Development and translation of thiometallate sulfide donors using a porcine model of coronary occlusion and reperfusion

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ABSTRACT

Sulfide-releasing compounds reduce reperfusion injury by decreasing mitochondria-derived reactive oxygen species production. We previously characterised ammonium tetrathiomolybdate (ATTM), a clinically used copper chelator, as a sulfide donor in rodents. Here we assessed translation to large mammals prior to clinical testing. In healthy pigs an intravenous ATTM dose escalation revealed a reproducible pharmacokinetic/pharmacodynamic (PK/PD) relationship with minimal adverse clinical or biochemical events. In a myocardial infarction (1-h occlusion of the left anterior descending coronary artery)-reperfusion model, intravenous ATTM or saline was commenced just prior to reperfusion. ATTM protected the heart (24-h histological examination) in a drug-exposure-dependent manner ($r^2 = 0.58$, $p < 0.05$). Blood troponin T levels were significantly ($p < 0.05$) lower in ATTM-treated animals while myocardial glutathione peroxidase activity, an antioxidant selenoprotein, was elevated ($p < 0.05$). Overall, our study represents a significant advance in the development of sulfides as therapeutics and underlines the potential of ATTM as a novel adjunct therapy for reperfusion injury. Mechanistically, our study suggests that modulating selenoprotein activity could represent an additional mode of action of sulfide-releasing drugs.

1. Introduction

Ischaemic heart disease remains a leading cause of global mortality [1]. Although early mechanical arterial revascularization is accepted as the most effective reperfusion modality for ST-elevation myocardial infarction, significant myocardial injury can still occur despite effective restoration of coronary blood flow [2]. The resulting reperfusion injury is a complex pathological process mediated by (i) cellular injury from excessive production of mitochondrial and extra-mitochondrial reactive oxygen species (ROS), rapid intracellular pH correction, calcium handling abnormalities and mitochondrial permeability transition pore (mPTP) opening, (ii) microvascular injury through impaired vasomotion, endothelial dysfunction and platelet aggregation, and (iii) inflammation, both resulting from, and further perpetuating, cellular and microvascular injury [3,4]. The net result is cardiomyocyte cell death [5]. Multiple pharmacological [6–9] and non-pharmacological [10,11] strategies have been trialled in humans to combat reperfusion injury. None, however, have proved consistently successful to achieve adoption in routine clinical practice.

Thiometallates were first synthesised nearly two centuries ago [12]; the most frequently used, both medicinally and as a chemical adjuvant, is the ammonium salt of tetrathiomolybdate (ATTM; $[NH_4]_2MoS_4$). ATTM is used clinically as an oral copper chelator, showing utility against copper toxicity in sheep [13] and Wilson's disease in humans [14]. In preclinical studies, ATTM conferred protection against various fibrotic, degenerative, malignant, and auto-immune conditions via its action as a copper chelator [15]. We [16] and others [17] more recently demonstrated that ATTM is a slow-release sulfide donor.

Hydrogen sulfide (comprising anionic hydrosulfide [HS⁻] and gaseous H_2S, referred to collectively herein as 'sulfide') is an important

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physiological mediator [18], putative therapeutic [19], and is classified as the third endogenous gasotransmitter [20]. Our primary interest in exogenous sulfide delivery relates to its ability to rapidly reduce mitochondrial respiration through partial inhibition of cytochrome C oxidase [16], and thus excessive generation of mitochondrial ROS. Readily available biochemical markers such as lactate and acid/base balance conveniently inform on the pharmacodynamic effects of mitochondrial modulation by ATTM [16,21]. Temporarily constraining oxidative phosphorylation with sulfide triggers a greater reliance on glycolytic ATP that induces hyperlactataemia, while both glycolysis and net ATP hydrolysis release H^+ ions generating a metabolic acidaemia.

We demonstrated cardio- and neuroprotection, and survival benefit using ATTM in small rodent models when given at the onset of reperfusion following myocardial infarction, stroke, and haemorrhagic shock, respectively [16,22]. The drug was well-tolerated with minimal off-target effects, likely due to ion-channel mediated cellular uptake and release of sulfide intracellularly, rather than decomposition within the circulation [21]. Notwithstanding investigation in a porcine peripheral vascular disease model [23], a Phase II safety study [24], and early studies using sulfide generators [25,26], translation of other sulfide therapeutics to large mammals has proven elusive. Thus, an assessment of the efficacy and safety of ATTM in a large animal model is warranted, as recommended by recent expert consensus guidelines [27]. We hypothesized that ATTM could confer cardioprotection in a relevant porcine model of acute myocardial infarction and reperfusion. To further explore sulfur/selenium interactions, myocardial glutathione peroxidase activity (an antioxidant selenoprotein) was investigated as an additional putative mechanism of effect.

2. Materials and methods

2.1. Materials

ATTM was synthesised by Almac Sciences (Craigavon, County Armagh, UK) as previously described [28] and validated, with Sigma Aldrich (Gillingham, Kent, UK) material as an internal standard, by *in vitro* (sulfide-release) and *in vivo* (rat PK/PD) assays. Unless stated, all other drugs/reagents were purchased from Sigma Aldrich.

2.2. Ethics statement

Animal studies were performed at University College London (PPL 70/8290; rat studies) or the Translational Biomedical Research Centre (TBRC), University of Bristol (UoB) (PPL 70/8975 and PP4585512; pig studies), following UK Home Office guidelines under the Animals (Scientific Procedures) Act 1986 and local ethics committee approval at both institutions. TBRC is a Good Laboratory Practice preclinical facility. Studies are reported in compliance with ARRIVE 2 guidelines [29].

2.3. Rodent PK/PD studies

Briefly, male Wistar rats (300 g; 2 months old; Charles River, Margate, Kent) were anaesthetised with isoflurane (Abbott, Maidenhead, Berks, UK), placed on a heated mat (Harvard Apparatus, Cambridge, Cambs, UK) to maintain euthermia (rectal temperature; 37 °C), and cannulated to allow monitoring of mean arterial blood pressure and IV administration of fluids and drugs. Urine output was measured via a bladder catheter. Animals were then administered sodium pentobarbitone (10 mg kg^{-1} IV; Pentoject; Animalcare, York, UK) to allow tracheal intubation and mechanical ventilation (Physiosuite, Kent Scientific, Torrington, CT, USA). Following 1-h stabilisation, increasing IV bolus doses of ATTM (1–100 mg kg^{-1}) from Almac or Sigma-Aldrich were administered. Measurements were collected at baseline, then as follows after each IV dose: blood pressure within 30 s, cardiac function (by echocardiography) within 1 min, blood sampling to determine plasma drug concentration at 2 min, and arterial blood gas analysis to measure

lactate, and acid/base changes at 27 min. Doses were escalated half-hourly. After the highest dose, the half-life of ATTM was assessed with regular blood sampling up to 1-h post-dose. Animals were then euthanised using IV sodium pentobarbitone.

