4PBA Restores Signalling of a Cysteine-substituted Mutant BMPR2 Receptor Found in Patients with PAH

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ABSTRACT

Mutations in the gene encoding the bone morphogenetic protein type 2 receptor, *BMPR2*, are the major cause of heritable pulmonary arterial hypertension (HPAH). Point mutations in the *BMPR2* ligand binding domain involving cysteine residues (such as C118W) are causative of PAH and predicted to cause protein misfolding. Using heterologous overexpression systems, we showed previously that these mutations lead to retention of *BMPR2* in the endoplasmic reticulum, but are partially rescued by chemical chaperones. Here we determine whether the chemical chaperone, 4-PBA, restores *BMPR2* signalling in primary cells and in a knock-in mouse harbouring a C118W mutation. First, we confirmed dysfunctional BMP signalling in dermal fibroblasts isolated from a PAH family segregating the *BMPR2* C118W mutation. Following BMP4 treatment, the induction of downstream signalling targets, Smad1/5, ID1 and ID2, was significantly reduced in C118W mutant cells. Treatment with 4-PBA significantly rescued Smad1/5, ID1 and ID2 expression. Pulmonary artery smooth muscle cells (PASMCs) isolated from the lungs of heterozygous mice harbouring the *Bmpr2* C118W mutation exhibited significantly increased proliferation. In the presence of 4-PBA, hyperproliferation was dramatically reduced. Furthermore, *in vivo*, 4-PBA treatment of *Bmpr2* C118W mice partially rescued *Bmpr2* expression, restored downstream signalling and improved vascular remodelling. These findings demonstrate in primary cells and in a knock-in mouse that the repurposed small molecule chemical chaperone, 4-PBA, might be a promising precision medicine approach to treat PAH in patients with specific subtypes of *BMPR2* mutation involving cysteine substitutions in the ligand binding domain.

INTRODUCTION

Idiopathic and heritable pulmonary arterial hypertension (PAH) are rare conditions affecting 1-2 individuals per million each year with a prevalence of 10-15 per million(1). The condition is progressive and characterised by a marked elevation of pulmonary arterial pressure and right ventricular hypertrophy, often resulting in death from right heart failure. Increased pulmonary vascular resistance results from extensive remodelling of the small pulmonary arteries due to adventitial, medial and intimal thickening resulting from fibroblast, smooth muscle and endothelial cell proliferation(2). Approximately 70% of families with PAH (HPAH), and 15-30% of apparently sporadic idiopathic PAH cases, are caused by heterozygous germline mutations in the bone morphogenetic protein type 2 receptor (BMPR2)(3-6). BMPR2 signalling involves the heterodimerization of the serine/threonine kinase type 2 receptor with BMP type I receptors at the cell surface. In the presence of BMP ligand, the constitutively active BMPR2 phosphorylates the type I receptor, which in turn phosphorylates downstream signalling proteins known as receptor-mediated Smads1, 5 and 8. These Smads complex with a common partner Smad, Smad4, and translocate to the nucleus where they drive the expression of target genes including the family of Inhibitors of DNA-binding (Ids 1-4)(7). Our group and others have shown previously that *BMPR2* mutations cause loss-of-function with reduced downstream Smad1/5/8 signalling, reduced induction of BMP target genes, and altered growth responses to BMPs(8, 9).

Although the majority of heterozygous *BMPR2* mutations lead to haploinsufficiency, approximately 25-30% of mutations comprise missense mutations in highly conserved amino acids in functionally important domains(10). We have previously reported in heterologous overexpression studies that point mutations causing cysteine substitutions within the ligand-binding domain cause retention of the receptor in the endoplasmic reticulum (ER) and reduced trafficking of mutant BMPR2 to the cell surface(11). There are 10 cysteine residues residing in the extracellular ligand-binding domain of BMPR2 that form 5 disulphide bonds, essential to efficient 3-dimensional folding of the protein(12). Three of the disulphide bridges in the extracellular domain are highly conserved and are critical for the three-finger toxin fold(13). It is possible that the buried disulphide bridges, C34-C66 and C118W-C123,

in BMPR2 are important for the correct folding rate of the protein(12). Thus, a mutation in one of these cysteine residues is likely to reduce the efficiency of folding. It is also possible that the free sulphydryl group generated by a cysteine substitution leads to interaction with chaperone components of the ER or associated BMP type I receptors leading to inefficient trafficking(12). In support of this, we previously showed that cysteine missense mutations in BMPR2 prevented normal trafficking of type I receptors(14).

Research from our laboratory has demonstrated that the cysteine-substituted extracellular domain mutant of BMPR2, C118W, possesses active kinase signalling(11), and is responsive to BMP ligands if correctly trafficked to the cell surface(14). Improved trafficking of mis-localised proteins has previously been explored in conditions such as nephrogenic diabetes insipidus and cystic fibrosis. Treatment using molecular or chemical chaperones (pharmacoperones) has been used to correct the misfolding of the vasopressin receptor(15-17) and cystic fibrosis transmembrane regulator (CFTR)(18-21). Furthermore, randomised clinical trials in cystic fibrosis using the chemical chaperone, sodium 4-phenylbutyrate (4PBA) improved the function of misfolded CFTR and provided some clinical benefits(22, 23). Previously, we examined the beneficial impact of chemical chaperones, in transient and stable overexpression cell lines, on the trafficking of mutant BMPR2(C118W)(14). 4PBA increased BMPR2(C118W) cell surface levels and improved downstream Smad signalling. Furthermore, Frump and colleagues investigated trafficking defects in mouse endothelial cells possessing an in-frame deletion of exon 2 in BMPR2(24). Similar, to the observation with cysteine substituted BMPR2, this mutation exhibited defective trafficking to the cell surface. Treatment with 4PBA rescued trafficking of the receptor to the cell surface. In addition, treatment with 4PBA improved downstream signalling in mouse pulmonary endothelial cells(24).

