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THEMED ISSUE ARTICLE

Multidrug resistance proteins preferentially regulate natriuretic peptide-driven cGMP signalling in the heart and vasculature

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Background and Purpose: cGMP underpins the bioactivity of NO and natriuretic peptides and is key to cardiovascular homeostasis. cGMP-driven responses are terminated primarily by PDEs, but cellular efflux via multidrug resistance proteins (MRPs) might contribute. Herein, the effect of pharmacological blockade of MRPs on cGMP signalling in the heart and vasculature was investigated *in vitro* and *in vivo*.

Experimental Approach: Proliferation of human coronary artery smooth muscle cells (hCASMCs), vasorelaxation of murine aorta and reductions in mean arterial BP (MABP) in response to NO donors or natriuretic peptides were determined in the absence and presence of the MRP inhibitor MK571. The ability of MRP inhibition to reverse morphological and contractile deficits in a murine model of pressure overload-induced heart failure was also explored.

Key Results: MK571 attenuated hCASMC growth and enhanced the anti-proliferative effects of NO and atrial natriuretic peptide (ANP). MRP blockade caused concentration-dependent relaxations of murine aorta and augmented responses to ANP (and to a lesser extent NO). MK571 did not decrease MABP *per se* but enhanced the hypotensive actions of ANP and improved structural and functional indices of disease severity in experimental heart failure. These beneficial actions of MRP inhibition were associated with a greater intracellular:extracellular cGMP ratio *in vitro* and *in vivo*.

Conclusions and Implications: MRP blockade promotes the cardiovascular functions of natriuretic peptides *in vitro* and *in vivo*, with more modest effects on NO. MRP inhibition may have therapeutic utility in cardiovascular diseases triggered by dysfunctional cGMP signalling, particularly those associated with altered natriuretic peptide bioactivity.

Abbreviations: ABC, ATP-binding cassette; ANP, atrial natriuretic peptide; Deta-NO, diethylenetriamine-NONOate; hCASMC, human coronary artery smooth muscle cell; HR, heart rate; KO, knockout; LV, left ventricle; MABP, mean arterial BP; MK571, 5-(3-(2-(7-chloroquinolin-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid; MRP, multidrug resistance protein; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3,-*a*] quinoxalin-1-one; SNP, sodium nitroprusside; Sper-NO, spermine-NONOate; VASP, vasodilator stimulated phosphoprotein; VSMC, vascular smooth muscle cell.

KEYWORDS
ATP-binding cassette transporter, cGMP, heart failure, multidrug resistance protein, natriuretic peptide, NO, vasorelaxation

1 | INTRODUCTION

cGMP is a common intracellular second messenger responsible for conveying many of the cardiovascular actions of **NO** and natriuretic peptides (Kuhn, 2016; Papapetropoulos et al., 2015). Generated by NO-sensitive (**GC-1** and **GC-2**) and natriuretic peptide-sensitive (**GC-A** and **GC-B**) GCs, this ubiquitous molecule possesses a number of cytoprotective roles including vasodilator, anti-leukocyte, anti-platelet, anti-fibrotic and anti-hypertrophic actions (Preedy et al., 2020). Cellular levels of cGMP are dynamically regulated in a spatial and temporal fashion, the result of a balance between GC-mediated formation and enzymatic hydrolysis by **PDEs**. Indeed, targeting of these synthetic (e.g. sGC stimulators and NO donors) and degradative (e.g. **PDE5** inhibitors) pathways has proven clinically effective in a number of cardiovascular diseases including heart failure and pulmonary arterial hypertension (Galie et al., 2005; Ghofrani et al., 2013; Roberts et al., 1992). The discrete localisation of these synthetic and degradatory enzymes enables compartmentalisation of cGMP-regulated processes to distinct cellular localities. This phenomenon has been established largely in cardiomyocytes and vascular smooth muscle cells (VSMCs) through the utilisation of cGMP-specific FRET sensors (Calamera et al., 2019; Russwurm & Koesling, 2018; Sinha et al., 2013; Subramanian et al., 2018; Thunemann et al., 2013) but also by functional pharmacological assessment of selelective GC ligands (i.e. NO vs. natriuretic peptides) and PDE inhibitors (Baliga et al., 2018; Bubb et al., 2014; Kokkonen & Kass, 2017; Moltzau et al., 2014; Zhao et al., 1999). Intriguingly, these cutting-edge approaches have also given rise to the possibility that cellular efflux plays a further, active role in terminating the biological actions of cGMP (Krawutschke et al., 2015) (and also in intercellular transfer, Menges et al., 2019). Indeed, appreciation of the compartmentalisation and cellular efflux of sibling **cAMP** *in vitro* and *in vivo* is comparatively well established (Sassi et al., 2008; Sinha et al., 2013; Vettel et al., 2017; Zoccarato et al., 2015).

Cellular efflux of cyclic nucleotides is thought to depend on the action of **multidrug resistance proteins** (MRPs) (Jedlitschky et al., 2000; Schuetz et al., 1999). These energy-dependent, transmembrane transporters belong to the superfamily of large **adenosine-5′-triphosphate (ATP)-binding cassette (ABC) proteins**, which include 10 MRPs, two sulfonylurea receptors and the cystic fibrosis transmembrane-conductance regulator (Dean et al., 2001; Deeley et al., 2006). ABC transporters are unidirectional (i.e. transport from the intracellular to the extracellular space) and ATP dependent and have a hugely diverse substrate profile that includes xenobiotics and therapeutic agents (Dean et al., 2001; Deeley et al., 2006). Two MRP isozymes are thought to primarily underpin efflux of cGMP and cAMP; **MRP4** (ABCC4) and **MRP5 (ABCC5)**, along with a modest contribution from **MRP8 (ABCC11**; Slot et al., 2011). Both transporters are widely expressed in the cardiovascular system, being found in VSMCs, endothelial cells and cardiomyocytes (Cheepala et al., 2013; Slot et al., 2011), and are inhibited by **MK571**. Indeed, this pharmacological tool, alongside MRP4$^{-/-}$ and MRP5$^{-/-}$ mice, has been used to establish cellular cGMP efflux as a key mechanism for regulation of cGMP signalling. For example, MRP4 expression is significantly increased in both VSMCs and endothelial cells at the mRNA and protein levels in lungs from patients with pulmonary hypertension (PH) compared with controls and is the same in a murine model of pulmonary hypertension. In accord, administration of MK571 or MRP4 (*Abcc4*) gene deletion causes resistance to hypoxia-induced pulmonary hypertension (Hara et al., 2011). Notably, the effect of MRP inhibition is accentuated in the presence of sildenafil, suggesting that in the absence of PDE degradation, MRP activity is significantly elevated and takes on a more substantial role in cGMP inactivation. Indeed, genetic deletion of MRP4 leads to significantly increased heart weight, cardiomyocyte size and left ventricular (LV) wall thickness with a concomitant increase in cardiac **atrial natriuretic peptide (ANP; *Nppa*)** gene expression (Sassi et al., 2012). Such mice

What is already known

- Cyclic nucleotide compartmentalisation spatially and temporally constrains cGMP and cAMP signalling.
- cGMP and cAMP cellular efflux may represent an important mechanism for regulating cyclic nucleotide signalling.

What this study adds

- Pharmacological inhibition of multidrug resistance proteins (MRPs) promotes cGMP signalling in the heart and vasculature.
- cGMP production by membrane-spanning GCs, triggered by natriuretic peptides, is governed preferentially by MRP activity.

What is the clinical significance

- Augmenting cGMP signalling is established to be beneficial in a number of cardiovascular diseases.
- MRPs inhibition might represent alone or adjunct therapy to optimise the therapeutic benefits of cGMP.

also exhibit an extended bleeding time demonstrating that MRP4 promotes platelet aggregation by decreasing intracellular cAMP and cGMP (Borgognone & Pulcinelli, 2012). Interestingly, MRP5 expressio is increased in the hearts of patients suffering from ischaemic cardiomyopathy and dilated cardiomyopathy, suggesting that this MRP isozymes may contribute to the pathology and that inhibition of MRP5 might be an attractive target to increase beneficial intracellular cyclic nucleotide concentrations (Dazert et al., 2003) Yet, MRP5$^{-/-}$ mice do not have an overt phenotype, a finding that is also true for MRP4$^{-/-}$/MRP5$^{-/-}$ animals (Borst et al., 2007). This is at odds with the adverse cardiovascular phenotype in MRP4$^{-/-}$ mice (Sassi et al., 2012). Therefore, a greater understanding of the physiological and pathological role(s) of MRPs in the regulation of cyclic nucleotide efflux is needed if these transporters are to be established as key players in terminating cGMP signalling and, consequently, as potential target in promoting the cytoprotective functions of cGMP for therapeutic benefit in cardiovascular disease.

Herein, in order to establish (patho)physiological role(s) for MRPs in regulating cGMP efflux and signal termination in the cardiovascular system, we have used functional pharmacological characterisation of vascular smooth muscle proliferation, isolated blood vessel reactivity, acute BP responses and an experimental model of heart failure in the absence of presence of MRP inhibition.

2 | METHODS

2.1 | Materials

ANP (Cambridge Bioscience, Cambridge, UK), spermine-NONOate (Sper-NO; Sigma Aldrich, Poole, UK), MK571 (MK; Sigma Aldrich), **isoprenaline** (Iso; Sigma Aldrich), **phenylephrine** (Sigma Aldrich) and **ACh** (Sigma Aldrich) were dissolved in deionised, distilled water (ddH_2O). For acute BP studies, **sodium nitroprusside (SNP**; Sigma Aldrich), diethylenetriamine/NO adduct (Deta-NO; Sigma Aldrich) and MK571 were dissolved in saline (0.9%; Baxter, Newbury, UK). For telemetry studies, MK571 was dissolved in the drinking water. **Probenecid** (Sigma Aldrich) and **ODQ** (1*H*-[1,2,4]oxadiazolo[4,3,-*a*] quinoxalin-1-one; synthesised in-house) were dissolved in DMSO (Sigma Aldrich), and identical DMSO concentrations added to control cells/tissues.

Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). All animal procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act of 1986 and were approved by a local animal welfare and ethical review board. Animals were housed in a temperature-controlled environment with a 12-h light–dark cycle. Food and water were accessible ad libitum. For *in vivo* experimentation, animals were randomly assigned to interventions and the experimenter was blinded to treatment. For cell- and tissue-based studies, interventions were randomly assigned but the experimenter was not blinded to treatment.

2.2 | Cell proliferation

Human coronary artery smooth muscle cells (hCASMCs; Passages 5–7; Lonza, Basel, Switzerland) were seeded onto six-well plates at a density of 30,000 cells per well. Cells were initially grown for 24 h in routine cell culture medium (Smooth Muscle Growth Medium-2; Lonza) with SmGM™-2 BulletKit containing FBS (5%), human EGF insulin, human fibroblastic growth factor and gentamicin/amphotericin B (proprietary concentrations). Subsequently, the cells were serum starved (FBS: 0.5%) for 48 h to synchronise cell cycles. Cells were then incubated in medium containing 5% serum in the presence of vehicle (sterile ddH_2O) or pharmacological treatment, and cells (stained with trypan blue diluted 1:1 to identify dead cells) counted at 0-, 24-, 48-, 72- and 96-h intervals using a haemocytometer. Individual plates were set up with the same treatments, in duplicate wells, one for each time point. Cells seeded at an initial density of 30,000 cells per well grew at an exponential rate under control conditions without becoming confluent (Figure 1). This proliferative profile was explored in the absence and presence of MK571 (30 nM to 30 μM), ANP (100 nM), the NO donor diethylenetriamine-NONOate (Deta-NO; 10 μM) or combinations thereof.

2.3 | cGMP assay

Intracellular and extracellular cGMP concentrations were measured by ELISA (GE Healthcare, Hatfield, UK) following 24 h of treatment in the same cells used for the proliferation assays described above. Each well was treated with the non-selective PDE inhibitor **IBMX** (100 μM; Sigma Aldrich) for 30 min prior to cell and supernatant harvest to prevent breakdown of cGMP during cell processing. Cell pellet (for intracellular cGMP) and supernatant (for extracellular cGMP) were frozen and stored at −80°C until use. *In vivo* cGMP measurements were determined using an identical ELISA (as above), determined in whole heart homogenates (for intracellular cGMP) and plasma (for extracellular cGMP), treated with IBMX (100 μM; as above) and frozen and stored at −80°C until use. cAMP concentrations were also evaluated in heart homogenates and plasma by ELISA (GE Healthcare). Extraction of cyclic nucleotides and ELISA were conducted as the per manufacturer's instructions.

2.4 | Organ bath pharmacology

Mice (C57/BL6; male and female; 20–30 g) were killed by cervical dislocation. The thoracic aortae were carefully removed, cleaned of connective tissue and cut into three to four ring segments of approximately 4 mm in length. Aortic rings were mounted in 10-ml organ baths containing Krebs-bicarbonate buffer (composition [mM]: Na^+ 143; K^+ 5.9; Ca^{2+} 2.5; Mg^{2+} 1.2; Cl^- 128; HCO_3^- 25; HPO_4^{2-} 1.2; SO_4^{2-} 1.2; D-glucose 11) and gassed with carbogen (95% O_2/5% CO_2; British Oxygen Company [BOC], Guildford, UK). Tension was initially set at 0.3 g and reset at intervals following an equilibration period of

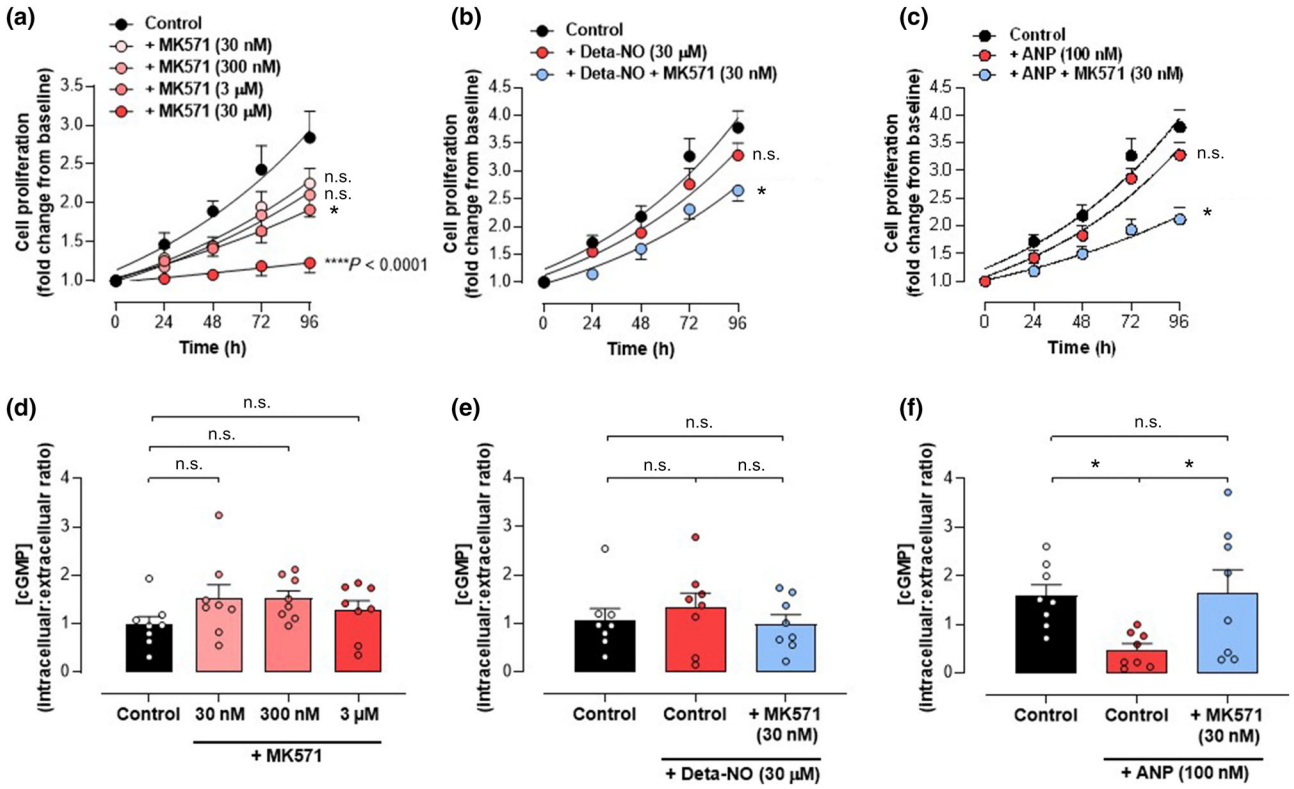

FIGURE 1 Proliferation (a–c) and intracellular:extracellular cGMP ratio (d–f) in human coronary artery smooth muscle cells in the absence (control) or presence of MK571 (30 nM to 30 μM; a), Deta-NONOate (Deta-NO; 30 μM; b) or atrial natriuretic peptide (ANP; 100 nM; c), or combination of Deta-NO or ANP (at the same concentrations) with MK571 (30 nM; b, c). Data are shown as mean ± SEM. Statistical analysis by one-way ANOVA with Šídák post hoc test. *P <0.05 (adjusted for multiplicity)

approximately 1 h during which time fresh Krebs-bicarbonate buffer was replaced every 15 min. After equilibration, the rings were primed with three separate additions of KCl (48 mM; Sigma Aldrich), at each addition maximum tension was observed (3 min) before being washed out by the addition of fresh Krebs-bicarbonate buffer at 10 min intervals for a total of 30 min. Cumulative concentrations of the α-adrenoceptor agonist phenylephrine (1 nM to 3 μM) were then added until a maximum contraction was observed. Another washout period was performed before vessels were contracted to an EC_{80} concentration of phenylephrine. Once this response had stabilised, a single addition of the endothelium-dependent dilator ACh (1 μM) was added to the bath to assess the integrity of the endothelium. If the contractions to phenylephrine were not maintained, or ACh produced relaxations of less than 50% of the phenylephrine-tone, tissues were discarded. After another wash period, the vessels were again contracted with phenylephrine (EC_{80}) and cumulative concentration response curves to MK571 (100 nM to 30 μM) or probenecid (1 μM to 1 mM) constructed; responses to MK571 were also studied in the presence of the NO-sensitive GC (GC-1/2) antagonist ODQ (5 μM) or the G-kinase inhibitor KT5823 (2 μM). In further studies, tissues were pre-contracted with an EC_{80} concentration of phenylephrine and then concentration–response relationships for ACh (1 nM to 3 μM), ANP (1 pM to 300 nM), the NO donor Sper-NO (1 nM to 30 μM) or the β-adrenoceptor agonist isoprenaline (300 pM to 300 nM) determined in the absence and presence of a subthreshold concentration (3 μM; 15 min pre-incubation) of MK571.

2.5 | Acute BP measurement in anaesthetised mice

Mice (C57/BL6; male and female; 20–30 g) were anaesthetised with 1.5% isoflurane (Abbott Laboratories Ltd, Queenborough, UK) in O_2 and placed supine on a thermostatically controlled heating blanket (37.0°C ± 0.5°C). To measure BP, the left common carotid artery was isolated and a fluid-filled (heparin; 100 U·ml^{-1} diluted in 0.9% saline), 0.28-mm internal diameter cannula (Critchley Electrical Products Pty Ltd, Castle Hill, Australia) introduced into the artery. BP was measured using an in-line P23 XL transducer (Viggo-Spectramed, California, USA) and PowerLab system, calibrated beforehand and recorded using LabChart (ADInstruments, Castle Hill, Australia). The jugular vein was cannulated for drug administration. The arterial cannula was flushed once with heparinised saline (heparin; 100 U·ml^{-1} diluted in 0.9% saline). After a minimum 10-min equilibration or until continuous stable pressure was observed, mice were given a weight adjusted (1 μl/1 g of body

weight) i.v. bolus injection of MK571 (0.001–3 mg·kg^{-1}). The 3 mg·kg^{-1} dose was chosen as subthreshold. Subsequently BP was measured following i.v. bolus administration of the NO donor SNP (1–10 μg·kg^{-1}) or ANP (1–100 μg·kg^{-1}) in the absence and presence of MK571 (3 mg·kg^{-1}; pre-incubated for 30 min).