2.4. Porcine studies

Pigs were bred by a local farm affiliated to the TBRC. Thirty female Large White pigs, six months old, body weight 67 ± 7 kg (mean ± standard deviation), were enrolled across two cohorts into three consecutive studies (Fig. 1A). A more detailed description of husbandry, anaesthesia and instrumentation can be found in the Supplementary material. Briefly, animals (fasted overnight) received intramuscular premedication (ketamine, 10 mg kg^{-1}; dexmedetomidine, 15 µg kg^{-1}; Vetoquinol UK Limited, Towcester, Northants, UK). Anaesthesia was induced with IV propofol (incremental doses of 1 mg kg^{-1}; Zoetis UK Limited, London, UK) until successful orotracheal intubation (Smiths Medical International, Luton, Beds, UK), then maintained using 1–2% isoflurane (Henry Schein, Dumfries, UK). Animals were mechanically ventilated (Datex-Ohmeda Aisys, GE Healthcare, Helsinki, Finland) using the following settings: fraction of inspired oxygen, 0.21–1; tidal volume, 10 ml kg^{-1}; respiratory rate, 20 breaths per min; positive end-expiratory pressure, 0–3 cm H$_2$O. Ringer's lactate (4 ml kg^{-1} h^{-1}; B. Braun Melsungen AG, Melsungen, Germany) provided intravenously via a right femoral vein cannula (Introcan Safety 24G x 3/4''; B. Braun)
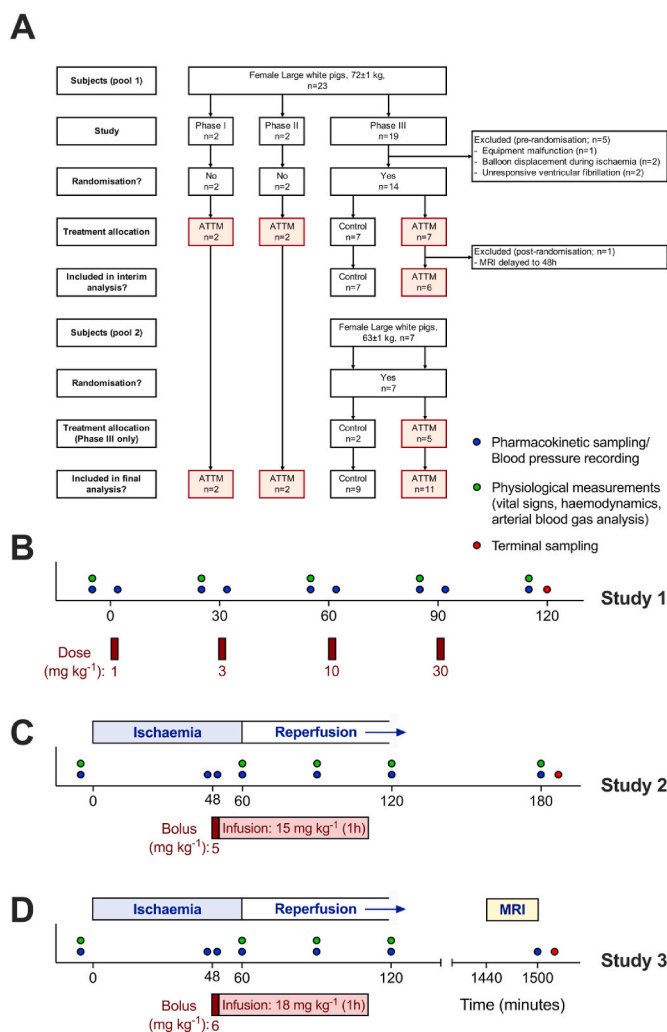

Fig. 1. Experimental design. (A) depicts study phases, defined exclusions, and sample sizes. Panels B–D show the protocols for studies 1–3, respectively.

maintained euvolaemia. Peripheral capillary oxygen saturation (SpO$_2$) was monitored by a pulse oximeter sited on the tail. Animals were allowed to stabilise for a minimum of 30 min following instrumentation.

2.4.1. Study 1 – dose escalation in healthy pigs

In non-recovery experiments, two anaesthetised, instrumented and mechanically ventilated pigs were administered increasing doses of ATTM as a 2-min intravenous bolus (1–30 mg kg^{-1}) at half hourly intervals (Fig. 1B) to ascertain the therapeutic window in this species. Following instrumentation and stabilisation, baseline measurements were taken, including physiological variables and sampling of 1 ml arterial blood for analysis of pH, blood gases, glucose, lactate, electrolytes, and acid-base balance. ATTM (1, 3, 10 and 30 mg kg^{-1} dissolved in phosphate-buffered saline; PBS) was delivered as a slow intravenous bolus (0.1 ml kg^{-1}) over 2 min (Fig. 1B). An arterial blood sample taken at the end of each bolus (2 min) was used to determine the maximum plasma concentration (C$_{MAX}$). At 25 min, arterial blood samples were withdrawn for blood gas and pharmacokinetic analyses. On completion, animals were euthanised by cardioplegic arrest (described below) with hearts harvested using clinical standards [30].

2.4.2. Study 2 – safety study of ATTM in pigs subjected to acute myocardial infarction

The safety profile of ATTM administration during myocardial ischaemia/reperfusion (I/R) injury was assessed in two anaesthetised, instrumented, mechanically ventilated pigs in non-recovery experiments. Animals were pre-treated with acetylsalicylic acid (75 mg orally with food 5–7 days before experimentation; Accord, Barnstaple, Devon, UK), anaesthetised, heparinised, and instrumented as previously reported [30]. To minimise the risk of peri-procedural malignant arrhythmia, infusions of amiodarone 300 mg (Hameln Pharma, Hameln, Germany) and magnesium sulfate 8 mmol (AS Kalceks, Riga, Latvia) were administered over 2-h via a femoral venous catheter, commencing once access was gained. Following left and right coronary angiography, a 0.014-inch interventional guide wire (Runthrough floppy, Terumo, Tokyo, Japan) was navigated to the distal left anterior descending (LAD) coronary artery. An appropriately sized balloon (2.5–3.5 mm Emerge, Boston Scientific, Marlborough, MA, USA) was positioned immediately beyond the first major diagonal branch. Prior to balloon inflation, a series of baseline measurements were made, with blood removed for arterial blood gas and pharmacokinetic analyses, as described for Study 1.

Myocardial ischaemia was induced for 1 h by balloon catheter inflation (Fig. 1C). In the event of electrical or haemodynamic compromise during ischaemia, resuscitation through direct current (DC) cardioversion, manual chest compression and/or inotropic support was provided, as required. If resuscitation attempts were unsuccessful, or ischaemia was disrupted through balloon dislodgement, the experiment was terminated. At 48 min from the onset of ischaemia, a slow intravenous bolus of ATTM (5 mg kg^{-1} in 0.1 ml kg^{-1} PBS) was administered over 2 min. Peak changes in blood pressures were monitored during this time. An arterial blood sample was taken after the bolus for pharmacokinetic analysis followed by a 1-h intravenous infusion of 15 mg kg^{-1} h^{-1} ATTM (in 0.3 ml kg^{-1} h^{-1}). The balloon was deflated 1-h after onset of ischaemia and animals monitored for a further 2-h post-reperfusion. Physiological evaluation and withdrawal of blood for blood gas and pharmacokinetic analyses commenced just prior to reperfusion, then at 30-, 60- and 120-min post-reperfusion. Animals were euthanised at experiment end, as below.