With a view to development of 4PBA as a precision medicine approach for patients with cysteine-substituted *BMPR2* mutations affecting the ligand binding domain, we sought to provide further evidence for target engagement of 4PBA on mutant BMPR2 in patient-derived cells and *in vivo* in a representative genetic mouse model.

MATERIALS AND METHODS

Cell Culture and Treatments. Human dermal fibroblasts from 3 individuals harbouring the BMPR2 C118W mutation were kindly provided by Dr. Eric Austin (Vanderbilt University, Nashville, TN, USA) and were cultured in DMEM (20% FBS and Antibiotic-Antimycotic (A/A; 100 U/mL penicillin, 100 mg/mL streptomycin and 0.25 mg/mL amphotericin B, Invitrogen)). Control dermal fibroblasts were a kind gift from Professor David Abraham (University College London, London UK). For experimental studies cells were used between passages 4 and 7. Mouse PASMCs were isolated from small pulmonary arteries, as described previously(25). HeLa cells were cultured in DMEM (10% FBS and A/A). All cell culture was conducted at 37 °C with 5% CO₂.

Human fibroblasts and mouse smooth muscle cells were quiesced in DMEM containing 0.1% FBS and A/A overnight prior to treatments. Recombinant human BMP4 was purchased from R&D Systems (Oxfordshire, UK). Cells were stimulated with BMP4 (10 ng/mL unless otherwise stated) for 4 or 24 hours. Sodium 4-phenylbutyrate (4PBA) was purchased from Sigma-Aldrich (Poole, Dorset, UK). Cells were treated overnight with 5 mM 4PBA (unless otherwise stated).

Plasmid transfection. The 5'-Myc-tagged BMPR2 wild-type and 5'-Myc-tagged mutant C118W constructs were prepared as previously described(14). Confirmation of receptor and tag sequences of the wild-type and mutant constructs was conducted using an ABI377 sequencer with the Applied Biosystems Big Dye terminator kit DNA sequencing.

Plasmid constructs were prepared using a Plasmid EndoFree Maxi Kit (Qiagen, West Sussex, UK) and transient transfections were performed using Lipofectamine 2000 transfection reagent (Invitrogen), according to the manufacturer's protocol. Cells were incubated for at least 24 hours before media exchange and transgene expression was assayed 48 hours post-transfection.

Cell proliferation. Mouse PASMC cells were seeded at 20,000 or 30,000 cells/well in 24-well plates and left to adhere overnight. Cells were then exposed to the stated treatments in DMEM containing

20% FBS and A/A and replenished every 48 hours. At the relevant time points, cells were trypsinised and counted on a haemocytometer using trypan blue exclusion to assess cell viability.

Immunoblotting. Frozen lung tissues were 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 prior to centrifugation for 15 minutes at 15,000 x g. Fibroblasts and PSMCs were lysed in lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS and 1x EDTA-free protease inhibitor cocktail). Using the Bio-Rad Lowry assay (Bio-Rad Laboratories, Hemel Hempstead, UK), protein concentrations were determined against a BSA standard. Cell lysates (20-100 µg protein) were separated by SDS-PAGE. Using semidry blotting proteins were transferred to polyvinylidene fluoride membranes (GE Healthcare, Buckinghamshire, UK). Membranes were blocked and probed with rabbit polyclonal antibodies for total Smad1; rabbit monoclonal antibodies toward phosphorylated Smad1/5 (clone 41D10, both Cell Signaling Technology, Danvers, MA); Id1 (clone 195-14); Id3 (clone 17-3, both CalBioReagents, San Mateo, CA) or mouse monoclonal antibodies against BMPR2 (clone 18/BMPR-II, BD Transduction Laboratories, Wokingham, Berkshire, UK). After washing, blots were incubated with secondary anti-mouse/rabbit horseradish peroxidase antibody (Dako, Cambridgeshire, UK) for 1 hour at room temperature. Where stated all blots were re-probed with a monoclonal antibody for either α -tubulin (clone DM1A, Sigma-Aldrich) or β -actin (clone AC-15, Sigma-Aldrich) as a loading control. Enhanced chemiluminescence was used to detect proteins (GE Healthcare) and Image J software was used to assess densitometry.

Cell surface biotinylation. As detailed previously, cell surface biotinylation was conducted as follows(26). Following plasmid transfection HeLa cells were cultured for 48 hours prior to treatment. After 4PBA treatment, cells were placed on ice prior to washing twice with ice-cold PBS. Cells were washed once with PBS pH 8 containing 1 mM MgCl₂ and 0.1 mM CaCl₂ (PBS-pH8), prior to incubation for 10 minutes at 4 °C with freshly prepared EZ-Link™ NHS-SS-biotin at 3 mg/ml (Perbio Science) in PBS-pH8. Biotinylation was terminated by quenching any unbound NHS-SS biotin by washing twice with PBS containing 1% BSA. Followed by 10 minutes incubation on ice. After washing with PBS cells were lysed with 1 ml of PBS (containing 1% Triton X100 and 1x EDTA-free protease

inhibitor cocktail). 900 µl of lysate was incubated with avidin-agarose beads (Pierce Protein Research Products, Rockford, IL, USA) by rotation overnight at 4 °C. Total protein concentration was assessed using the remaining 100 µl. Prior to SDS-PAGE, the biotin–avidin agarose complexes were centrifuged and washed three times with lysis buffer before resuspending in 5× sample loading buffer and boiling for 5 minutes.

