Chemistry, pharmacology and cellular uptake mechanisms of thiometallate sulphide donors

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Abstract

Background and Purpose: A clinical need exists for targeted, safe, and effective sulfide donors. We recently reported that ammonium tetrathiomolybdate (ATTM) belongs to a new class of sulfide-releasing drugs. Here, we investigated the cellular uptake mechanisms of this drug class compared to sodium hydrosulfide (NaHS) and the effects of a thiometallate tungsten congener of ATTM, ammonium tetrathiotungstate (ATTT).

Experimental Approach: In vitro H_2S release was determined by headspace gas sampling of vials containing dissolved thiometallates. Thiometallate and NaHS bioactivity was assessed by spectrophotometry-derived sulfhaemoglobin formation. Cellular uptake dependence on the anion exchange protein (AE)-1 was investigated in human red blood cells. ATTM/glutathione interactions were assessed by LC–MS/MS. Rodent pharmacokinetic and pharmacodynamic studies focused on haemodynamics and inhibition of aerobic respiration.

Key Results: ATTM and ATTT both exhibit temperature-, pH-, and thioldependence of sulfide release. ATTM/glutathione interactions revealed the generation of inorganic and organic persulfides and polysulfides. ATTM showed greater ex vivo and in vivo bioactivity over ATTT, notwithstanding similar pharmacokinetic profiles. Cellular uptake mechanisms of the two drug classes are distinct; thiometallates show dependence on AE-1, while hydrosulfide itself was unaffected by inhibition of this pathway.

Conclusions and Implications: The cellular uptake of thiometallates relies upon a plasma membrane ion channel. This advances our pharmacological knowledge of this drug class, and further supports their utility as cell-targeted sulfide donor therapies. Our results indicate that, as a more stable form, ATTT is better suited as a copper chelator. ATTM, a superior sulfide donor, may additionally participate in intracellular redox recycling.

Introduction

Hydrogen sulfide (comprising H2S and HS−) is the third endogenous gasotransmitter alongside NO and carbon monoxide and acts as a signaling molecule across numerous physiological systems (Kimura, 2014; Szabo et al., 2014). Given exogenously in preclinical studies, sulfide protected against diverse pathological conditions, ranging from circulatory (Wang, Wang, Guo, & Zhu, 2011), neurodegenerative (Zhang & Bian, 2014) and arthritic (Wu et al., 2016) disorders to diabetes (Wu et al., 2009), pain (Di Cesare Mannelli et al., 2017), and cancer (Lee et al., 2014). Our particular interest in sulfide relates to its ability to transiently reduce cellular respiration by inhibition of mitochondrial cytochrome C oxidase (Szabo et al., 2014). This, and the consequent reduction of mitochondria-derived ROS production, confers protection in states of ischaemia/reperfusion injury, hypoxia, and circulatory shock (Blackstone & Roth, 2007; Elrod et al., 2007; Morrison et al., 2008).

Notwithstanding more than a decade of promising preclinical research, only a handful of sulfide-based therapies have entered clinical trials (Wallace, Vaughan, Dicay, MacNaughton, & de Nucci, 2017); none have yet proven successful in randomised phase 2/3 studies. Numerous delivery methods have been investigated, focusing initially on the use of simple sulfur salts such as sodium sulfide (Na2S). However, these salts release sulfide in a rapid, uncontrolled fashion with resulting implications for safety and efficacy. More recently, drug design of sulfide-based therapeutics has advanced significantly, with identification of numerous slow-release sulfide “donors” and sulfur-hybrid molecules. These aim for more controlled sulfide delivery and improved targeting to its intended site of action (Szabo & Papapetropoulos, 2017).

We and others recently reported that inorganic thiometallates represent a new class of sulfide-releasing drugs (Dyson et al., 2017; Xu et al., 2016) that confer protection against ischaemia/reperfusion injury (Dyson et al., 2017). These thiometallates consist of a transition metal core and four covalently bound sulfur atoms, and cleavage of the metal–sulfur bonds enables these molecules to act as slow-release sulfide donors. The archetypical thiometallate, tetrathiomolybdate, was first synthesised nearly 200 years ago (Berzelius, 1826). The ammonium salt, ATTM ([NH4]2MoS4), has proven efficacy as a copper chelator, having been developed and used off-label for the treatment of Wilson's disease (Brewer et al., 2009). Notably, the rate(s) of hydrolysis of molecules from this class depend on numerous intrinsic and extrinsic factors (Lee, Schulman, Stiefel, & Lee, 2007); this ultimately determines their suitability as sulfide donors or copper chelators.