2.4.3. Study 3 – efficacy of ATTM in pigs subjected to acute myocardial infarction

This study comprised a randomised assessment of ATTM versus control (PBS). Our power calculation, targeting a 50% relative reduction in infarct size (power, 0.8; α, 0.05), estimated that a sample size of nine animals per group would be sufficient to demonstrate clinically and

statistically significant differences. Final group sizes were 9 control and 11 ATTM-treated animals. Methodology was essentially as described for Study 2, with some exceptions: upon using a femoral artery access route, two instances of coronary balloon dislodgement occurred during DC cardioversion resuscitation attempts; hence, the left carotid artery was subsequently cannulated to enhance catheter stability. Based on pharmacokinetic outcomes of Study 2, the ATTM dose level was increased by 20% (to a 6 mg kg^{-1} bolus and 18 mg kg^{-1} 1-h infusion). Control-treated animals received equivalent volumes of PBS. A background infusion of 4 ml kg^{-1} h^{-1} Ringer's lactate maintained euvolaemia. Animals were monitored, and blood sampled for up to 1-h post-reperfusion and then returned to their pen to recover from anaesthesia. Post-surgical analgesia consisted of intravenous buprenorphine (0.02 mg kg^{-1}; Ceva Animal Health, Amersham, Bucks, UK) and meloxicam (0.4 mg kg^{-1}; Boehringer Ingelheim, Ingelheim/Rhein, Germany). The animals received a meal at the end of the day after their first procedure before being fasted prior to day 2. Water was available *ad libitum* throughout. The following day (at 24 ± 2 h post-reperfusion), animals were re-anaesthetised and underwent cardiac magnetic resonance imaging (MRI; described in Supplementary material). Area at risk (AAR) using MRI-derived early gadolinium enhancement (EGE) images was determined as previously described and validated against microspheres, SPECT and T2 mapping; here, a two-standard deviation (2SD) thresholding was applied [31]. An arterial blood sample was collected for pharmacokinetic analysis.

2.5. Cardioplegic arrest and organ harvest

At experiment end, under general anaesthesia, median sternotomy was performed, and heparin administered to achieve an activated coagulation time >250 s. Next, the superior and inferior vena cava and the aorta were clamped in sequence and 500 ml of cold (4 °C) cardioplegia solution was administered into the aortic root at high pressure (>200 mmHg) to achieve standstill. This solution comprised magnesium chloride (1.193 g), potassium chloride (1.193 g), and procaine hydrochloride (272.8 mg) per 20 ml at clinical grade diluted into 1 L of normal saline (Martindale Pharmaceuticals, Romford, Essex, UK) [32]. The arrested hearts were promptly removed and preserved in cardioplegia solution at −20°C for histological and biochemical analyses.

2.6. Histology and blood/tissue analyses

Hearts were minimally defrosted at room temperature (within cardioplegia solution for 1-h), then sliced into 8 mm sections from the apex to the left atrium. Excluding the apex, five of the first six slices were incubated (at 37°C) with 0.1% tetrazolium chloride for 30 min, then fixed with 10% formalin for a further 30 min. Open access software (Image J, 1.52a; National Institutes of Health, Bethesda, MD, USA) was used to quantify the area of viable (stained deep pink/purple with tetrazolium chloride) and non-viable (light grey or dark haemorrhagic) tissue.

The remaining tissue slice was used to analyse antioxidant enzyme activities. Tissue samples were obtained by biopsy from the ischaemic penumbra (mapped from the histology of adjacent slices) and an area of left ventricle remote from injury (tissue not at risk). Samples were ground in liquid nitrogen using a mortar and pestle then homogenised within an ice-cold biopsy preserving solution [33]. Glutathione peroxidase (GPx) and catalase activity were respectively measured in supernatant derived from the tissue homogenate using standard methodology [34], or a commercially available enzyme activity kit (ab83464) according to the manufacturer's instructions (Abcam, Cambridge, Cambs, UK). Protein concentrations were determined using a bicinchoninic acid (BCA) assay (Thermo Scientific, Waltham, MA, USA).

Troponin T was measured in plasma obtained from animals at 1-h post-reperfusion using a high-sensitivity ELISA kit (Cloud-Clone Corp, Katy, TX, USA), performed in duplicate and following the

manufacturer's instructions.

2.7. Pharmacokinetic analyses

Conveniently, coloration of ATTM allows its quantification in plasma using a simple calorimetric assay. Arterial blood drawn into heparin-containing tubes was centrifuged at $1000 \times g$ for 10 min at 4°C. The resulting plasma supernatant was stored at -80°C until subsequent analysis. Absorbance by plasma ATTM (at 468 nm) was assessed using a microplate reader and BioTek (Gen5) software (Synergy 2, North Star Scientific, Sandy, Beds, UK) [21]. Plasma concentrations were derived by comparison against ATTM standard curves in the range 0–500 µmol 1^{-1}.

2.8. Data and statistics

For rat PK/PD and pig efficacy studies, treatments were randomly allocated using an online tool (random.org). Administration of ATTM could not be conducted fully blinded due to distinctive red/black coloration of the molecule. However, all biochemistry and histology analyses were undertaken by investigators unaware of treatment allocation. Normality testing was performed using the Shapiro–Wilk test. Data are presented as individual points, mean $\pm$ standard error of the mean, or median, quartiles and range, as appropriate. Pharmacokinetic data were analysed using one- or two-phase decay curves and the least squares fitting method. Correlations were performed using linear regression. Parametric data were analysed using a repeated measures two-way ANOVA followed by Šídák's multiple comparisons test. Nonparametric data were analysed using the Mann-Whitney test. All analyses were two-tailed and performed using Prism 9.0 software (GraphPad, San Diego, CA, USA). Multiplicity adjusted p-values <0.05 were considered statistically significant.

3. Results

3.1. In vitro and in vivo validation of custom synthesised ATTM

ATTM showed appropriate *in vitro* H_2S gas release (Suppl. Fig. 1A; 2–4 ppm over 1 h). While gas release increased over time, its level remained within our predefined limit (4 ppm) based on previous experience using this compound [21]. Expected bioactivity in rats was observed, including dose-dependent increases in blood lactate levels and metabolic acidaemia (Suppl. Fig. 2).