RNA Preparation and Quantitative RT-PCR. Following the manufacturer's protocol, total RNA was extracted using the RNeasy Mini Kit with DNase digestion (Qiagen) and cDNA prepared from ~1 µg of RNA using the High Capacity Reverse Transcriptase kit (Applied Biosystems, California, USA). All qPCR reactions were prepared in MicroAmp® optical 384-well reaction plates (Applied Biosystems). cDNA (50 ng/µl) was mixed with SYBR®Green Jumpstart™ Taq Readymix™ (Sigma-Aldrich), ROX reference dye (Invitrogen) and custom sense/anti-sense primers (all 200 nM) with reactions amplified on a QuantStudio 6Flex Real-Time PCR system (Applied Biosystems). Primers for human: *BMPR2*, *HPRT* (encoding hypoxanthine phosphoribosyl transferase), *ID1*, *ID2* and *SMAD9* were all designed using Primer3 (<http://primer3.sourceforge.net/>).

***Bmpr2*^{C118W} knock-in mouse model.** In accordance with the UK Animals (Scientific Procedures) Act 1986 and approved under Home Office Project License 80/2460 all animal work was conducted as follows. Based upon our past experience with rodent PAH models group sizes were determined using estimates of variance and minimum detectable differences. Mice were assigned unique animal identification numbers and randomized allowing investigators to be blinded when performing all cardiopulmonary phenotyping procedures and histological analyses.

The *Bmpr2*^{C118W} knock-in mouse model was generated by GenOway (Lyon, France). Supplementary Figure 1 depicts an overview of the strategy used to generate the C118W mutant. The murine *Bmpr2* locus (Supplementary Figure 1A) and targeting construct carrying the wild-type exon 3 in a sense orientation (3' sense) and a C118W mutated version of exon 3 in an antisense orientation (3' a-sense) (Supplementary Figure 1B). The construct also carried an FRT flanked Neomycin positive selection cassette and heterotypic Lox sites were positioned to allow inversion of the wild-type 3' sense and

mutated 3' a-sense sequences, reversing their orientation following the activity of CRE recombinase. The targeted locus following homologous recombination via the homology arms flanking the targeting cassette (Supplementary Figure 1C). Targeting was carried out in mouse embryonic stem cells and these targeted cells were used to create chimeras, which were bred to produce heterozygous and homozygous mice following standard procedures. Supplementary Figure 1D shows the *Bmpr2* locus in C118W mutant mice following deletion of the Neomycin cassette (by crossing to a *Rosa26:FLIPase* mouse) and crossing to a *Rosa26:Cre* transgenic mouse. Following recombination of lox sites via the action of CRE recombinase, the opposite directional positioning of the *Lox511* sites resulted in the inversion of the wild-type 3' sense and mutated 3' a-sense sequences, bringing the mutated exon 3 into the correct sense orientation matching the rest of the locus (Supplementary Figure 1E). The action of CRE recombinase on the LoxP sites resulted in deletion of the wild-type exon 3 from the *Bmpr2* locus. The locus now produces *Bmpr2* transcripts carrying the C118W mutation rather than wild-type transcripts. Genotyping and sequencing confirmed that heterozygous mice had the expected 'knock-in' manipulation of the *Bmpr2* locus (Supplementary Figure 1F). After Sanger DNA Sequencing, *Bmpr2* and *Bmpr2*^{C118W} sequences were visualised using SnapGene® (GSL Biotech LLC, Chicago, IL, USA).

Littermate female controls (*Bmpr2*) and C118W knock-in (*Bmpr2*^{C118W}) mice between 7-11 months of age were randomized into two groups. One group received 4PBA by oral gavage at 500 mg/kg⁻¹ twice daily (Cayman Chemical, Ann Arbor, Michigan, USA) for 2 weeks, diluted in Dulbecco's phosphate-buffered saline (Sigma-Aldrich) and the other group received vehicle alone.

Mice were anesthetized with isoflurane inhalation. Catheterisation of the right ventricle was undertaken via cannulation of the right internal jugular vein using a Millar SPR-139 catheter (Millar Instruments, Houston, TX) connected to PowerLab hardware utilising LabChart 8 (ADInstruments, Oxford, UK). Assessment of right ventricular hypertrophy (RVH) was performed by dissecting the right ventricle (RV) free wall from the left ventricle plus septum (LV+S) prior to weighing separately with the degree of RVH determined from the ratio of RV/LV+S.

Immediately following haemodynamic assessment, mice were exsanguinated and the lungs were removed for further analysis. Lungs were either fixed *in situ* in the distended state by infusion of 10% buffered formalin into the pulmonary artery (at 25 mmHg pressure) via the trachea and overnight fixation in 4% formalin prior to embedding in paraffin, or immediately frozen in liquid nitrogen for protein isolation.

Assessment of Pulmonary Vascular Muscularization in Mouse Tissues. In order to assess pulmonary arteriolar muscularization, sections of fixed mouse lung tissue (5 μm in thickness) were labelled with monoclonal mouse-anti-smooth muscle α -actin (SMA) (clone 1A4, Dako, Glostrup, Denmark), followed by polyclonal goat anti-mouse HRP. To detect staining the Dako ARK™ kit (Dako) was used in accordance with the manufacturer's instructions. Antibody staining was visualised using 3-3' diaminobenzidine (DAB)–hydrochloride as substrate-chromogen and counterstained with Carrazzi haematoxylin.

