serum free medium with 10μM chloroquine.
Online Figure IV. Hydroxychloroquine inhibits cell proliferation in rat pulmonary artery smooth muscle cells. Cell growth curves of rat pulmonary artery smooth muscle cells (PASMCs) treated with 10μM chloroquine (CLQ), 10 or 30μM hydroxychloroquine (HCQ) (A). Bar chart representing \(^{3}\text{H}\)-thymidine incorporation. Rat PASMCs were treated with 10μM CLQ, 10 or 30μM HCQ for 24 hours (B). * P<0.05, **P<0.01, ***P<0.001 compared with 10%FBS.
Online Figure V. Rat pulmonary smooth muscle cells were treated with concanamycin A (50nM) and chloroquine (10μM) overnight with appropriate vehicle controls (DMSO and water, respectively). Immunoblotting was performed with a BMPR-II antibody and blots were reprobed with α-tubulin. Bar chart showing BMPR-II fold change after lysosomal inhibition relative to appropriate vehicle control (n=3). **P<0.01 compared with control. ConA, concanamycin A; CLQ, chloroquine.