3.2. Study 1 – dose escalation in healthy pigs

A dose-dependent elevation in plasma ATTM levels was observed that was linear between 1 and 10 mg kg^{-1} but plateaued at 30 mg kg^{-1} (Fig. 2A) due to partial insolubility of the compound at the highest dose. Similar to our prior studies in rats, rapid removal of ATTM from the circulation was observed in pigs (Fig. 2B). There was a dose-dependent increase in arterial blood lactate levels related to mitochondrial inhibition (Fig. 2C), and a transient fall in blood pressure at higher dose levels, albeit still within an acceptable range (Fig. 2D). All other vital signs and physiological variables remained within normal ranges (Suppl. Figs. 3–6). The no-observed-effect-level (NOEL) was 3 mg kg^{-1}, equating to a plasma concentration of 100 µmol 1^{-1} in pigs. ATTM showed a strong PK/PD relationship with lactate ($r^2 = 0.86$, p < 0.05, Fig. 2E). The no-observed-adverse-effect-level (NOAEL) for the study was not determined; a transient fall in blood pressure recovered within 1–2 min (Fig. 2D) but was not considered an adverse event according to our pre-defined criteria (blood pressure $\leq$30 mmHg from baseline for 5 min and/or requirement for vasopressor support). Nonetheless, we considered the therapeutic window (Fig. 3A) to be between the NOEL value of 3 mg kg^{-1} and 10 mg kg^{-1} where changes in blood pressure were seen.
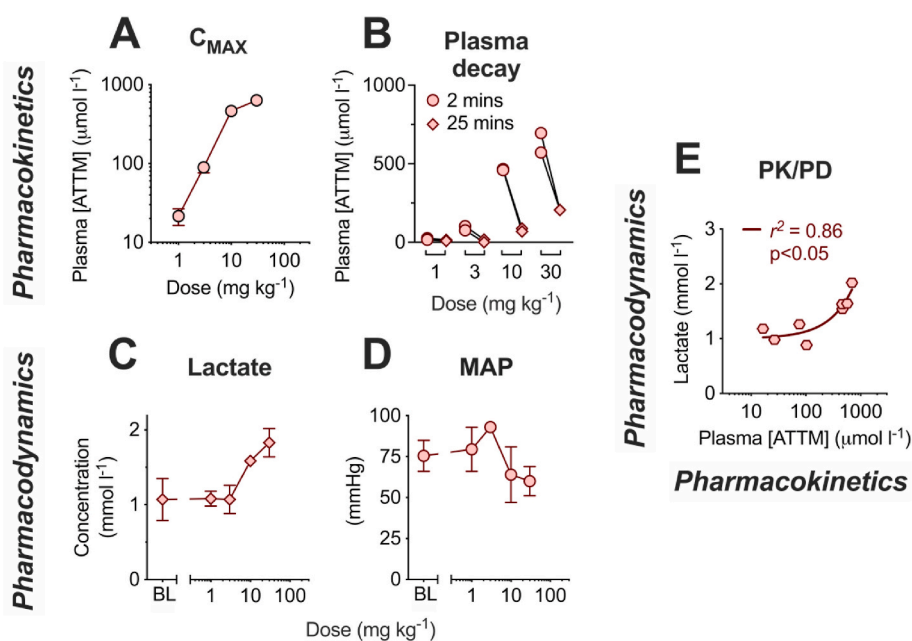
Fig. 2. Pharmacokinetics (PK) and pharmacodynamics (PD) in healthy pigs (Study 1). A and B show the maximal ATTM plasma concentration following IV bolus dosing (C$_{MAX}$), and plasma clearance prior to dose escalation (at 25-min), respectively. Lactate levels (25-min post-bolus) and blood pressure (1-2-min from bolus onset, recorded as the greatest observed change compared to pre-drug measurements) are shown in panels C and D. The PK/PD relationship using lactate as a dynamic biomarker is shown in panel E, with correlation performed using linear regression.
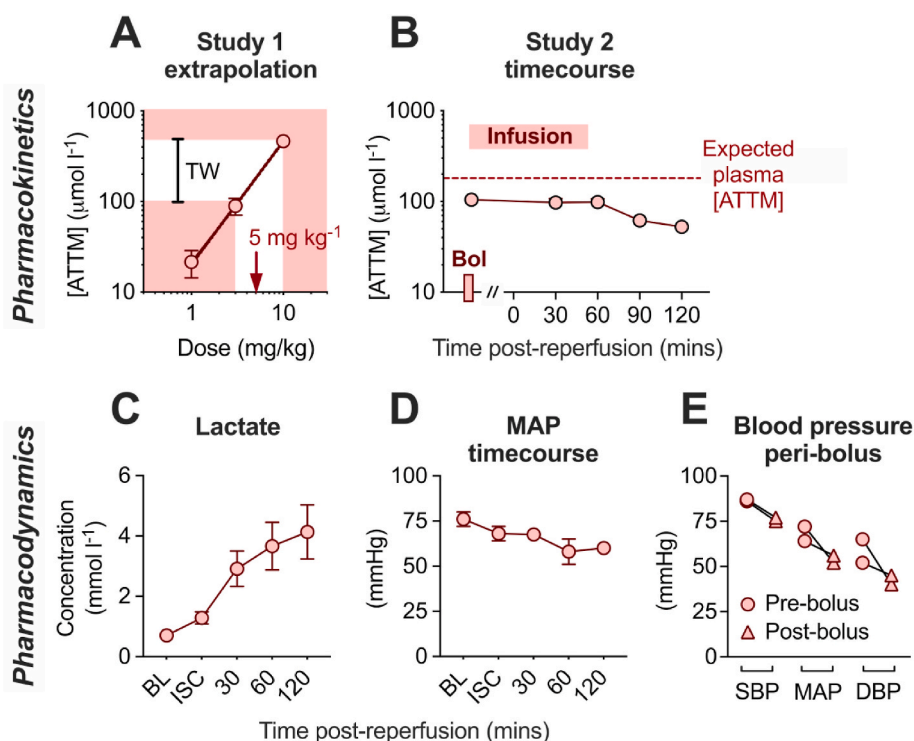
Fig. 3. Pharmacokinetics (PK) and pharmacodynamics (PD) in animals subjected to myocardial ischaemia/reperfusion injury (Study 2). Panel A shows construction of a therapeutic window (TW) derived from Study 1. Actual ATTM plasma levels following bolus (5 mg kg^{-1}) and infusion (15 mg kg^{-1}), respectively are shown in panel B; expected plasma levels are denoted by the dotted line. Arterial lactate levels and blood pressures either over the course of the experiment or maximum decrease peri-bolus are shown in panels C–E. BL, baseline; Bol, bolus; DBP, diastolic blood pressure; ISC, ischaemia; MAP, mean arterial pressure; SBP, systolic blood pressure.

3.3. Study 2 – safety study in pigs subjected to acute myocardial infarction

We opted for a 5 mg kg^{-1} ATTM intravenous loading dose (after 4/5 of ischaemia time; Fig. 1C) and pharmacokinetic computational modelling (MS Excel) suggested that plasma drug levels could be maintained by an infusion containing three times the bolus dose, administered subsequently over 1 h. Accordingly, intravenous administration of 5 mg kg^{-1} ATTM, followed by 15 mg kg^{-1} h^{-1} could maintain plasma levels over this duration however, peak plasma levels only reached the lower threshold of the therapeutic range (Fig. 3B). Notwithstanding this shortfall in exposure, ATTM treatment elevated plasma lactate levels (Fig. 3C), and only transient effects on blood pressure were observed (Fig. 3D–E). To target a plasma level more comfortably within the therapeutic range, we prospectively increased the dosing regimen by 20% in Study 3.