Pulmonary arteriolar muscularization was assessed by the identification of alveolar ducts and categorization of the accompanying intra-acinar artery as non-, partially or fully muscularized, by the degree of SMA immunostaining. A minimum of 20 vessels with diameters ranging from 25 to 75 μm were categorized as either fully, partially or non-muscularized.

Wall thickness was evaluated by the identification of small arteries (<100 μm) proximal to the terminal epithelial bronchioles. Using ImageJ the diameter and thickness of the artery was measured after immunostaining for SMA. Thickness measurements were taken in four different positions of the artery and a minimum of 10 arteries were assessed in each lung section.

Assessment of muscularisation and wall thickness were performed in a blinded fashion by a single researcher, to reduce operator variability, who was not aware of the group allocation of the samples being analysed.

Statistics. Using GraphPad Prism data were analysed by one-way/two-way ANOVA with post-hoc Tukey's HSD analysis or unpaired two-tailed Student's *t*-test (Mann-Whitney) where indicated. Data are presented as mean $\pm$ S.E.M and $P < 0.05$ was considered significant.

RESULTS

Dermal fibroblasts harbouring *BMPR2* C118W mutations exhibit impaired BMP signalling

Dermal fibroblasts were derived from three related individuals harbouring the *BMPR2* C118W mutation. At the time of isolation, two of these had been diagnosed with PAH (Table 1). We assessed downstream signalling in the presence of BMP ligand and compared the response to wild-type dermal fibroblasts. After 24 hours, Smad1/5 phosphorylation was significantly reduced in BMP4 treated C118W mutant cells at both 10 and 50 ng/ml of BMP4, but not after 4 hours BMP4 treatment (Figure 1A and B). Furthermore, downstream target gene expression was significantly reduced in the mutant cells treated with BMP4 for 24 hours. Both, ID1 and ID2 mRNA expression was significantly reduced compared to wild-type controls (Figure 1C and D).

Sodium 4-phenylbutyrate improves *BMPR2* C118W signalling in dermal fibroblasts

We had previously showed that 4PBA improved C118W trafficking and downstream signalling in heterologous overexpression studies(14). We therefore determined the efficacy of 4PBA on the cells derived from individuals harbouring the C118W mutation. A concentration response study was conducted in the presence of BMP4 to determine the effect of 4PBA on target gene expression. Dermal fibroblasts were treated with 10 ng/ml BMP4 as well as 0.5, 1 and 5 mM 4PBA for 24 hours. Firstly, the induction of ID1 and ID2 gene expression was significantly impaired in mutant cells compared to control dermal fibroblasts (Figure 2A and B). Treatment with 5 mM 4PBA rescued both ID1 and ID2 mRNA expression in the C118W mutant cells (Figure 2A and B). In particular, ID2 appeared to be subject to a dose response effect (Figure 2B). These studies agreed with our previous observations that 5 mM 4PBA was sufficient to enhance C118W trafficking in the heterologous expression studies(14).

BMPR2 mRNA expression was enhanced by BMP4 treatment (Figure 2C). However, no effect on BMPR2 gene expression was observed in the 4PBA treated cells (Figure 2C).

We further confirmed the ability of 4PBA to enhance downstream signalling in the mutant cells by assessing Smad1/5 phosphorylation. Both wild-type and C118W mutant cells were treated with 10 ng/ml BMP4 for 24 hours. As seen in the gene expression studies treatment with 4PBA at 5 mM significantly restored Smad1/5 phosphorylation in cells harbouring the C118W mutation (Figure 2D and E).

Unlike, the Smad1/5 phosphorylation, significant reduction in downstream targets ID1 and ID3 was observed after 4 hours BMP4 treatment in the C118W mutant cells (Supplementary Figure 1A and C). Although, ID2 wasn't significantly reduced there was a trend towards reduced signalling (Supplementary Figure 1B). In the presence of 4PBA at 5 mM all IDs expression was either rescued or enhanced (Supplementary Figure 1A, B and C).

Using heterologous overexpression of wild-type and mutant C118W BMPR2 we were able to confirm our previous findings that 4PBA increased trafficking to the cell surface using receptor biotinylation. Transfected cells were subjected to a timecourse of 4PBA treatment. After 1.5 hours, 4PBA increased myc-tagged mutant C118W receptor (Supplementary Figure 3). After 24 hours, the relative expression of the mutant receptor was significantly reduced compared to the wild-type control (Supplementary Figure 3).

4PBA improves BMPR2 expression and downstream signalling in *Bmpr2*^{C118W} mice

To further understand the implications of the loss of the cysteine residue in the extracellular domain, a knock-in mouse harbouring a heterozygous C118W mutation (*Bmpr2*^{C118W}) was generated. Previous studies in transfected cells have shown that mutations in the extracellular cysteine residues lead to a partially glycosylated precursor on immunoblotting(11, 27). We confirmed that this hypoglycosylated form was also present in the lungs of *Bmpr2*^{C118W} mice (Figure 3A), consistent with the mutant receptor undergoing incomplete post-translational modification. As expected, there were no differences in total