2.6 | Chronic BP measurement in conscious telemeterised mice

Mice (C57/BL6; male and female; 20–30 g) were implanted with a DSI PhysioTel® HD-X11 telemetry probe (Data Sciences International, Minneapolis, USA). Each probe was cleaned and sterilised according to the manufacturer's guidelines. Mice were anaesthetised with 5% isoflurane in O_2 and anaesthesia was maintained using 1.5%–2% isoflurane in O_2 with body temperature maintained at 37°C. The left common carotid artery was exposed, isolated and cleaned of any surrounding tissue. A small incision was made in the carotid, and the catheter inserted whilst submerged in saline to ensure no bubble form inside the catheter tip. The tip of the catheter was placed into the aortic arch, securely fastened and the transmitter body placed s.c. on the right flank. The incision was stitched and each animal received post-operative analgesia consisting of 0.3-μg Vetergesic® (buprenorphine; Abbott Laboratories Ltd) in 0.5-ml saline (s.c.). Animals were left to recover for a minimum of 7 days, after which haemodynamic recordings were taken for 64 h over the weekend to minimise noise disturbance. Mean arterial BP (MABP), heart rate (HR) and activity were recorded for 2 min at 15-min intervals using Dataquest Art Acquisition System (Data Sciences International). Baseline haemodynamic measurements were taken 1 week before dosing. The daily drinking water consumption was measured each day, and, where appropriate, water bottles were replaced with bottles containing MK571 at a concentration that entailed each mouse received 25 mg·kg^{-1}·day^{-1} (based on a mean consumption of 4 ml·mouse^{-1}·day^{-1}). A 24-h time period was used for analysis starting from 1 pm Saturday and ending 1 pm Sunday (12 h light/dark), with dosing commencing 24 h before the start of this time period.

2.7 | Pressure overload-induced heart failure

Abdominal aortic constriction (AAC) was performed at the suprarenal level to induce pressure overload. Mice (C57/BL6; male and female; 20–30 g) were anaesthetised (1.5% isoflurane in O_2) and the analgesic buprenorphine was administered (0.1 mg·kg^{-1}; i.m.; Centeur, UK). Mice were placed under an operating microscope in the supine position with body temperature maintained at 37°C. An incision was made in the abdominal cavity and the abdominal aorta was separated from the surrounding tissue at the suprarenal level by moderate dissection. Aortic constriction was performed by tying a 4–0 surgical thread against a 25-gauge needle between the superior mesenteric and renal arteries. This produces a ~30% constriction of the luminal diameter. For sham operations, the 4–0 surgical thread was passed under the aorta and removed without tying it against the needle. To close internal and external incisions, 6.0 absorbable and non-absorbable sutures were used, respectively, and local anaesthetic (lidocaine hydrochloride; 2.0% w/v; Dechra, UK) was administered. Mice were monitored regularly for 3 days post-surgery. MK571 (25 mg·kg^{-1}·day^{-1}) was delivered in the drinking water (changed every other day, based on average water consumption) starting at Day 21 following abdominal aortic constriction and maintained throughout the remaining 3 weeks of study.

To track changes in cardiac morphology and function *in vivo*, M-mode echocardiograms were obtained from anaesthetised mice at baseline, 3 and 6 weeks post-abdominal aortic constriction using a VisualSonics Vevo 3100 echocardiographic system and a 40-MHz transducer. Mice were anaesthetised (1.5% isoflurane in O_2) and placed in the supine position with body temperature maintained at 37°C. LV internal diameter (LVID) at diastole (d) and systole (s) was measured from short-axis M-mode images. LV ejection fraction (LVEF, %) was calculated as follows:- $\text{LVEF}\ (\%) = [(\text{LVIDd})^3 - (\text{LVIDs})^3]/(\text{LVIDd})^3 \times 100$. Values were averaged from 3 beats. ECG, HR, and respiration rate were also recorded. Animals were closely monitored durign recovery from anaesthesia and, if necessary, provided with extra supportive care (e.g. thermoregulatory support).

2.8 | Histology, staining and imaging

For murine studies, the isolated left ventricles were cut transversely below the mitral valves, fixed in 10% formalin for 24 h and then stored in 70% ethanol before embedding in paraffin wax and sectioning (6-μm transverse sections). Human heart samples from 'healthy' (non-failing) control patients and those with heart failure (i.e. ischaemic cardiomyopathy or dilated cardiomyopathy) were obtained under ethical approval from the Institutional Review Boards at the University of Pennsylvania and the Gift-of-Life Donor Program (Pennsylvania, USA), and the kind gift of Dr. K. Margulies and Dr. K Bedi.

2.9 | Picrosirius red staining

Tissue slides were dewaxed, rehydrated and stained with Picrosirius Red (0.1% w/v) to visualise collagen fibres in the heart. Images were obtained using a Nanozoomer S210 Slide Scanner (×40 objective, Hamamatsu, Japan) and analysed by threshold analysis using ImageJ (National Institutes of Health).

2.10 | Quantitative RT-PCR and immunoblotting

Left ventricles were snap frozen, broken down using a pestle and mortar and then homogenised using QIAshredder technology. RNA was

extracted using a Fibrosis Tissue extraction kit (as above; Qiagen) and quantified using a NanoDrop spectrophotometer (ThermoFisher Scientific, MA, USA). One microgram of RNA was converted to cDNA by reverse transcription (High Capacity RNA-to-cDNA Kit; Applied Biosystems, Life Technologies Ltd, UK). Specific primers for hypertrophic or fibrotic markers (detailed in Table 1) and housekeeping gene ribosomal protein L19 (RPL19) were added to cDNA template and SyBr Green quantitative PCR mix (PowerUp™ SYBR Green master mix, Fisher Scientific, UK). Ten nanograms of cDNA from each sample was amplified using quantitative real-time PCR over 45 cycles, as per the manufacturer's instructions in the thermal cycler (CFX96 Connect Realtime System, Bio-Rad, UK). Samples were subjected to melting curve analysis to confirm amplification specificity. mRNA expression was analysed by expressing the cycle threshold (Ct) value as $2^{-\Delta\Delta Ct}$, relative to the levels of *RPL19*, and further normalised as a fold change to control.

Standard SDS-PAGE protocols were employed to identify MRP4 protein expression using anti-MRP4 (1:500, ab15602, Abcam) and anti-vasodilator stimulated phosphoprotein (total VASP; 1:1000, ab205952, Abcam), anti-phospho-VASP^{157}(1:1000, clone 3111, Cell Signaling) and anti-phospho-VASP^{239} (1:1000, clone 3114, Cell Signaling) used as an index of myocardial cGMP/G-kinase signalling activity (Butt et al., 1994). GAPDH (1:50,000; ThermoFisher Scientific) was used as loading control. The Immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.11 | Statistical analyses

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). All data are reported as mean ± SEM, where *n* denotes the number of mice used in each group. Statistical analyses were conducted using GraphPad Prism (Version 8; GraphPad Software, USA). For the comparison of two groups of data, a two-tailed, unpaired Student's *t*-test was used. When comparing three or more groups of data, either a one-way or two-way ANOVA followed by a Šidák multiple comparisons test was used with adjustment for multiplicity. A *P*-value < 0.05 was considered statistically significant, and the *P*-values presented in each figure indicate all comparisons undertaken. Post hoc tests were conducted only if *F* in ANOVA achieved $P < 0.05$. Sample sizes subjected to statistical analysis were at least five animals per group ($n = 5$, where *n* is the number of independent values).

2.12 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

TABLE 1 Primer sequences for RT-PCR analyses

Target	Sequence (5′ → 3′)
Mouse αSMA	Forward: ACTACTGCCGAGCGTGA Reverse: ATAGGTGGTTTCGTGGATGC
Mouse ANP	Forward: GGATTTCAAGAACCTGCTAGACC Reverse: GCAGAGCCCTCAGTTTGCT
Mouse Col1α1	Forward: TCTGACTGGAAGAGCGGAGAG Reverse: AGACGGCTGAGTAGGGAACA
Mouse Col1α2	Forward: TGGATACGCGGACTCTGTTG Reverse: CCCTTTCGTACTGATCCCGATT
Mouse Col3α1	Forward: GGGAGGAATGGGTGGCTATC Reverse: CTGGGCCTTTGATACCTGGA
Mouse fibronectin	Forward: CCGGTGGCTGTCAGTCAGA Reverse: CCGTTCCCACTGCTGATTTATC
Mouse MMP-2	Forward: TGTCGCCCCTAAAACAGACA Reverse: CAGGTGTGTAACCAATGATCCTGT
Mouse MRP4	Forward: CCGTCCTTTTTGGCATAGCG Reverse: CGAGGAGCAACGTCTGGATG
Human MRP4	Forward: CCCGTGTACCAGGAGGTGAA Reverse: GGGATTGAGCCACCAGAAGAA
Mouse MRP5	Forward: CCGTGGAGAGGATCAACCAC Reverse: CCAAAGAGGACTTCCCTGACC
Human MRP5	Forward: TGGAATCTGTGGCAGTGTGG Reverse: CTCCTCGCTCTCCAATCTCC
Human and mouse RPL19	Forward: GGTTGCCTCTAGTGTCCTCC Reverse: TTGGCGATTTCATTGGTCTCA
Mouse SERCA2a	Forward: TGGAACCTTTGCCGCTCATTT Reverse: CAGAGGCTGGTAGATGTGTT
Mouse TGF-β1	Forward: TCAGACATTCGGGAAGCAGT Reverse: GCCCTGTATTCCGTCTCCTTG

3 | RESULTS

3.1 | Smooth muscle cell proliferation

MK571 (30 nM to 30 μM) concentration-dependently inhibited human coronary artery smooth muscle cell (hCASMC) proliferation compared with control (Figure 1a); 30-μM MK571 completely arrested cell growth but also changed cell morphology and lowered viability. Based on these data, a subthreshold concentration of MK571 (30 nM) was selected to study effects of MRP inhibition on the anti-proliferative effects of NO and ANP. Deta-NO (10 μM) and ANP (100 nM) were chosen due to their ability to elicit submaximal inhibition of human VSMC proliferation (Bubb et al., 2014). Deta-NO and ANP alone did not significantly alter hCASMC proliferation, although a tendency for both to reduce mean fold change in cell number was observed (Figure 1b,c). However, when the subthreshold concentration of MK571 (30 nM) was combined with either Deta-NO or ANP, a significantly enhanced inhibitory effect on hCASMC proliferation was observed compared with control (Figure 1b,c). Moreover, MK571 combined with ANP significantly inhibited hCASMC growth compared with ANP alone (Figure 1c), although a similar potentiation was not seen with MK571 plus Deta-NO (Figure 1b).