3.4. Study 3 – efficacy study in pigs subjected to acute myocardial infarction

We initially enrolled 19 animals, with 14 randomised to treatment, and 13 eligible for analysis following a 24-h recovery period (Fig. 1A). During the myocardial ischaemia phase, two animals exhibited unresponsive ventricular fibrillation, and two suffered balloon dislodgement during resuscitation attempts; all four were euthanised immediately. Another animal was culled after instrumentation due to equipment malfunction, while a further (ATTM-treated) animal was excluded from analysis due to delay in follow-up. We took the opportunity to perform a blinded interim pharmacokinetic analysis, and this revealed suboptimal exposure in some animals (i.e., lower than the cut-off for the therapeutic window; 100 μmol l^{-1}) due to inadequate drug dissolution and thus reduced dose administration. To overcome this, we included a second cohort of animals, randomised 2:1 in favour of the active treatment. We

administered ATTM at the same dose level (6 mg kg^{-1} loading bolus + 18 mg kg^{-1} infusion over 1 h; Fig. 1C) but took extra care to ensure the drug was adequately dissolved by vortexing for an extra 30 s and checking the vial to ensure no material remained. Thereafter, all animals recovered uneventfully from anaesthesia and showed no overt clinical signs except for one (ATTM-treated) animal that exhibited emesis (one episode) shortly after recovery from anaesthesia. Due to coloration of the molecule, ATTM-treated animals were identifiable by an orange 'tan' and pigmented urine due to renal excretion; both resolved by 24-h.

All animals followed up to 24-h post-myocardial infarction were included in the final batched analysis. The median ATTM plasma level at reperfusion in ATTM-treated animals was 125 μmol l^{-1}; this was maintained up to 1-h post-reperfusion, albeit with variability between animals (Fig. 4A). Plasma ATTM clearance at 24-h post-reperfusion was 92%. Arterial blood lactate levels, monitored as a marker of ATTM bioactivity, were significantly higher in ATTM-treated animals at 1-h post-reperfusion (Fig. 4B) and showed good correlation with the ATTM plasma level at reperfusion ($r^2 = 0.44$, $p < 0.05$, Fig. 4C). ATTM bolus administration caused a transient (<1 min) fall in blood pressure (Fig. 4D), similar to that observed in Study 2. Except for this peri-bolus period, blood pressures were similar in both groups over the course of the experiment (Fig. 4E).

A comparison of all (n = 11) ATTM-treated animals (including sub-therapeutic dosing, <100 μmol l^{-1} at reperfusion, n = 4) indicated improvement against controls (27% relative reduction in infarct size measured histologically by tetrazolium chloride staining). This was not statistically significant (p = 0.08; Fig. 5A) according to the power calculation. However, comparison of animals with therapeutic ATTM plasma levels (i.e., >100 μmol l^{-1}, n = 7) at the point of reperfusion showed greater protection (41% relative reduction in infarct size vs control, $p < 0.05$, Fig. 5B, left panel). A significant correlation ($r^2 = 0.58$, $p < 0.05$) was observed between ATTM plasma levels (at reperfusion) in all animals and subsequent infarct size (Fig. 5B, right panel).
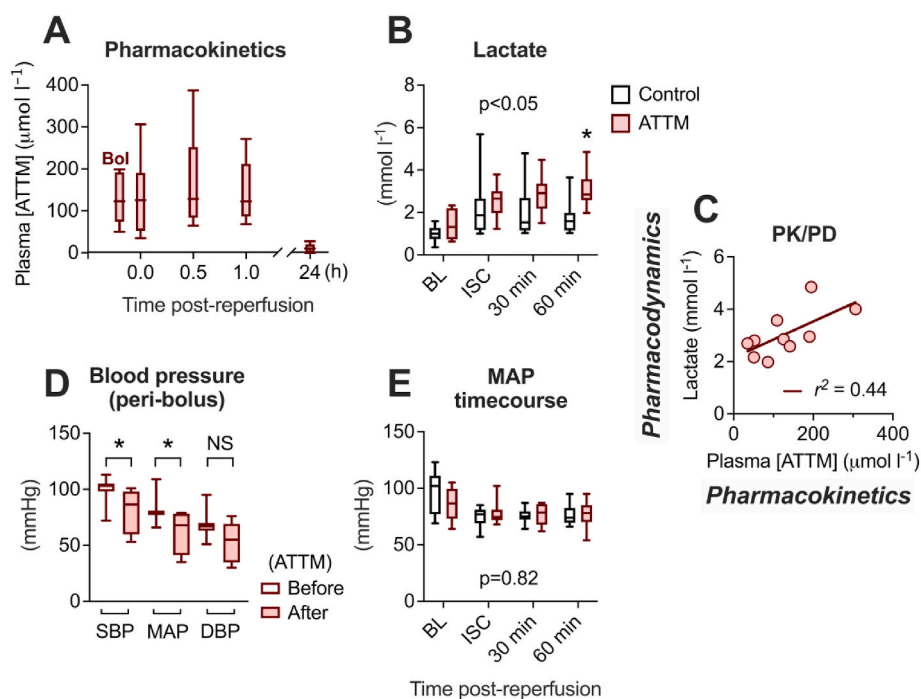
Fig. 4. Pharmacokinetics (PK) and pharmacodynamics (PD) in animals subjected to myocardial ischaemia/reperfusion injury (Study 3). Plasma ATTM levels post-bolus (Bol), immediately before reperfusion (time zero) and post-reperfusion are shown in panel A. Lactate levels are shown in B; the actual P-value is the result of the overall ANOVA. The PK/PD relationship of plasma ATTM levels at reperfusion and subsequent lactate levels (1-h later) are shown in C, analysed using linear regression. Blood pressures, either peri-bolus or over the course of the experiment, are shown in panels D and E, respectively. *$p < 0.05$ vs control using a 2-way repeated measures ANOVA followed by Šídák's multiple comparisons test in 'B', and a Wilcoxon matched-pairs signed rank test in 'D'. BL, baseline; DBP, diastolic blood pressure; ISC, ischaemia; MAP, mean arterial pressure; NS, not significant; SBP, systolic blood pressure.

Representative histological slices are presented in Fig. 5D. We performed cardiac magnetic resonance imaging (cMRI) but could not gain meaningful results for infarct size due to poor signal quality in most animals (data not shown); this likely relates to the short (24-h) follow-up time and was verified by two cMRI experts blinded to treatment allocation. The overall AAR as a percentage of left ventricular mass was 42 $\pm$ 7% (mean $\pm$ standard deviation) and was similar between groups (Supplementary Fig. 7).

3.5. Histology and blood/tissue analyses

At 1-h post-reperfusion, Troponin T levels were significantly (p < 0.05 vs control) lower in all ATTM-treated animals (Fig. 5C). Glutathione peroxidase activity was significantly increased in tissue from both regions in ATTM-treated animals (p < 0.05 vs control; Fig. 6A–B). By contrast, catalase (that similarly detoxifies hydrogen peroxide) showed significantly decreased activity in the penumbra (p < 0.05 vs control; Fig. 6C) yet remained unchanged in the remote region (Fig. 6D). A tissue slice denoting indicative regions of penumbra and remote left ventricle (myocardium not at risk) is shown in Fig. 6E. We did not test samples from the infarcted core as this tissue was considered non-salvageable.