levels of BMPR2 protein between mutant animals and littermate controls (Figure 3B). Furthermore, as previously reported(24), the wild type BMPR2 undergoes a mobility shift after treatment with PNGase-F (Supplementary Figure 4), whereas it was resistant to deglycosylation by Endo-H glycosidase, this is consistent with the majority of the protein being located in the post-Golgi compartment. In contrast, the mutant C118W BMPR2 appeared to be more sensitive to Endo-H indicating that the cysteine substitution mutant BMPR2 would be retained in the ER (Supplementary Figure 4). A mobility shift in BMPR2 was also observed in PASMCs isolated from the lungs of the *Bmpr2*^{C118W} mice (Figure 3C). However, unlike in the total lung lysates from the C118W mutant animals there appears to be three bands potentially corresponding to the glycosylated, partially glycosylated and unglycosylated form of the receptor. The majority of the observed mutant Bmpr2 was the partially glycosylated form (Figure 3C). As previously stated by others, cysteine mutations in the ligand binding domain predominantly express the faster migrating bands, such as the partially glycosylated and unglycosylated forms(28). Again, no difference was observed in total Bmpr2 protein expression between control and *Bmpr2*^{C118W} derived mouse PASMCs (Figure 3D).

Unlike other previously reported BMPR2 genetic models of PAH, such as the R899X knock-in or transgene models(29, 30), the *Bmpr2*^{C118W} mice did not spontaneously develop a robust PAH phenotype. No differences in right ventricular systolic pressure (RVSP) and right ventricular hypertrophy were identified (Supplementary Figure 5A and B).

To determine whether 4PBA exposure could rescue the processing of *Bmpr2*^{C118W}, mutant and control mice were treated with 4PBA (500 mg/kg), or PBS, twice daily by oral gavage for two weeks. Bodyweight of all animal cohorts remained consistent throughout the duration of the study (Supplementary Figure 6A). As before no changes in right ventricular hypertrophy were observed in mutant mice treated with PBS or 4PBA (Supplementary Figure 6B).

We investigated whether 4PBA improved downstream BMP signalling in the mutant mouse model. Both Id1 and Id3 expression were significantly reduced in the lungs of the mice harbouring the C118W mutation (Figure 5A and B). No differences in BMPR2 expression was observed (Figure 5A and B).

Both Id1 and Id3 expression were increased by 4PBA treatment in the *Bmpr2*^{C118W} mice although Id3 did not quite reach significance (Figure 4A and B). Somewhat surprisingly the overall protein expression of Bmpr2 was significantly improved by 4PBA treatment (Figure 4A and B). Nevertheless, in *Bmpr2*^{C118W} animals treated with 4PBA expression of the partially glycosylated band was significantly increased with no significant change in the lower unglycosylated band (Figure 5A and B).

We assessed the vascular remodelling in the *Bmpr2* and *Bmpr2*^{C118W} animals treated with PBS or 4PBA by examining pulmonary artery wall thickness as a percentage of luminal area and muscularisation as a percentage of the alveolar and duct arteries. Firstly, the pulmonary artery wall thickness was unaffected in the mutant animals treated with either PBS or 4PBA (Supplementary Figure 6C). Although not statistically significant, there was a trend towards an increase in alveolar duct artery muscularisation in *Bmpr2*^{C118W} mice compared littermate controls (Figure 6A). However, treatment with 4PBA reduced muscularisation in mutant mice with a statistically significant increase in non-muscularised vessels and decrease in partially muscularised vessels (Figure 6B and C, respectively). Fully muscularised vessels were not significantly changed (Figure 6D).

The hyperproliferative phenotype of *Bmpr2*^{C118W} PASMCs is rescued by 4PBA

We have previously demonstrated that hyperproliferation of PASMCs is a hallmark of the heritable and idiopathic PAH (8). We therefore investigated the proliferative status of isolated PASMCs. Similar to the observations in total lung lysates, Id3 expression was significantly reduced in PASMCs derived from the *Bmpr2*^{C118W} mice (Figure 7A and B). PASMCs from mice harbouring the C118W mutation proliferated to a greater extent than control cells (Figure 7C). Next, PASMCs were treated with 1 mM 4PBA or PBS prior to cell growth assessment and drug exposure was maintained throughout the time course. Again, C118W mutant cells were hyperproliferative compared to cells from wild-type littermates (Figure 7D; open circles). However, in the presence of 4PBA, proliferation was restored to the rate of control cells (Figure 7D; closed circles). Interestingly, 4PBA treatment did not restore the growth rate of another disease associated BMPR2 mutation (R899X) indicating the specificity of the

chemical chaperone treatment on specific subtypes of *BMPR2* mutation in the extracellular domain (Supplementary Figure 7).

DISCUSSION

Mutations in *BMPR2* are the most common genetic cause of pulmonary arterial hypertension(5, 31). Although the majority of *BMPR2* mutations lead to a state of haploinsufficiency, missense substitutions of cysteine residues in the extracellular domain comprise an important class of mutations. We have previously shown that these mutations result in the retention of the receptor in the endoplasmic reticulum(11, 14). Chemical chaperones such as glycerol, thapsigargin and sodium 4-phenylbutyrate (4PBA) rescued the trapped receptor and improved downstream signalling in heterologous overexpression systems(14). Here, we investigated the efficacy of 4PBA, a drug already in clinical use, in human primary cells harbouring the representative human disease causing C118W mutation, as well as in a mouse knock-in model harbouring the same mutation.