3.2 | Effect of multidrug resistance protein (MRP) inhibition on intracellular and extracellular cGMP concentrations

To investigate whether MRP inhibition augments VSMC growth through effects on cGMP dynamics/transport, intracellular and extracellular cGMP concentrations were measured in hCASMC at the 24-h time point, under identical conditions employed for the proliferation assays described above. Human CASMC grown under control conditions showed an essentially equal intracellular and extracellular level of cGMP (Figure 1d). In hCASMCs treated with MK571 (30 nM to 3 μM), the intracellular and extracellular cGMP concentrations did not differ significantly from control (Figure 1d). Following 24-h treatment with Deta-NO (10 μM) in the absence or presence of a subthreshold concentration of MK571 (30 nM), the intracellular:extracellular cGMP ratio did not significantly change, although there was a tendency for this to increase in the presence of Deta-NO (Figure 1e). However, 24-h treatment with ANP (100 nM) produced a clear reduction in the intracellular:extracellular cGMP ratio that was significantly restored in the presence of MK571 (Figure 1f; absolute cGMP levels for all conditions are depicted in Figure S1). These findings suggest that efflux of cGMP through MRP4/MRP5 (i.e. MK571-inhibitable MRPs) regulates the cellular response of hCASMC to NO donors and ANP, but that activation of the membrane-spanning GC-A by ANP is subject to significant cGMP efflux that is sensitive to MRP blockade.

3.3 | Effect of MRP inhibition on vascular reactivity

To determine if cell-based findings were mirrored in a functional pharmacological fashion in whole blood vessels, studies were performed on mouse thoracic aorta. Here, the structurally-distinct MRP inhibitors MK571 (100 nM to 30 μM) and probenecid (1 μM to 1 mM) produced concentration-dependent relaxations of phenylephrine-induced tone, with the highest concentrations of each resulting in 100% relaxation (Figure 2a,b). In addition, the concentration-dependent relaxations to MK571 (100 nM to 30 μM) were significantly shifted to the right in the presence of the NO-sensitive GC inhibitor ODQ (5 μM) and the G-kinase inhibitor KT5823 (2 μM; Figure 2a) confirming the cGMP dependency of these relaxations.

A subthreshold concentration of MK571 (3 μM) was chosen to assess the effects of MRP inhibition on vasorelaxant responses. The endothelium-dependent vasorelaxant ACh exerted concentration-dependent relaxations of phenylephrine pre-contracted mouse aorta that were not significantly different in the presence of MK571 (Table 2 and Figure 2c). The NO donor, Sper-NO, ANP, CNP and the β-adrenoceptor agonist isoprenaline all induced concentration-dependent relaxations of phenylephrine tone that were sensitive to MRP inhibition. The greatest effect of MRP inhibition on cGMP-driven responses was observed on vasorelaxation to ANP; MK571 significantly increased the potency of both vasodilators by approximately fourfold (Table 2 and Figure 2d). Echoing the observations

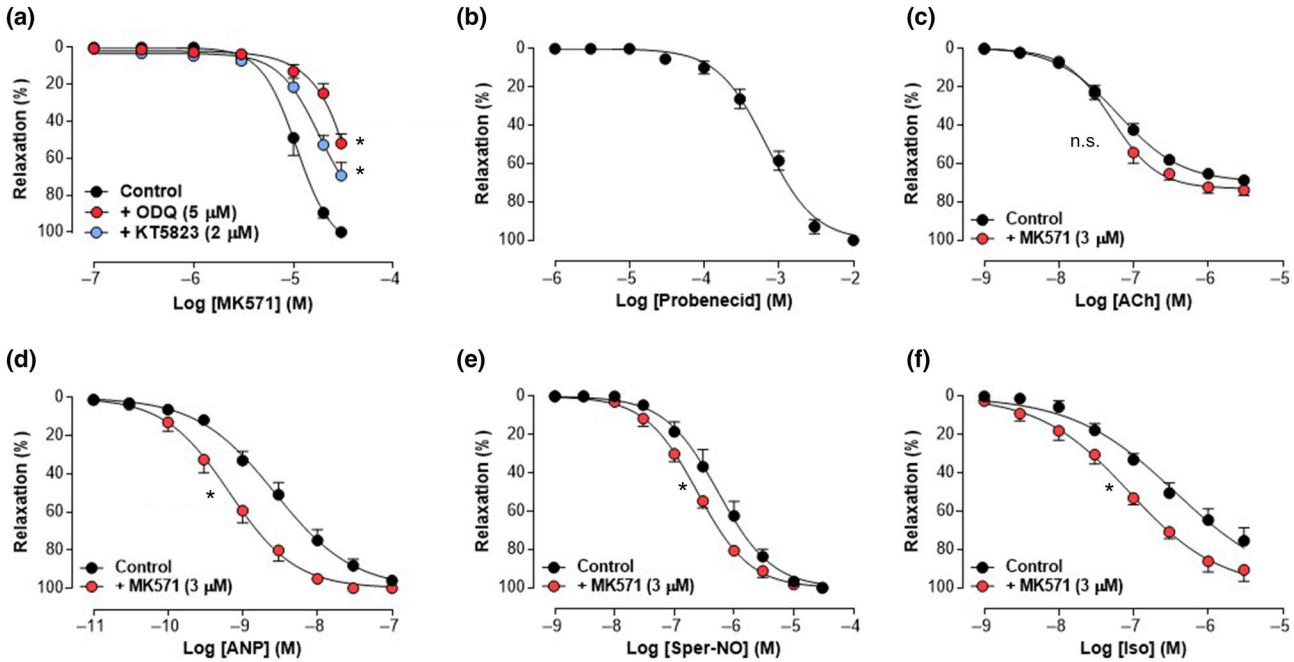

FIGURE 2 Vasorelaxant responses to MK571 (100 nM to 30 μM; a) and probenecid (1 μM to 10 mM; b) alone or MK571 in the presence of the GC-1/2 inhibitor, ODQ (5 μM) or G-kinase inhibitor KT5823 (2 μM; a), and to ACh (1 nM to 3 μM; c), atrial natriuretic peptide (ANP; 0.01–100 nM; d), spermine-NONOate (Sper-NO; 1 nM to 30 μM; e) and isoprenaline (Iso; 1 nM to 3 μM; f) in the absence and presence of MK571 (3 μM) in mouse isolated aorta. Data are shown as mean ± SEM. Statistical analysis by two-way ANOVA across the entire concentration–response curve. *P <0.05 (adjusted for multiplicity)

TABLE 2 Absolute and fold change in EC_{50} values for vasorelaxant responses to ACh, atrial natriuretic peptide (ANP), spermine-NONOate (Sper-NO) and isoprenaline (Iso) in mouse isolated aorta in the absence and presence of the multidrug resistance protein (MRP) inhibitor MK571 (3 μM)

	Log EC_{50} (M)			
	Control	+ MK571 (3 μM)	Fold shift	*P* value
ACh	−7.206 ± 0.103	−7.296 ± 0.111	1.23	> 0.05
ANP	−8.570 ± 0.089	−9.175 ± 0.093	4.03	*< 0.05
Sper-NO	−6.264 ± 0.093	−6.612 ± 0.057	2.23	*< 0.05
Iso	−6.441 ± 0.120	−7.056 ± 0.109	4.12	*< 0.05