4. Discussion

Testing of prospective cardioprotective agents in large mammals is a necessary development step advocated by recent expert consensus guidelines [27]. ATTM treatment in our large animal model of myocardial infarction and reperfusion injury replicate our earlier findings of organ protection in rodent models [16,22], with the proviso that therapeutic dosing must be administered. The significant correlation between infarct size and plasma ATTM levels at reperfusion, observation of cardioprotection in animals administered sufficient quantities of ATTM, and the decrease in troponin T levels, a marker of myocardial

injury, support this claim. In addition, myocardial antioxidant selenoprotein activity was elevated. Administration of ATTM was well tolerated; animals were clinically well on recovery from anaesthesia with only one instance of emesis (in an ATTM-treated animal) observed. Importantly, in these studies we replicated a clinical scenario where treatment would be instituted *peri*-recanalisation.

ATTM has been used clinically as an oral copper chelator for over three decades [35], while a bis-choline salt has been investigated more recently for Wilson's disease and as an anti-cancer agent [36]. These precedents show that ATTM has a good safety profile with manageable adverse effects related to copper depletion. We considered that copper chelator effects are not relevant in our acute study as it takes six weeks of daily oral dosing of ATTM to deplete systemic copper levels [37]. Our work focuses on the use of ATTM as a sulfide donor and its utility as a novel intravenous adjunct therapy during reperfusion. Historically, translation of sulfides from rodents to larger mammals has shown variable effects. For example, application of gaseous H_2S or basic salts (e.g., sodium sulfide; Na_2S) was effective in rodents [38–40], yet induction of hypometabolism and/or improved outcomes in larger mammals (sheep and pig) yielded variable results [41–44]. It was thus imperative to assess the safety and efficacy of ATTM in a large animal model undergoing a clinically relevant insult and, crucially, to assess PK/PD to ensure adequate drug exposure.

Our first study in pigs comprised a dose escalation in healthy animals during which a therapeutic window was established and a PK/PD evaluation performed. A transient (<1 min) dose-dependent period of hypotension was observed, as anticipated from the literature [45] and our previous experience using sulfide-releasing drugs [16]. This timeline is consistent with a direct effect of free sulfide on vascular tone. Pharmacological vasopressor support was not required, and the magnitude/duration of hypotension did not meet our pre-defined criteria as an adverse event. Coloration of ATTM allows pharmacokinetic evaluation in plasma samples using a simple colorimetric assay [16,21].
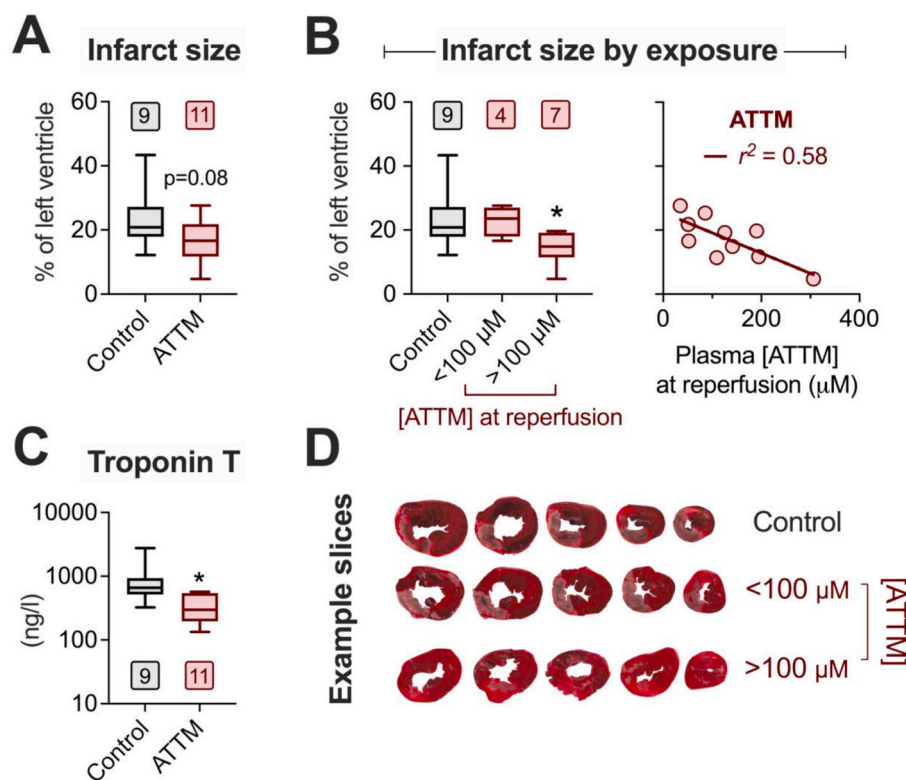
Fig. 5. Impact of ATTM following myocardial I/R injury (Study 3). Infarct size measured histologically is shown in panel An as a comparison of overall treatment effect (including subtherapeutic). Infarct size following subdivision of animals based on the ATTM plasma concentration at the point of reperfusion is shown in B. Four animals were considered subtherapeutic, denoted by a plasma ATTM level at reperfusion $<100\ \mu mol\ l^{-1}$. High sensitivity plasma troponin T levels and example histologically stained heart slices are shown in C and D, respectively. Numbered squares denote the sample size. *$p < 0.05$ vs control. In A, the stated P-value is derived from a parametric, unpaired, 2-tailed *t*-test. In B, data were analysed using a Kruskal-Wallis test followed by Dunn's multiple comparisons test (left panel), and correlation performed using linear regression (right panel). In C, data were analysed using a Mann-Whitney test.

Pharmacodynamic evaluation relies on sulfide liberated by thio-metallates acting at the level of the mitochondrion via partial inhibition of cytochrome C oxidase [16]. This results in a greater reliance on glycolysis to generate ATP, thereby increasing net lactate production. Furthermore, increases in both glycolytic activity and net ATP hydrolysis contribute to metabolic acidaemia. Importantly, the ATTM-induced rise in lactate levels in our previous rat studies was not due to organ hypoperfusion since cardiac output remained unchanged and tissue oxygen tension, an indicator of the interstitial oxygen supply/demand balance, was elevated [16]. In the pig studies reported here, ATTM induced a mild hyperlactataemia; this biomarker enables the modelling of a consistent (across the three studies) PK/PD relationship. This feature can be readily adopted in any future clinical trials.