As observed by others(24, 28), the glycosylation status of BMPR2 was affected by the cysteine substitution with a lower molecular weight receptor observed in lung homogenates from *Bmpr2*^{C118W} mice. A lower molecular weight band was observed suggesting a non-glycosylated form of the receptor. Ordinarily, wild-type BMPR2 comprises a mature glycosylated protein and partially glycosylated precursor. Previous research has reported that cysteine mutations cause the predominant form of the receptor to be the precursor(28). Using the N-linked glycosidases Endo-H and PNGase-F, we sought to determine whether the *Bmpr2*^{C118W} mutant receptor was able to traffic through the endoplasmic reticulum and Golgi apparatus. N-linked glycoproteins processed in the ER are sensitive to Endo-H glycosidase, but having passed through the Golgi become resistant(32). However, PNGase-F does not discriminate against the maturation status of the N-linked glycoproteins when cleaving glycan residues. Glycosidase treatment suggested that the mutant form of the receptor would be trapped in the ER as it was sensitive to Endo-H. We also observed the predominant lower molecular weight form of BMPR2 in pulmonary artery smooth muscle cells (PASMCs) isolated from mice expressing the C118W mutation.

We subjected control and mutant *Bmpr2* C118W animals to 4PBA treatment to investigate whether BMPR2 trafficking and signalling could be restored in a genetic mouse model. 4PBA was chosen for these studies because it is orally bioavailable and already in use in the clinic. In mice, 4PBA reduced the expression of the partially glycosylated precursor of BMPR2. Furthermore, expression of downstream signalling molecules was substantially increased. Therefore, it is likely that release of misfolded and partially glycosylated mutant BMPR2 protein was sufficient to rescue important downstream effectors.

4PBA has been used principally in the treatment of urea cycle disorders as an ammonia scavenger having been approved by the FDA in 1996(33, 34). However, its role as a chemical chaperone has seen it proposed as a treatment for cystic fibrosis(22, 23), because the most common deletion of phenylalanine at amino acid 508 leads to retention of the cystic fibrosis transmembrane regulator (Δ F508-CFTR) in the endoplasmic reticulum(35-37). Mutant CFTR retains the ability to transport chloride suggesting that correction of defective trafficking would be important therapeutic target(38). Rubenstein and colleagues postulated that improved trafficking by 4PBA could be due to differential effects on the ER stress induced heat shock protein homologs, Hsc70 and Hsp70. Although, highly homologous Hsc70 and Hsp70 appear to have differing cellular effects(39, 40). In respect of Δ F508-CFTR it was proposed that Hsc70 inhibits maturation and promotes endoplasmic reticulum associated degradation (ERAD)(40-42). Whereas, overexpression of Hsp70 appeared to increase maturation of mutant CFTR(43). 4PBA treatment decreased Hsc70 gene and protein expression, but increased Hsp70 expression and interaction with CFTR in Δ F508-CFTR epithelial cells(40, 41, 43, 44).

The unfolded protein response (UPR) and ER stress have been implicated in pulmonary hypertension. The potent vasoactive peptide endothelin-1 (ET-1), widely implicated in PAH patients, stimulates the production of pro-inflammatory molecules associated with activation of the UPR in PASMCs(45). A selective inhibitor of ER stress induced apoptosis, salubrinal, partially attenuated these effects. Furthermore, monocrotaline induced pulmonary hypertension was partially attenuated by salubrinal treatment(46). In the lungs of chronic hypoxia-induced mice many of the molecules associated with ER stress and the UPR were upregulated(47). Furthermore, PASMCs exposed to hypoxia elevated a number

of UPR associated genes. In two experimental models of PAH, chronic hypoxia mice and monocrotaline rats, treatment with 4PBA significantly reduced RVSP, mean pulmonary artery pressure (PAP) and vascular remodelling(47, 48). In both models, many molecules associated with UPR and ER-stress were significantly reduced(47, 48). Recently, 4PBA has been proposed as a pan-HDAC inhibitor in cancer. Treatment of human lung adenocarcinomas induced growth arrest and apoptosis(49). In glioblastoma cell lines selective inhibition was observed on proliferation and cell morphology(50). Furthermore, the 4PBA reduced the expression of anti-apoptotic genes, Bcl-2/Bcl-X_L.

Although the precise mechanism of action of 4PBA is unclear, recent evidence identified the COPII coat as a target suggesting that 4PBA reduces the stringency of retention in the ER by the inhibition of packaging p24 proteins into COPII vesicles(51).

The lack of a haemodynamic phenotype in the C118W mouse was disappointing but not unexpected given the minimal pulmonary hypertension reported in the *Bmpr2* heterozygous null mouse(25). We have previously reported a consistent but mild degree of pulmonary hypertension in another knock-in mouse harbouring the human disease causing R899X mutation(29). Furthermore, no statistical changes in vascular remodelling were observed between C118W mutant animals and littermate controls. However, there were clear differences in the muscularisation status of vessels associated with alveolar ducts when treated with 4PBA. Non- and partially muscularised vessels increased and decreased, respectively, in mutant C118W mice after 4PBA treatment. We are confident that the mouse *Bmpr2* C118W mutation is deleterious because, similar to homozygous null mice, homozygous offspring were never observed (data not shown) indicating early embryonic lethality(52).

Hyperproliferation of PASMCs is a hallmark of the pathology of PAH. We have previously shown that PASMCs isolated from individuals with a *BMPR-II* mutation had increased proliferation(8). Furthermore, our research has shown that the Ids are critical regulators of proliferation in *BMPR-II* deficiency(53). Loss of functional *BMPR2* reduces Id expression in PASMCs contributing to a dysregulation of proliferation. PASMCs isolated from C118W mutant mice exhibited both dysfunctional Id3 signalling and increased proliferation compared to control PASMCs. Importantly,

consistent with restoration of signalling, 4PBA treatment was able to restore normal proliferation rates in mutant cells.