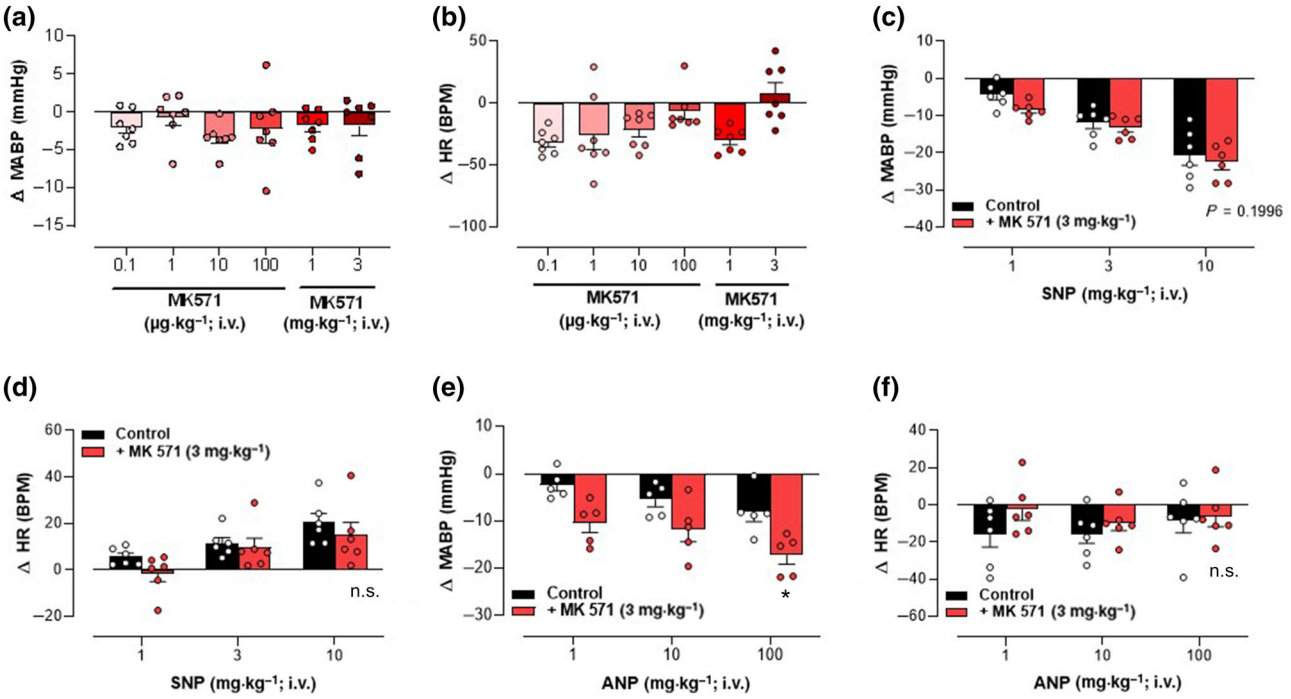

FIGURE 3 Changes in mean arterial BP (MABP; a, c, e) and heart rate (b, d, f) in anaesthetised mice following bolus doses of MK571 (0.1 μg·kg^{-1} to 3 mg·kg^{-1}, i.v.; a, b), or bolus doses of sodium nitroprusside (SNP; 1–10 mg·kg^{-1}, i.v.; c, d) or atrial natriuretic peptide (ANP; 1–10 μg·kg^{-1}, i.v.; e, f) in the absence and presence of MK571 (3 mg·kg^{-1}). Data are shown as mean ± SEM. Statistical analysis by two-way ANOVA across the entire dose–response curve. *P <0.05 (adjusted for multiplicity)

from the cell-based studies (above), the potency of Sper-NO was enhanced to a lesser degree when combined with MK571 (Table 2 and Figure 2e). Finally, MK571 significantly increased the potency of isoprenaline (Table 2 and Figure 2f), confirming a dual action on cGMP- and cAMP-mediated vasorelaxant responses.

3.4 | Effect of MRP inhibition on acute changes in BP

As a whole, in vitro observations suggest that MRP4 and/or MRP5 play a key role in regulating cGMP-driven responses in the vasculature and that this phenomenon is more important in with respect to natriuretic peptide/particulate GC signalling compared with NO-sensitive GC/cGMP processes. To determine if such an influence was also functionally apparent *in vivo*, the effect of MRP inhibition on NO- and ANP-induced acute changes in BP was explored in anaesthetised mice.

Administration of MK571 at doses ranging from 0.1 μg·kg^{-1} to 3 mg·kg^{-1} caused little or no change in MABP or HR (Figure 3a,b). Accordingly, the highest dose (3 mg·kg^{-1}) of MK571 was used in subsequent experiments to assess the effect of MRP inhibition on haemodynamic responses to NO and ANP. The NO donor SNP (1, 3 and 10 μg·kg^{-1}; i.v. bolus) and ANP (1, 10 and 100 μg·kg^{-1}; i.v. bolus) were chosen due to their ability to elicit dose-dependent reductions in MABP (Madhani et al., 2006). SNP produced a dose-dependent decrease in MABP, which was not significantly altered when combined with MK571 (Figure 3c). SNP also elicited a dose-dependent increase in HR, but this was not changed by MK571 (Figure 3d). ANP also produced dose-dependent decreases in MABP, however in this

case, the hypotensive response to ANP was significantly enhanced in the presence of MK571 (Figure 3e). The effects of ANP on HR were not significantly different following MK571 administration (Figure 3f). These data imply that MRP inhibition has a preferential ability to promote ANP/GC-A/cGMP signalling in the vasculature compared with NO/GC-1/cGMP pathway, but that basally, MRP inhibition does not significantly alter cGMP-driven vasodilation, perhaps arguing that in healthy animals intracellular levels of cGMP are not sufficient to be affected by MRP-driven efflux.

3.5 | Effect of MRP inhibition on chronic changes in blood pressure

In vivo studies in anaesthetised mice suggested that MRP inhibition acutely enhances the hypotensive activity of ANP, but not NO, substantiating earlier *in vitro* observations. Investigations were therefore undertaken to establish whether MK571 was able to modulate BP in conscious telemetered mice *in vivo* over a 24-h period. Mice had a normal circadian rhythm with respect to MABP, HR and activity during the 24-h time period (Figure 4a–f). Notably, there were no significant differences in MABP, HR or activity over the 24-h period (or during either the 12-h light or dark phase) in the absence or presence of MK571 (Figure 4a–f). These findings are in accord with the acute BP measurements in that at rest (i.e. healthy mice) MRP inhibition does not play a significant role in vascular cGMP-dependent signalling.

3.6 | Effect of MK571 on experimental heart failure

Because MRP inhibition appeared to preferentially augment ANP-elicited cGMP responses in the vasculature *in vitro* and *in vivo*, it was hypothesised that cGMP efflux via MRPs might play a key role in cGMP-dependent signalling *in vivo* in a setting of high background natriuretic peptide levels. Thus, a well-established model of pressure overload-induced heat failure (i.e. abdominal aortic constriction; Moyes et al., 2020) was employed as this condition is associated with significant elevated circulating natriuretic peptide concentrations in rodents and patients (Potter et al., 2009; Wang et al., 2004; Wei et al., 1993).

Pressure overload produced the expected increase in LV wall thickness and LV dilatation, and reduced contractility (i.e. fractional shortening and ejection fraction), characteristics of an heart failure phenotype (although this did not result in a significant reduction in cardiac output or stroke volume, Figures 5 and 6). Remarkably, the vast majority of these indices of disease severity were reversed in the presence of MK571, manifesting as both improved cardiac morphology and enhanced myocardial contractility (Figure 5a–f). This was mirrored by qPCR analysis of the cardiac expression of pro-hypertrophic and pro-fibrotic markers which were increased in response to pressure overload, with the exception of **SERCA2**, which was decreased in line with a previous report (Nagai et al., 1989). These effects were reversed in the presence of MK571 (Figure S2). The anti-fibrotic actions of MRP inhibition were confirmed by assessment of collagen deposition (Figure S3). Importantly, MK571 did not significantly reduce MABP following abdominal aortic constriction, indicating that the beneficial actions of MRP inhibition are not due to indirect effects on BP.

3.7 | Expression of MRP4 and MRP5 in heart failure

Molecular analysis revealed contrasting effects of pressure overload on MRP subtype expression. MRP4 mRNA levels trended towards a reduction in experimental heart failure, and this was corroborated at the protein level (Figure S4). However, expression of MRP5 mRNA was significantly increased in response to abdominal aortic constriction (Figure S4). These changes were largely mirrored in tissue from patients with heart failure with the exception of MRP4 expression in ischaemic patients, suggesting that this mouse model closely mimics the human medical condition (Figure S4).

3.8 | Effect of MK571 on cGMP levels *in vivo* following pressure overload

To evaluate whether the advantageous outcome associated with MK571 administration in experimental heart failure was dependent on promoting tissue cGMP levels, measurements of cardiac and plasma cyclic nucleotides were conducted. cAMP levels in the heart and plasma exhibited little or no change following abdominal aortic constriction in the absence and presence of MK571 (Figure 7a–c). In contrast, abdominal aortic constriction tended to cause an increase in cardiac cGMP levels and markedly elevated circulating cGMP levels, perhaps indicative of cardiac production of cGMP but significant cellular efflux (Figure 7c–e). Intriguingly, in the presence of MK571, cardiac cGMP levels were maintained at levels commensurate with abdominal aortic constriction alone, but plasma cGMP concentrations were significantly reduced (Figure 7c–e), intimating a positive shift in the intracellular:extracellular cGMP balance. The functional importance of cGMP in mediating the beneficial effects of MK571 *in vivo* was further corroborated by studies showing that phosphorylation of VASP at Ser^{157} and Ser^{239} was significantly increased in hearts from animals exposed to abdominal aortic constriction treated with MK571 versus mice with abdominal aortic constriction alone (Figure S5).

4 | DISCUSSION

Cellular efflux has been postulated to contribute to compartmentalisation of cGMP signalling. This has been postulated based on cell- and tissue-based systems and understood to involve, at least in part, MRP4 and/or MRP5, although gap junctions may also