The concept of using a sulfide-releasing agent for protection against reperfusion injury is well-established [46]. During ischaemia, tissue viability in the area at risk will be either irrecoverable or salvageable, particularly within the surrounding penumbra. On revascularization, salvageable cells are subjected to an excess of oxygen and nutrient supply, driving oxidative phosphorylation, and generating large quantities of ROS, particularly by mitochondria [47,48]. The utility of sulfide donors in this context likely relates to their ability to rapidly attenuate excessive ROS generation by slowing reactivation and/or preventing overactivity of the electron transport chain [16]. An *in vitro* anoxia/reoxygenation model demonstrating decreased mitochondrial superoxide levels with ATTM treatment is consistent with this working hypothesis [16]. Similarly, S-nitrosation of a specific Complex I cysteine residue could temper reactivation of mitochondria during reperfusion, decreasing ROS production and oxidative damage [49]. Prevention of ROS production through metabolic modulation is analogous to the use of therapeutic hypothermia ('targeted temperature management') for

out-of-hospital cardiac arrest [50] and revascularization after myocardial infarction [10]. Aggressive patient cooling did show outcome utility for anterior myocardial infarction [10]; however, this method remains challenging and not universally applicable, with target temperatures often not achieved for several hours. A pharmacological means of safely reducing metabolism (and ROS production) would be faster, easier, and likely more effective [16].

While our mechanistic focus is on the modulation of metabolism and ROS generation, it should be noted that reperfusion injury is complex, comprising cardiomyocyte and microvascular injury, and inflammatory responses [3]. When ATP production recovers, intracellular pH rapidly corrects. However, excessive/uncoordinated myofibrillar contraction occurs in response to increased calcium cycling between sarcoplasmic reticulum and cytosol [51]. ROS- and Ca^{2+}-induced cellular injury cause opening of the mitochondrial permeability transition pore (mPTP) and downstream apoptotic signalling [5]. The relative contribution of cellular and microvascular injury to reperfusion injury pathophysiology remains unresolved [3]. While prevention of reperfusion injury remains a major therapeutic target, attempts to confer cardioprotection in clinical populations have yielded either little efficacy, or an inability to convert promising results in small trials to positive outcomes in large multicentre studies. These include ischaemic post-conditioning or remote ischaemic pre/peri/post conditioning [11], and multiple pharmacological interventions. Several have been targeted to mitochondria, including cyclosporin A and TRO40303, both aiming to prevent mPTP opening, and MTP-131, a cardiolipin-targeted compound that optimizes mitochondrial energetics through supercomplex formation. Despite promising preclinical evaluations, these compounds failed to confer benefit in pivotal clinical trials [6–8]. We have reasoned that by directly slowing ROS production, the mechanism of action of ATTM differs from
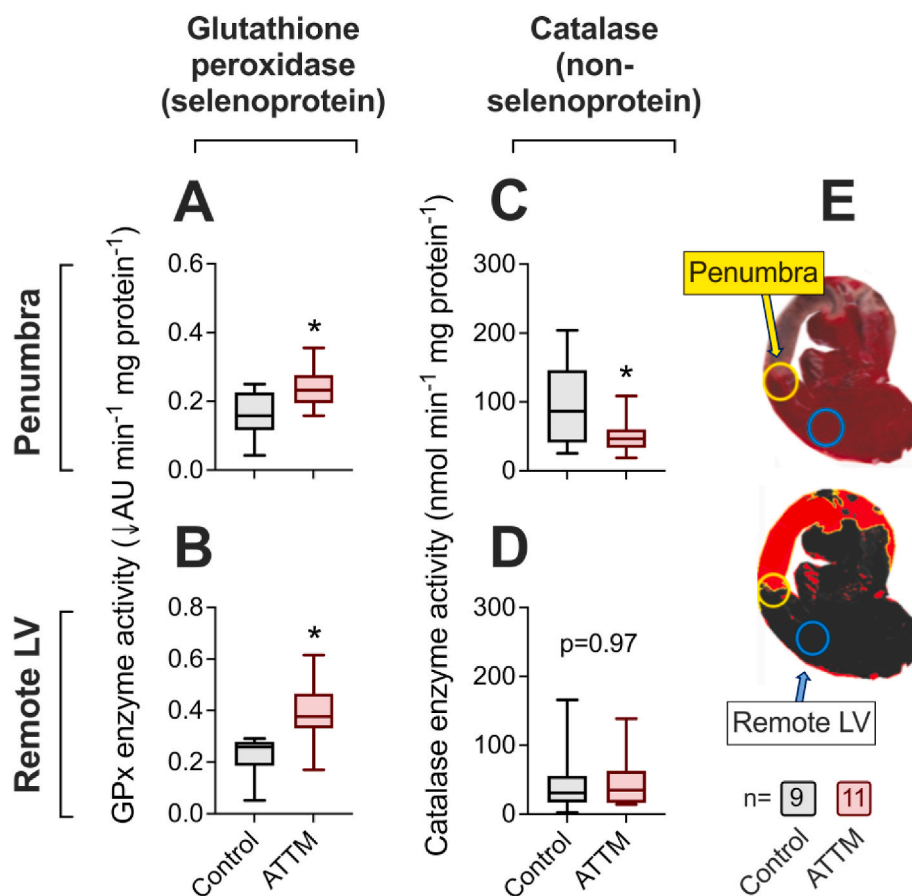
Fig. 6. Impact of ATTM on tissue antioxidant enzyme activity (Study 3). Glutathione peroxidase activity is shown in panels A and B. Tissue originating in the ischaemic penumbra is shown in A, while B shows enzyme activity in a location distant to the area at risk (remote LV). Corresponding catalase activity in the same tissue samples is shown in panels C and D. The ischaemic penumbra was located by matching histologically stained slices with an unstained tissue slice, depicted in E. AU, arbitrary units; GPx, glutathione peroxidase; LV, left ventricle. *$p < 0.05$ vs control using parametric, unpaired t-tests in A and C, and non-parametric t-tests (Mann Whitney test) in B and D. In D the stated p-value is the result of the *t*-test.

these other cardioprotective strategies, being upstream of either scavenging reactive species or preventing their secondary effects (e.g., activation of apoptotic or inflammatory pathways).

Notably, while excessive ROS production that overwhelms antioxidant defences is clearly detrimental, lower levels of ROS contribute to redox signalling both in health and disease [52], and towards the beneficial effects of some cardioprotective interventions [46]. The efficacy demonstrated with conditioning protocols may be due in part to ROS signalling and activation of pro-survival kinases since its effects can be inhibited with antioxidant therapy [53]. Numerous stressors such as hypoxia, calorie restriction, physical activity and mitochondrial inhibitors may act through (lower level) mitochondrial ROS generation to induce transcription of antioxidant and other detoxifying enzymes [54]. We studied the activity of two antioxidant enzymes in myocardium taken from the ischaemic penumbra and a remote (not within the left coronary artery perfusion zone) area of left ventricle. Glutathione peroxidases are a family of antioxidant enzymes that catalyse detoxification of peroxides by thiols. Although the assay used could not discriminate between GPx isoforms, selenium-containing GPx-1 predominates and is abundant in myocardium. The importance of GPx-1 in myocardial ischaemia-reperfusion injury has been documented, with transgenic GPx-1-overexpressing mice more resistant to myocardial infarction and GPx-1$^{-/-}$ mice more susceptible to injury [55]. Our study revealed that GPx activity was elevated in both myocardial regions studied (ischaemic penumbra and unaffected tissue), suggesting this response relates to ATTM-derived sulfide activity and is not triggered by decreases in oxygen tension. We postulate this relates to a sulfide-induced increase in selenocysteine lyase activity, an enzyme that catalyses hydrogen selenide production yielding greater quantities of selenium-containing GPx [56]. Additional benefit may derive from a direct sulfide ROS scavenging effect, sulfide-derived anti-inflammatory effects (including reduction in neutrophil infiltration), and/or indirect effects such as increased intracellular acidity enabling more rapid reduction of oxidised glutathione (GSSG) back to its reduced form (GSH) [18,57].