Taken together, we provide proof-of-concept for the restoration of BMPR2 protein expression and signalling by 4PBA *in vitro* in primary human cells and *in vivo* in a mouse knock-in genetic model harbouring a cysteine-substituted mutation in the extracellular domain of BMPR2. 4PBA reduced the hyperproliferation of mutant mice PASMCs and *in vivo* increased the proportion of non-muscular small pulmonary arteries. Given that 4PBA is already in clinical use in other disorders, this drug represents a repurposing and precision medicine opportunity for patients with specific types of *BMPR2* mutations.

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FIGURE LEGENDS

Figure 1. Dermal fibroblasts from BMPR2 C118W patients have reduced BMP signalling.

(A) Wild-type (n=3) and C118W mutant (n=3) dermal fibroblasts were treated with BMP4 at 1, 10, 50 ng/ml for 4 or 24 hours. Protein lysates were immunoblotted for phosphorylated Smad1/5 and total Smad1. Blots were re-probed for α -tubulin as a loading control. (B) Densitometry of the ratio between phosphorylated Smad1/5 and total Smad1. (C and D) Wild-type (n=3) and C118W mutant (n=3) dermal fibroblasts were treated with BMP4 at 10 ng/ml for 24 hours. *ID1*(C) and *ID2*(D) mRNA expression was assessed and normalised to *HPRT*. Two-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. Error bars represent mean $\pm$ s.e.m.

Figure 2. 4PBA improves BMP signalling in BMPR2 C118W patients.

(A, B and C) Wild-type (n=3) and C118W mutant (n=3) dermal fibroblasts were treated with 4PBA at 0.5, 1 and 5 mM with or without BMP4 (10 ng/ml) treatment for 24 hours. *ID1*(A), *ID2*(B) and *BMPR2*(C) mRNA expression was assessed and normalised to *HPRT*. (D) Wild-type (n=3) and C118W mutant (n=3) dermal fibroblasts were treated with 4PBA (5 mM) and BMP4 (10 ng/ml) for 24 hours. Protein lysates were immunoblotted for phosphorylated Smad1/5 and total Smad1. Blots were re-probed for α -tubulin as a loading control. (E) Densitometry of the ratio between phosphorylated Smad1/5 and total Smad1. Two-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Error bars represent mean $\pm$ s.e.m.

Figure 3. *Bmpr2*^{C118W} mice possess a lower molecular weight receptor and reduced BMP signalling.

(A) Immunoblot of BMPR-II in mouse lungs isolated from wild-type (*Bmpr2*) and C118W mutant (*Bmpr2*^{C118W}). Reprobed for α -tubulin to ensure equal loading. (B) Densitometry of BMPR-II immunoblot. (C) Immunoblot of BMPR-II in mouse PSMCs isolated from *Bmpr2* and *Bmpr2*^{C118W} animals. Reprobed for α -tubulin to ensure equal loading. (D) Densitometry of BMPR-II immunoblot.

Figure 4. 4PBA improves BMP signalling in *Bmpr2*^{C118W} mice.

(A) Lungs were isolated from wild-type (*Bmpr2*) and C118W mutant (*Bmpr2*^{C118W}) mice treated daily with PBS or 4PBA (1 mg/kg) for 2 weeks (n=4). Immunoblots of BMPR-II, Id1 and Id3. Reprobed for α -tubulin to ensure equal loading.

(B) Densitometry of BMPR-II, Id1 and Id3 immunoblots. Two-way ANOVA. * $P \leq 0.05$. Error bars represent mean $\pm$ s.e.m.

Figure 5. 4PBA increases expression of the higher molecular weight BMPR-II band in *Bmpr2*^{C118W} mice. (A) Lungs were isolated from C118W mutant (*Bmpr2*^{C118W}) mice treated daily with PBS or 4PBA (1 mg/kg) for 2 weeks (n=4). Immunoblots of BMPR-II and reprobed for α -tubulin to ensure equal loading. (B) Densitometry of higher and lower molecular weight bands of BMPR-II. Students' *t*-test. * $P \leq 0.05$. Error bars represent mean $\pm$ s.e.m.

Figure 6. 4PBA treatment improves muscularisation in *Bmpr2*^{C118W} mice. Wild-type (*Bmpr2*) and C118W mutant (*Bmpr2*^{C118W}) were treated with saline (PBS) or 4PBA (500 mg/kg), twice daily by oral gavage for two weeks. (A) Quantification of non, partially and fully muscularised vessels as a percentage of total alveolar and duct arteries of the following groups - *Bmpr2* +PBS (n=4); *Bmpr2*^{C118W} +PBS (n=6); *Bmpr2*^{C118W} +4PBA (n=6). (B, C and D) Statistical assessment of non, partially and fully muscularised vessels in *Bmpr2*^{C118W} mice treated with PBS or 4PBA. Students' *t*-test. * $P \leq 0.05$. Error bars represent mean $\pm$ s.e.m.

Figure 7. 4PBA improves BMP signalling and reduces hyperproliferation in *Bmpr2*^{C118W} PASMCs. PASMCs isolated from *Bmpr2* and *Bmpr2*^{C118W} mice. (A) Immunoblot of Id3 and reprobed for α -tubulin to ensure equal loading. (B) Densitometry of Id3 immunoblot. (C) Proliferation assessment in mouse PASMCs from *Bmpr2* and *Bmpr2*^{C118W} animals over 4 days. (D) Proliferation of mouse PASMCs from *Bmpr2* and *Bmpr2*^{C118W} animals. After 24 hours cells were treated with 4PBA (1 mM) prior to replenishing treatment every 48 hours. Two-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Error bars represent mean $\pm$ s.e.m.