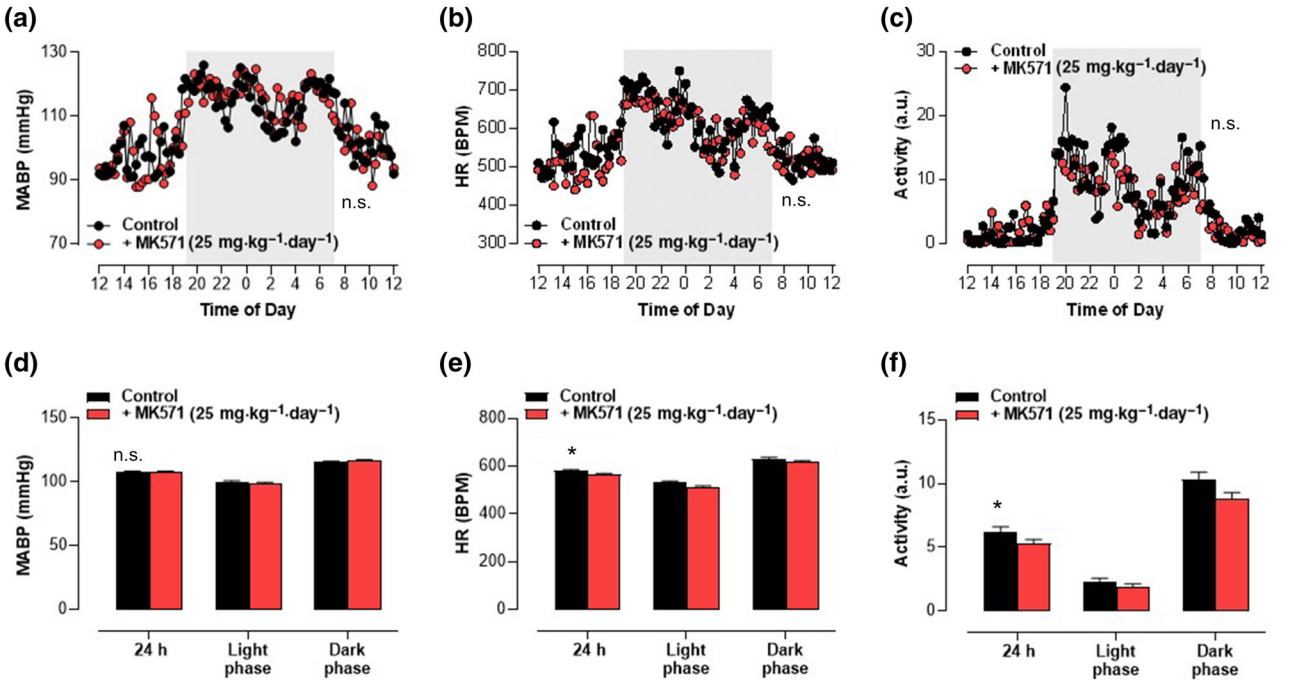

FIGURE 4 Twenty-four hours (a–c) and mean (d–f) radiotelemetry evaluation of mean arterial BP (MABP; a, d), heart rate (HR; b, e) and activity (c, f) in conscious mice in the absence and presence of MK571 (25 mg·kg^{-1}·day^{-1}). Data are shown as mean ± SEM (error bars omitted for clarity in a–c). Statistical analysis by two-way ANOVA across all time points. *P <0.05 (adjusted for multiplicity)

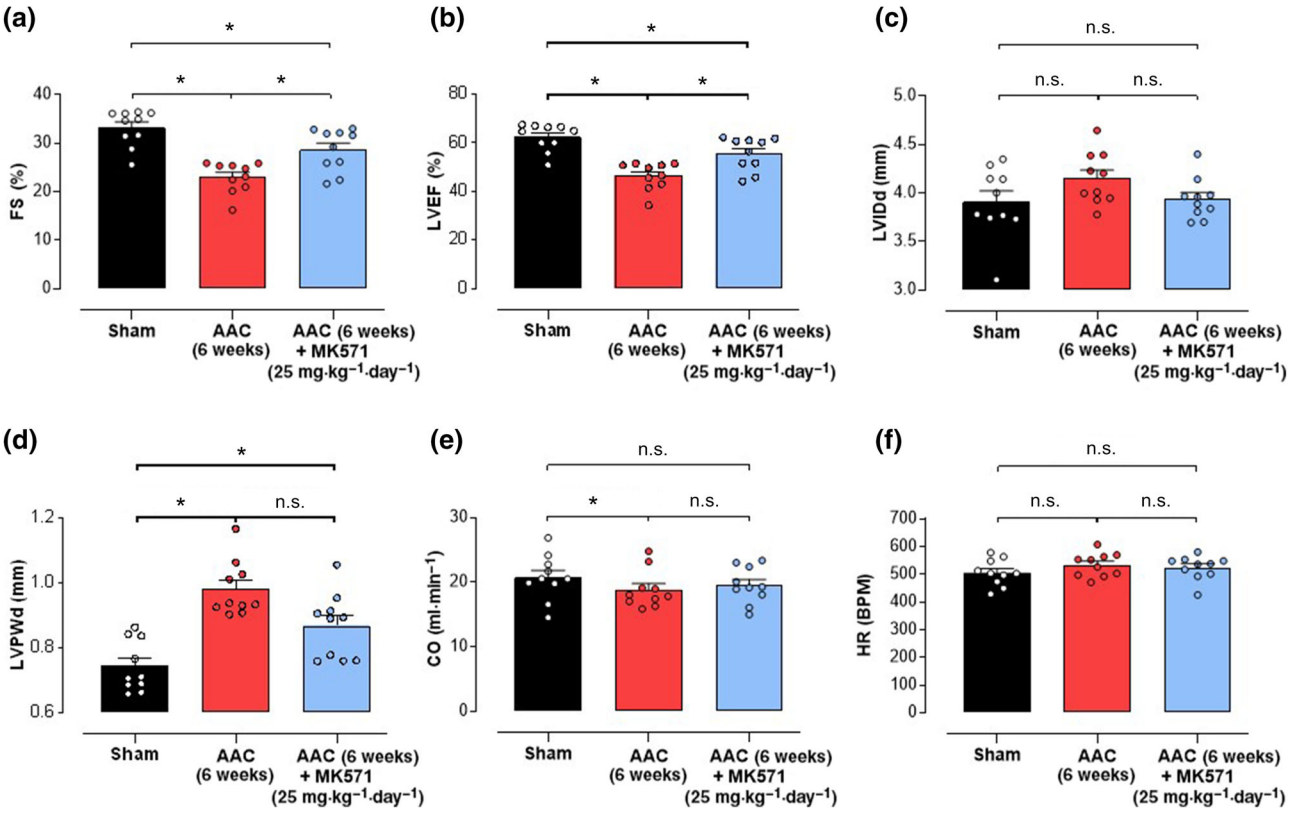

FIGURE 5 Fractional shortening (FS; a), left ventricular ejection fraction (LVEF; b), left ventricular internal diameter at diastole (LVIDd; c), left ventricular posterior wall diameter at diastole (LVPWDd; d), cardiac output (CO; e) and heart rate (HR; f) in sham mice and animals undergoing abdominal aortic constriction (AAC; 6 weeks) in the absence and presence of MK571 (25 mg·kg^{-1}·day^{-1}; p.o.). Data are shown as mean ± SEM. Statistical analysis by one-way ANOVA with Šídák post hoc test. *P <0.05 (adjusted for multiplicity)

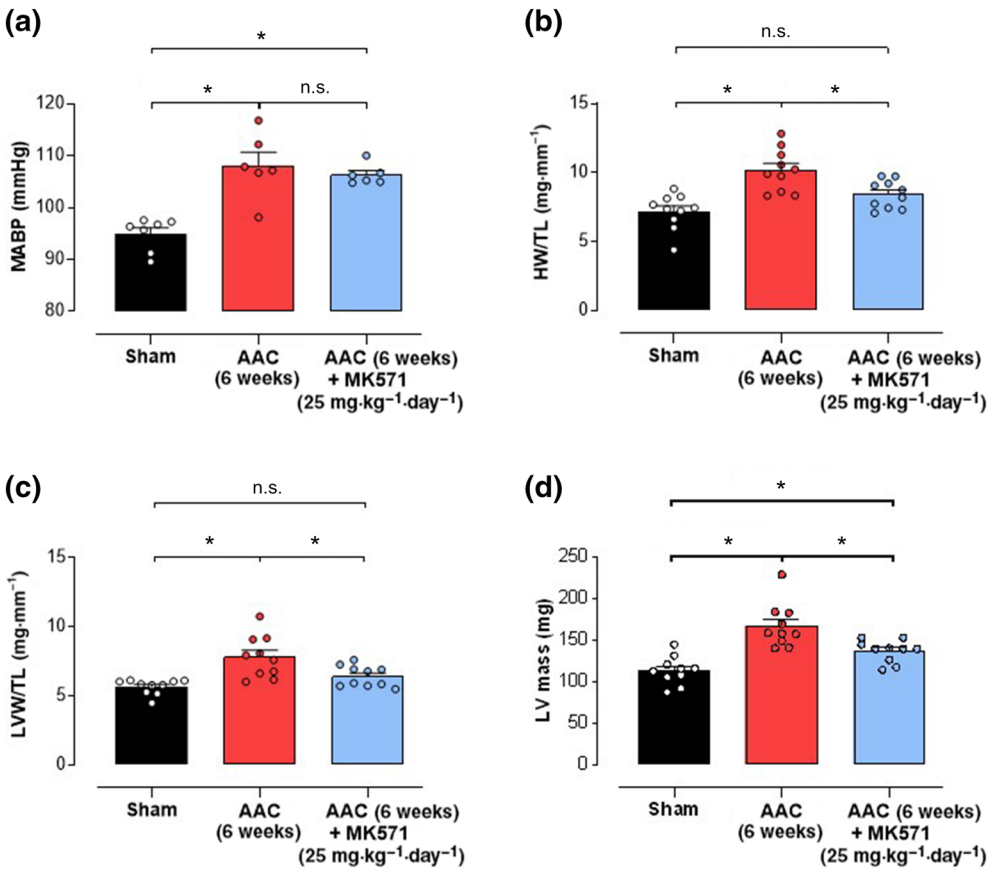

FIGURE 6 Mean arterial BP (MABP; a), heart weight to tibia length ratio (HW/TL; b), left ventricular weight to tibia length ratio (LVW/TL; c) and left ventricular mass (LV mass; d) in sham mice and animals undergoing abdominal aortic constriction (AAC; 6 weeks) in the absence and presence of MK571 (25 $mg \cdot kg^{-1} \cdot day^{-1}$; p.o.). Data are shown as mean ± SEM. Statistical analysis by one-way ANOVA with Šídák post hoc test. *P <0.05 (adjusted for multiplicity)

fulfil this remit, Bevans et al., 1998. However, there is a paucity of information regarding the physiological and pathological role(s) of extracellular transport of cGMP in governing vascular and cardiac function. Herein, we establish that multidrug resistance proteins (MRPs) play an overt role terminating cGMP signalling in blood vessels and the heart, regulating vascular smooth muscle proliferation, vessel reactivity, BP and the severity of cardiac damage in experimental heart failure. Interestingly, the sphere of action of MRPs appears to be directed towards membrane-localised natriuretic peptide-driven cGMP synthesis, with a lesser influence on NO-sensitive GC-generated cGMP pathways. These observations help define a central role for cGMP efflux via MRPs as a mechanism to turn off cGMP-dependent response and pharmacological inhibitors of this process may hold promise in the treatment of cardiovascular disease akin to existing modulators of cGMP signalling (i.e. PDE inhibitors and neutral endopeptidase [NEP] inhibitors).