Notwithstanding more than a decade of attempts to translate sulfide-based therapeutics, few have shown utility in large mammals. SG-1002 has shown efficacy in a porcine model of peripheral artery disease [23] and could increase sulfide 'bioavailability' in eight heart failure patients [58]. ATB-346, an oral sulfur-non-steroidal anti-inflammatory drug conjugate showed significant gastrointestinal protection over the parent molecule (naproxen) [24]; of note, this drug does not act directly via sulfide liberation. Treatment with sulfide generators has also shown utility in porcine models of myocardial ischaemia/reperfusion injury [25,26], however these drugs cannot be developed as medicines via intravascular delivery. The obvious challenge with sulfide-based therapies is ensuring appropriate levels of sulfide release (and bioactivity) in the correct compartment. ATTM possess important properties compared to other sulfide-releasing drugs that make it particularly favourable for treating reperfusion injury [16,21]. We previously demonstrated that ATTM rapidly leaves the circulation and can gain cellular entry (as MoS_4^{2-}) using non-specific anion transporters, thus acting as a cell-targeted therapy [21]. Furthermore, ATTM releases sulfide in a linear fashion over time using an *in vitro* sulfide release test; over 1 h, we estimated that approximately 1/300 sulfur atoms are liberated as sulfide

compared (on a molar basis) to the sulfide generator, NaHS [16]. However, we also found that ATTM is thiol-activated (by both oxidised and reduced thiols at physiological concentrations), thus enabling more (approximately 6-times according to our *in vitro* tests) sulfide to be liberated intracellularly, close to the intended site of action [16]. Finally, sulfide release from ATTM is dependent upon both temperature and acidity, consistent with hydrolysis of the metal-sulfur bonds, and will preferentially dissociate in more acidic, ischaemic cells that have a greater need for treatment [16].

4.1. Limitations

With each consecutive study, we observed lower-than-expected (and intended) plasma ATTM levels in some animals. This led to us increasing the dose level for Study three and contributed to the necessary inclusion of a second cohort of animals. During the study, we noted an increase in sulfide release using our *in vitro* test. Although the level of this release was within our predefined criteria (4 ppm), our development work points to trace quantities of residual solvent as causative of this effect. It can also contribute to polymerisation of the molecule to aqueous insoluble products e.g., Mo_2S_8 or Mo_2S_7 ($+H_2S/HS^-$), thus requiring higher quantities of drug for detection and bioactivity. While this has complicated interpretation of the primary endpoint in the current study, an unexpected benefit from the subsequent variability in plasma levels is clear demonstration of an exposure-dependent effect. Indeed, multiple trials of promising new therapeutics for reperfusion injury have failed, translating from small rodent studies to large mammals or clinical proof-of-concept. A fundamental aspect of trial design, not performed in many of these studies, is to enhance the likelihood of success through optimal dosing. Since conducting this study, we have prioritised improvements in stability and solubility in our development work.

A further limitation of our study was the short follow-up time for MRI. A more protracted assessment (4–6 weeks) would have enabled superior MRI images of established infarcts and functional measures of cardiac performance. At the 24-h timepoint most cases were confounded by measurement difficulties due to unresolved tissue inflammation and oedema. With the exception of early gadolinium enhancement-derived AAR measurements, we have removed the MRI data from this manuscript but have kept the methodology in the Supplementary material for reference. This issue provides an important learning point for researchers wishing to engage in similar studies.

We used histological determination of infarct size as our primary endpoint, a standard methodology for the appraisal of potential cardioprotective agents [59]. We acknowledge that this could not be expressed as a fraction of histologically derived AAR as we opted to perform an MRI 24 h later rather than reintroducing an intracoronary balloon catheter under fluoroscopy. However, the similar MRI-derived AAR across groups and significant decrease in troponin levels lends strong additional support to a beneficial effect of ATTM in this model.

In our studies, we opted to replicate a clinical scenario where treatment could be instituted *peri*-recanalisation. This approach is based on the scenario that patients with ongoing myocardial infarction out-of-hospital are rushed to hospital for primary percutaneous coronary intervention (PPCI). This would allow ATTM to be started *peri*-revascularization and continued up to 1 h after PPCI in a tertiary specialist centre. More work is needed to ascertain the impact of ATTM in different clinical scenarios e.g., late presentation of myocardial infarction, or in patients presenting with some degree of reflow. The impact of delayed ATTM administration (e.g., after reperfusion) should also be assessed. However, the results shown here do provide encouragement and rationale for further study on the ATTM dosing schedule and its timing.

A final limitation is the use of only female pigs. This was done to minimise intra-experimental variability, increase the robustness of our results, and to aid in animal husbandry. Mixed cohorts would be preferable regarding translatability but may trigger potential bias in a relatively small group derived from alterations in growth, anatomical size, and hormonal differences.

4.2. Conclusion

While the primary endpoint was neutral overall due to some pigs receiving sub-therapeutic doses of ATTM, there was a significant correlation between myocardial infarct size and plasma ATTM concentration at the point of reperfusion. This suggests utility of a sulfide donor for the treatment of ischaemia/reperfusion injury in large mammals provided appropriate quantities of the drug are administered. Further studies are warranted to ascertain the impact of the proposed intervention on longer-term outcomes of cardiovascular function and remodelling based on clinical-grade imaging. However, the safety and utility reported in the present study, evidence of its operation via multiple mechanisms of action (now including an increase in abundance and/or activity of antioxidant selenoproteins), and a good safety record in humans, provide substantial encouragement for translation to the clinic as a novel adjunct treatment for reperfusion injury.

CRediT authorship contribution statement

Thomas W. Johnson: Writing – review & editing, Project administration, Methodology, Investigation. **James Holt:** Investigation, Formal analysis. **Anna Kleyman:** Investigation, Formal analysis. **Shengyu Zhou:** Investigation, Formal analysis. **Eva Sammut:** Investigation, Formal analysis. **Vito Domenico Bruno:** Methodology, Investigation, Formal analysis. **Charlotte Gaupp:** Investigation, Formal analysis. **Giacomo Stanzani:** Resources. **John Martin:** Resources. **Pietro Arina:** Formal analysis. **Julia Deutsch:** Project administration. **Raimondo Ascione:** Writing – review & editing, Supervision, Project administration, Methodology. **Mervyn Singer:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Alex Dyson:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

AD and MS are developing thiometallates for the treatment of reperfusion injury. JM, MS and AD hold patents on therapeutic uses of ammonium tetrathiomolybdate.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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