Figure 1

Figure 1

A

B

C

D

Figure 2

A

B

C

D

E

Figure 3

Figure 4

A

B

Figure 5

Figure 6

A

B

C

D

Figure 7

Figure 1

Figure 2

Figure 3

Figure 4

Figure 6

Figure 7

Online Data Supplement

Supplementary Figure 1. C118W mouse schematic. (A) The murine *Bmpr2* locus. (B) Targeting construct carrying the wild-type exon 3 in a sense orientation (3' sense) and a C118W mutated version of exon 3 in an antisense orientation (3' a-sense). FRT flanked Neomycin positive selection cassette and heterotypic Lox sites were positioned to allow inversion of the wild-type 3' sense and mutated 3' a-sense sequences, reversing their orientation following the activity of CRE recombinase. (C) *Bmpr2* targeted locus following homologous recombination via the homology arms flanking the targeting cassette. (D) *Bmpr2*^{C118W} locus following deletion of the Neomycin cassette and crossing to a *Rosa26:Cre* transgenic mouse. (E) Neo-excised wild-type and C118W knock-in allele following recombination of lox sites via the action of CRE recombinase. Inversion of the wild-type 3' sense and mutated 3' a-sense sequences, bringing the mutated exon 3 into the correct sense orientation matching the rest of the locus. Deletion of the wild-type exon 3 from the *Bmpr2* locus following CRE recombinase. (F) *In vivo* expression of *Bmpr2*^{C118W}. Confirmation of genotype of heterozygous mice had the expected 'knock-in' manipulation of the *Bmpr2* locus.

Supplementary Figure 2. Dermal fibroblasts from BMPR2 C118W patients have reduced BMP signalling. Control (n=3) and C118W mutant (n=3) dermal fibroblasts were pre-treated 4PBA (5 mM) overnight followed by BMP4 treatment (10 ng/ml) for 4 hours. *ID1*, *ID2* and *ID3* mRNA expression was assessed and normalised to *B2M*. Two-way ANOVA. *P≤0.05, **P≤0.01. Error bars represent mean ± s.e.m.

Supplementary Figure 3. 4PBA increases surface expression of mutant BMPR2. HeLa cells overexpressing myc-tagged wild-type BMPR2 or mutant C118W were treated with 4PBA at 0, 0.5, 1.5, 6, 12 and 24 hours prior to surface protein biotinylation. BMPR2 expression was assessed in the biotinylation pulldown assay and whole cell lysate (WCL) by immunoblotting (n=2).

Supplementary Figure 4. BMPR-II from *Bmpr2*^{C118W} mice is differentially deglycosylated. Lung lysates isolated from wild-type (*Bmpr2*) and C118W mutant (*Bmpr2*^{C118W}) animals. Lysates deglycosylated with Endo-H or PNGase-F as indicated and immunoblotted for BMPR-II.

Supplementary Figure 5. Characterisation of *Bmpr2*^{C118W} mice. Assessment of right ventricular systolic pressure (RVSP) and right ventricular hypertrophy (Fulton index (RV/LV+S)). (A) RVSP in wild-type (*Bmpr2*; n= 9) and C118W mutant (*Bmpr2*^{C118W}; n=11) mice. (B) Fulton index in *Bmpr2* (n=14) and *Bmpr2*^{C118W} (n=15) mice.

Supplementary Figure 6. Characterisation of 4PBA treatment study. Wild-type (*Bmpr2*) and C118W mutant (*Bmpr2*^{C118W}) were treated with saline (PBS) or 4PBA (500 mg/kg), twice daily by oral gavage for two weeks. (A) Bodyweight assessment of the following groups - *Bmpr2* +PBS (n=4); *Bmpr2* +4PBA (n=6); *Bmpr2*^{C118W} +PBS (n=6); *Bmpr2*^{C118W} +4PBA (n=6). (B) Right ventricular hypertrophy (Fulton index (RV/LV+S) of the following groups - *Bmpr2* +PBS (n=4); *Bmpr2* +4PBA (n=6); *Bmpr2*^{C118W} +PBS (n=6); *Bmpr2*^{C118W} +4PBA (n=6). (C) Quantification of pulmonary artery wall thickness as a percentage of luminal area of the following groups - *Bmpr2* +PBS (n=4); *Bmpr2* +4PBA (n=6); *Bmpr2*^{C118W} +PBS (n=6); *Bmpr2*^{C118W} +4PBA (n=6). Error bars represent mean ± s.e.m.

Supplementary Figure 7. Hyperproliferation in *Bmpr2*^{R899X} PASMCs was unaffected by 4PBA treatment. PASMCs isolated from *Bmpr2* and *Bmpr2*^{R899X} mice. Proliferation of mouse PASMCs from *Bmpr2* and *Bmpr2*^{R899X} animals. After 24 hours cells were treated with 4PBA (5 mM) prior to replenishing treatment every 48 hours. Two-way ANOVA. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001. Error bars represent mean ± s.e.m.

Supp Figure 1

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Figure 3

Table 1: Patient demographics of C118W dermal fibroblasts.

Age	Age at diagnosis	Gender	Ethnicity	mPAP (mmHg)	CI (L/min/m ²)	PVR (WU)	Treatment
62	57	M	Caucasian	61	N/A	13.1	Sildenafil Bosentan Prostacyclin (Deceased)
44	-	M	Caucasian	N/A	N/A	N/A	N/A
19	10	F	Caucasian	57	2.2	17.4	Sildenafil Bosentan Prostacyclin (Lung Tx)