To elucidate potential functional role(s) of MRPs in the cGMP-mediated inhibition of hCASMC proliferation, the MRP4/MRP5 inhibitor MK571 (Chen et al., 2002; Jedlitschky et al., 2012; Reid et al., 2003) was employed. MK571 produced a concentration-dependent reduction in hCASMC growth in the absence of exogenous cGMP-elevating agents. Because healthy VSMCs do not express **NO synthase** or natriuretic peptides, it is likely this innate anti-proliferative action of MK571 is a consequence of reducing export of basal cGMP production by the inherent GCs, a phenomenon well established for the NO-sensitive enzymes, but less so for the particulate isozymes (Potter & Garbers, 1992; Wolin et al., 1982). Moreover, a concentration of MK571 that did not significantly reduce proliferation *per se* augmented the anti-proliferative effects of subthreshold concentrations of both NO and ANP. Such findings imply that cellular efflux is a key mechanism regulating the anti-proliferative effects of cGMP, regardless of the synthesising GC (i.e. NO or natriuretic peptide sensitive). This concept was corroborated, to a certain degree, by measurements of intracellular and extracellular cGMP levels. Here, MRP inhibition alone tended to increase the intracellular:extracellular cGMP ratio, indicative of a reduction in transport out of the cell. However, the low basal cGMP concentrations entailed that such changes were modest at best. The NO donor, Deta-NO, tended to increase the mean intracellular cGMP concentrations, but MK571 was unable to increase this further. In contrast, the effect of MRP inhibition on ANP-mediated rises in cGMP was more clear cut. Interestingly, addition of ANP actually reduced the intracellular:extracellular cGMP ratio, driven by a subtle rise in intracellular levels but a dramatic increase in extracellular cGMP concentrations. In addition, in the presence of MK571, this balance was restored to a basal phenotype in which intracellular cGMP was significantly greater than extracellular cGMP. This pronounced effect of MRP inhibition on ANP responses fits with the particulate nature of this cognate GC, as synthesis of cGMP in the juxtamembrane region by GC-A should be in proximity to MRPs and thereby more overtly influenced by cGMP efflux. The lack of effect of MK571 and NO donor alone, or combination of the two, on cGMP

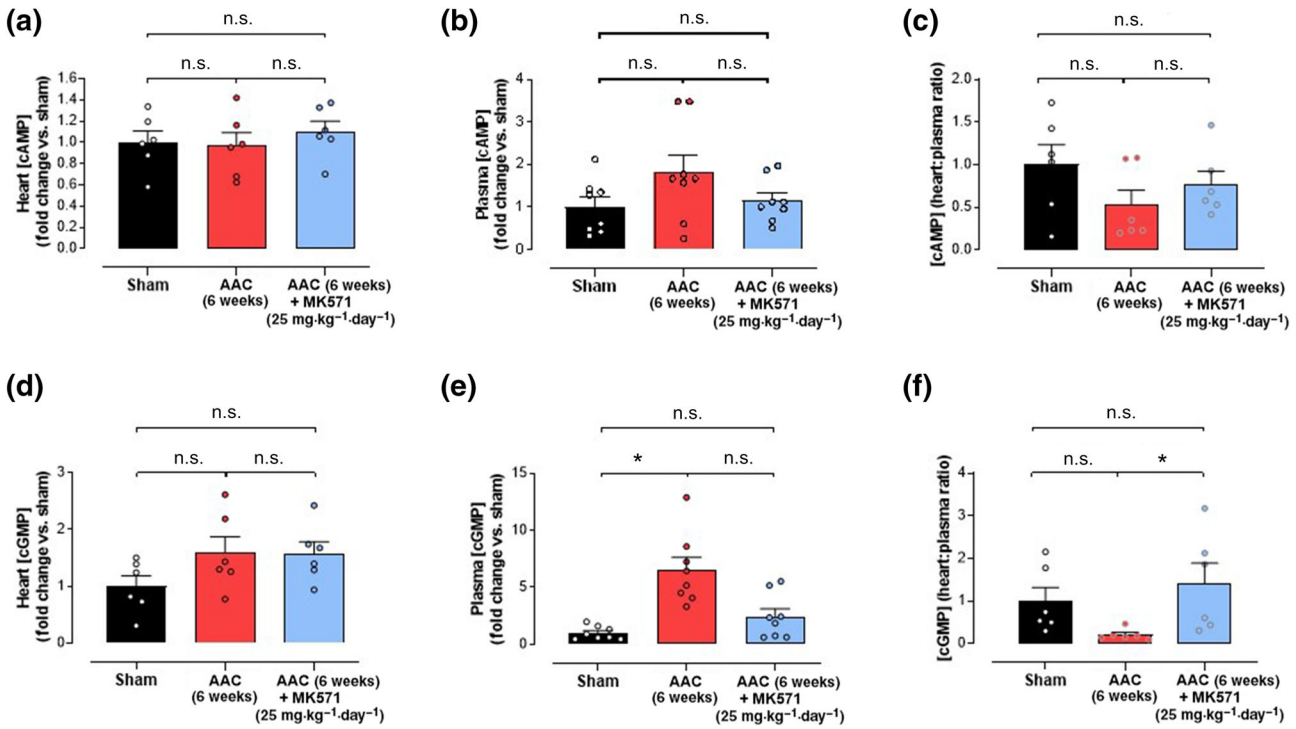

FIGURE 7 Fold change versus sham in heart (a, d) and plasma (b, e) cAMP or cGMP concentrations, and heart:plasma cAMP (c) or cGMP (d) ratio in animals undergoing abdominal aortic constriction (AAC; 6 weeks) in the absence and presence of MK571 (25 mg·kg^{-1}·day^{-1}; p.o.). Data are shown as mean ± SEM. Statistical analysis by one-way ANOVA with Šídák post hoc test. *P <0.05 (adjusted for multiplicity)

levels suggest that the NO-sensitive GC generates cGMP in a more diffuse, cytoplasmic region that is less sensitive to efflux via MRPs. These observations confirm and extend previous reports indicating that cGMP extrusion plays a critical role in terminating signalling in vascular tissue (Krawutschke et al., 2015), although herein it is demonstrated that natriuretic peptide-stimulated cGMP production is more susceptible to MRP-mediated regulation. Published work also supports the notion that cGMP produced by the membrane-spanning GCs is maintained in proximity to the membrane by PDE2 and PDE3, thereby accentuating the consequences of cellular efflux (Castro et al., 2006; Fischmeister et al., 2006; Krawutschke et al., 2015).

Echoing findings in cell-based experiments, MK571 and the structurally distinct MRP inhibitor probenecid (Hamet et al., 1989) elicited concentration-dependent relaxations of mouse aorta. Again, the ability of MRP inhibitors *per se* to relax vessels probably reflects intrinsic turnover of cGMP production and turnover as a result of NO production in the endothelium and GC-1/2 activity in the smooth muscle. This thesis is supported by inhibition of MK571-induced vasorelaxation by the NO-sensitive GC inhibitor, ODQ and the G-kinase inhibitor KT5823. In further studies, a subthreshold concentration of MK571 and probenecid significantly increased the potency of Sper-NO- and ANP-induced relaxations. Notably however, the potentiating effect of MRP inhibition was more pronounced against natriuretic peptide-dependent relaxations, paralleling observations in isolated hCASMCs. Oddly, MK571 was unable to potentiate vasorelaxant responses to ACh, which are solely dependent on NO release from the vascular endothelium in this tissue (Huang et al., 1995; Madhani et al., 2003; Scotland et al., 2005). Whether this is a consequence of cGMP signal compartmentalisation within the vascular smooth muscle remains to be determined. Specifically, that ACh-dependent NO release stimulates VSMC GC-1/2 but the cGMP generated is not of sufficient magnitude to be affected by MRPs, whereas cGMP production stimulated by exogenous NO (i.e. Sper-NO) is more global, activating the entire GC-1/2 enzyme pool. This interpretation is supported by previous findings that increasing concentrations of ACh do not elevate extracellular cGMP in rat aorta, in contrast to the NO donor, SNP (Schini et al., 1989). These data imply that greater cytosolic cGMP is generated by pharmacological concentrations of NO, spilling over into the membrane region and resulting in increased extrusion by MRPs. It should also be noted that the 3 μM (subthreshold) concentration of MK571 employed in these studies produced little or no effect on vascular tone *per se*, whereas higher concentrations had a significant vasorelaxant activity. However, at higher concentrations of MK571, it was not possible to maintain precontraction with phenylephrine. Indeed, an arguably larger vascular effect of MRP inhibition has been shown in murine aorta in response to the NO donor, S-nitrosoglutathione, using 10 μM MK571 (Krawutschke et al., 2015). Thus, although the increases in potency observed in Krawutschke et al. and in the present study are relatively modest, the beneficial effects of MRP inhibitors are almost certainly underestimates of the maximum attainable outcome, albeit difficult to dissect because of the inherent vasorelaxant activity of MRP

inhibitors. Additionally, MK571 is shown to potentiate vasorelaxant responses to the β-adrenoceptor agonist isoprenaline, to confirm that MPRs also functionally regulate cAMP signalling in the vasculature (Sassi et al., 2008).

Can this preferential activity of MRPs to regulate natriuretic peptide-sensitive GC signalling be explained by subcellular co-localisation? Currently, there are little or no data defining MRP protein–protein interactions with membrane-spanning GCs. However, MRP4 is localised in caveolin-1-enriched membrane fractions in vascular smooth muscle (Sassi et al., 2008) and both GC-A and GC-B co-localise with caveolins in the cardiovascular system (Chen et al., 2012; Doyle et al., 1997). In some situations, this phenomenon may also be of relevant to NO-sensitive GC because of its ability to translocate to the plasma membrane via the chaperone Hsp^{90} (Venema et al., 2003). In addition, a fraction of endogenous GC-1/2 within the heart is found in caveolin-3-positive caveolae (Tsai et al., 2012). When present in the caveolae, the enzyme is protected against oxidation that can occur from vascular injury, which inactivates the catalytic haem domain in the β subunit. Therefore, the enzyme remains active compared with its cytosolic counterparts (Tsai et al., 2012). Overall, this demonstrates that not only can MRP inhibition potentiate particulate GC produced cGMP but also in disease environments characterised by oxidative stress, MRP inhibition may also potentiate cGMP generated from membrane located sGC, amplifying the signal, thus expanding the therapeutic potential of MRP inhibitors.

The vasorelaxant effect of MK571 *per se* appears to be an isolated vessel phenomenon, because MRP inhibition did not significantly affect BP in anaesthetised or conscious mice, despite the compound having a relatively long plasma half-life (2–3 h in humans; Margolskee, 1991) and the dose used (25 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$) having previously been shown to effectively reverse experimental pulmonary hypertension (Hara et al., 2011). However, MK571 did exhibit a preference to potentiate GC-A activation as evidenced by significantly enhanced reductions in BP elicited by acute bolus doses of ANP (but not NO). This suggests in situations where endogenous circulating levels of NPs are increased (e.g. heart failure and pulmonary hypertension) that MRP inhibition will potentiate particulate GC activity, and the lack of an observable effect of MRP inhibition on MABP, in healthy mice, may be due to the low basal circulating concentrations of NPs. This should not be a surprise as the $MRP4^{-/-}$, $MRP5^{-/-}$ and $MRP4^{-/-}/MRP5^{-/-}$ mice lack an overt basal phenotype, suggesting that under healthy conditions, MRPs are not essential to vascular homeostasis (Borst et al., 2007). However, longer term MRP4 deficiency results in cardiac hypertrophy, as observed in aging $MRP4^{-/-}$ mice, which is not apparent in the same strain at 3 months (Sassi et al., 2012). Thus, in health, this mechanism may not be crucial to the maintenance of cardiovascular homeostasis *in vivo*, whereas its role may be triggered in cardiovascular disease characterised by enhanced circulating levels of natriuretic peptides (Potter et al., 2006).

To explore the latter hypothesis, the effect of MK571 on experimental heart failure, induced by pressure overload, was investigated. Abdominal aortic constriction produced significant systolic dysfunction, LV hypertrophy and LV dilatation (without any overt changes in cardiac output or HR). Remarkably, the vast majority of these indices of disease severity, including markers/drivers or hypertrophy and fibrosis, were reversed by the addition of MK571, demonstrating that inhibition of cGMP efflux can promote the well-established beneficial effects of this cyclic nucleotide in heart failure; presumably, based on the *in vitro* and *in vivo* findings described herein, this salutary action of MK571 is exerted through augmentation of natriuretic peptide/particulate GC signalling. The effect of MRP inhibition to promote tissue retention of cGMP was also apparent in this disease setting. Whereas cAMP levels remained largely unaltered by the pathology or presence of MK571, cardiac and circulating cGMP concentrations were elevated in response to abdominal aortic constriction. However, following MRP inhibition, the cardiac cGMP levels were maintained whereas the circulating concentrations were reduced considerably, thereby markedly improving the intracellular:extracellular cGMP ratio. It might be argued that MK571 did not increase cardiac cGMP above and beyond that observed in mice following abdominal aortic constriction alone, but the salutary actions of MRP inhibition in this regard would inherently lower natriuretic peptide activity, which is proportionate to disease severity, and therefore preservation of cardiac cGMP likely reflects a net increase to that expected. Moreover, myocardial VASP phosphorylation is significantly increased in the setting of pressure overload in the presence of MK571, intimating that functional G-kinase activity is promoted by MRP inhibition, an effect established to be beneficial in models of heart failure (Frantz et al., 2013; Patrucco et al., 2014).

Exploration of MRP expression in experimental heart failure and tissue from heart failure patients revealed a reduction in MRP4 levels, but a contrasting increase in MRP5, the latter matching a previous report (Dazert et al., 2003). Further investigation will be required to determine whether MRP4 or MRP5 plays a more important in regulating cGMP signalling in health and disease and to elucidate whether these expressional variations represent a host defence mechanism to promote cGMP signalling (i.e. MRP down-regulation) or potentially a pathogenic mechanism (i.e. MRP up-regulation). Such changes might also be argued to have opposing outcomes with respect to cAMP, because this cyclic nucleotide is predominantly detrimental in heart failure. Indeed, the cyclic nucleotide transporting specificity of MRP4 and MRP5 remains controversial. MRP4 is considered a predominantly cAMP transporting protein despite the fact that MRP4 inhibition results in elevated intracellular cGMP levels (Hara et al., 2011; Sassi et al., 2008). Indeed, MRP4 has a fivefold lower K_m for cGMP compared with cAMP, suggesting that it may actually have a preference for cGMP over cAMP (Chen et al., 2001). However, the V_{max} for cAMP extrusion is approximately twice as high as cGMP, raising the possibility that at lower cAMP concentrations, MRP4 transports cGMP, but at higher cAMP concentrations, cAMP extrusion is favoured. This theory appears to be supported in practice by studies that show increasing cAMP levels decrease cGMP efflux, presumably via competition (Hamet et al., 1989; Patel et al., 1995). In fact, it is possible that this competitive interaction underpins some of the crosstalk between the cGMP and cAMP systems, which is often thought to exclusively originate from the activity of PDE2 (cGMP

stimulated) and PDE3 (cGMP inhibited) (Conti & Beavo, 2007). In contrast, MRP5 is commonly accepted as a predominantly cGMP transporting protein because of its significantly lower K_m (180-fold) and V_{max} (20-fold) for cGMP compared with cAMP (Jedlitschky et al., 2000). In the vasculature, MRP4 and MRP5 are both expressed on endothelial cells and VSMCs, so in terms of localisation, both appear to be present to regulate cGMP efflux (Dazert et al., 2003; Sassi et al., 2008; Xu et al., 2004). Overall, these findings indicate that MRP4 and MRP5 are capable of regulating vascular homeostasis, with the inhibition of both proteins likely to elicit an additive, more advantageous effect. Elucidation of the precise role of each in cardiovascular homeostasis will almost certainly necessitate the use of $MRP4^{-/-}$ and $MRP5^{-/-}$ mice (Borst et al., 2007) because isozyme-selective pharmacological inhibitors do not exist. Indeed, a clear limitation of the present study is the reliance on suboptimal pharmacological tools in elucidating the physiological and pathological roles of MPRs with respect to cGMP transport. Further work using non-pharmacological means (i.e. *in vitro* gene knockdown and *in vivo* gene deletion) is warranted to substantiate the findings presented herein and to determine the consequences of altered MR4 and/or MRP5 expression/activity (including changes brought about by disease) on intracellular:extracellular cGMP ratios in the heart and vasculature, and the functional corollaries of this. Such investigations might help explain why observations from this study are at odds with some previous reports that concluded the majority of the beneficial effects of MRP inhibitors result from the augmentation of NO/cGMP signalling (Ahmed et al., 2007; Billiar et al., 1992; Patel et al., 1995). One explanation for this difference lies with the time and concentrations of the NO-sensitive GC agonist used. Thus, longer term exposure to supramaximal NO concentrations results in excessive cGMP production that spills over in to the juxtamembrane compartment and is susceptible to MRP extrusion. However, in a more physiological setting, we conclude that this phenomenon is largely exclusive to the particulate cyclase(s), which synthesise cGMP adjacent to the membrane.

In summary, these data support that concept that MRP inhibition has ability to promote cytoprotective cGMP signalling in the heart and vasculature, and provide additional evidence verifying the importance of MRPs in contributing to cGMP compartmentalisation. Since MRP inhibitors are already in the clinic (i.e. probenecid), repurposing of such molecules may offer a rapid and inexpensive means by which to enhance cGMP-targeted agents for cardiovascular diseases such as heart failure and pulmonary hypertension. Indeed, synergy between cGMP elevating agents (e.g. PDE and neutral endopeptidase inhibitors) has been well characterised in experimental models of pulmonary hypertension and patients with the disease (Baliga et al., 2008, 2014; Hobbs et al., 2019). This gives credence to the idea that combination of natriuretic peptide elevating agents (e.g. neutral endopeptidase inhibitors) with MRP inhibitors is also likely to be of supplementary therapeutic benefit. Furthermore, in the setting of pulmonary hypertension, blockade of both cGMP- and cAMP-transporting MRPs might also be argued to hold additional promise. However, the ultimate goal of utilising MRP inhibitors as drugs is fraught with difficulty. The selectivity of current inhibitors leaves much to be desired (e.g. MK571 blocks lymphotoxin signalling), MRP4/MRP5 are known to transport a number of key biological molecules (e.g. ADP, ATP, 5-hydroxytryptamine, steroids and eicosanoids) and both transporters have a widespread distribution, both within and outside the cardiovascular system (Zhou et al., 2008). Thus, despite the promise of MRP inhibition as an additional mechanism to pharmacologically augment the cardioprotective and vasoprotective actions of cGMP, far more refined and targeted interventions will be necessary to harness this effectively.

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AUTHOR CONTRIBUTIONS

All authors discussed the results and provided comments on the manuscript. R.G., M.P., J.D., V.L., A.M., C.P.-T., A.A., R.B. and A.H. contributed to the design and implementation of the research and to the analysis of the results. R.G., A.A. and A.H. wrote the manuscript.

CONFLICT OF INTEREST

A.J.H. is a scientific advisory board member/consultant for Palatin Technologies Inc. and Novo Nordisk and has received research support from Palatin Technologies Inc. for an unrelated project.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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