Given the potential utility of thiometallates for indications not necessarily related to the depletion of copper, we investigated other compounds within this class. Here, we report on the chemistry and pharmacology of the tungsten analogue, ATTT ([NH4]2WS4), in comparison to its lighter homologue and the "standard" sulfide generator, sodium hydrosulfide (NaHS). In view of the need for sulfide mimetics that are better targeted, we explored mechanisms of cellular uptake by both thiometallates and NaHS. We hypothesised that (a) thiometallates utilise non-selective anion channels to gain intracellular access due to their ionic nature in solution and (b) ATTT would represent a new member of the sulfide-releasing thiometallate drug class. Finally, we elaborate on two methods developed as part of this work, and provide a detailed description of our H_2S release assay and the measurement of sulfhaemoglobin, and present pilot data on the interaction of ATTM with oxidised and reduced glutathione

Methods

In vitro H_2S release

As noted above, free (biologically active and physiologically relevant) sulphide constitutes H_2S gas and the hydrosulphide (HS^-) anion. The assay, developed in-house and described in detail herein, relies on detection of free H_2S measured by a commercially available H_2S detector (Z900XP, Environmental Sensors, Boca Raton, FL, USA). The supporting information provides detail on the protocol. In brief, compound is dissolved in phosphate-buffered saline (PBS) to a 10x stock solution and diluted rapidly (0.5 into 4.5 ml) into airtight Falcon tubes (50 ml; Corning Science Mexico, Reynosa, Mexico) containing PBS. The PBS is typically pre-warmed to 37°C but can be adjusted as necessary (e.g. to different temperatures, pH levels, or to contain thiols, other adjuvants or matrices). The solution is then incubated in a water bath, typically for one hour at 37°C. Five ml of headspace gas is then withdrawn over 10 seconds and passed through the detector via a 3-way tap (closed to room air) to accommodate the syringe. This is attached to the detector inlet, as shown inset in Fig 1A.

Strict working conditions should be adhered to when using hydrogen sulphide. In our studies, we observed a maximal background level of 0.1 ppm (working with 1 M NaHS) which is well within EU limits of safety (short-term exposure limit of 15 ppm). Use of fume hoods and PPE are strongly recommended. The Z900XP H_2S detector displays a reading every 10 seconds. During the first series of experiments, we recorded both peak H_2S gas level (in parts per million; ppm), and values during the washout phase until the meter displayed zero. A direct correlation was seen when comparing the peak H_2S value (Fig 1A) against the sum of the washout readings (decay) over time (area under curve; AUC; Fig 1B). Therefore, the time to washout and the sum of values therein is directly proportional to the peak quantity of gas initially detected. As such, it

is appropriate to present only the peak H_2S gas level. In the current study, we adjusted the environment into which drug stocks were diluted as follows: temperature was set to either 4, 21, 37 or 50°C, and solution pH to 4.5, 7.4 or 10. As we have previously shown thiol-dependence of sulphide release with ATTM, a further set contained either reduced or oxidised glutathione (GSSH and GSSG, respectively; 5 mM final concentration for both). Concentration of the drug stock solutions (10-1000 mM) and time of incubation (30-180 mins) were also adjusted under standard conditions (pH 7.4, 37°C).

Sulphaemoglobin formation

To assess plasma membrane transport and subsequent intracellular biological activity, we examined the formation of sulphaemoglobin in blood spiked with either ATTT, ATTM or NaHS. The assay developed and described in detail herein is based on the various forms of haemoglobin (oxy-, deoxy-, carboxy-, met- and sulph-) having distinct absorbance signatures. Fig 2A shows that oxyhaemoglobin is highly absorbent at 541- and, in particular, 577 nm. The sulphaemoglobin spectrum, while flatter at these wavelengths, has an additional absorbance peak at 620 nm. Notably, there is no interference from ATTM or ATTT, or the colourless NaHS, at these wavelengths.

Absorbance of any given chromophore was calculated according to the Beer-Lambert law (Fig 2B, *Equation 1*), where *A* is absorbance at a particular wavelength (λ), *c* is concentration of the chromophore and *l* is the pathlength. and ε is an extinction co-efficient (known also as millimolar lineic absorbance) specific to the wavelength and chromophore(s) of interest. The contribution of ≥2 chromophores to absorbance can be derived according to *Equation 2* (Fig 2B). For oxy-, deoxy- and sulphaemoglobin, ε values at 577 and 620 nm are shown in Fig 2C, obtained from Zwart et al (1981).

Calculation of sulphaemoglobin can be broadly divided into three components. The first requires an assessment of the relative contributions of oxy- and deoxyhaemoglobin to spectrophotometer-derived total haemoglobin (tHb) measurements in either untreated or baseline samples. This was accomplished using Equation 2 (Fig 2B) and ε values at 577 nm (Fig 2C). To assess the fractions of oxy- and deoxyhaemoglobin, we used a blood gas analyser (ABL90 FLEX, Radiometer, Crawley, West Sussex, UK). In studies where blood gas analyses were not available, the following standard values were used for arterial blood: partial pressure of oxygen (13 kPa), deoxyhaemoglobin (5%), oxyhaemoglobin (95%). The second component concerns manual measurement of spectrophotometer-derived sulphaemoglobin (sHb) in treated samples, achieved using absorbance and ε values at 620 nm. The fraction of sulphaemoglobin was then calculated as sHb/tHb.

We used fresh venous whole blood obtained from consenting healthy volunteers, collected into ethylenediaminetetraacetic acid (EDTA)-containing (20 ml) syringes (final concentration; 2.5 mM). This venous blood (typically 15 ml) was subsequently transferred to 50 ml Falcon tubes and placed on a rotary shaker for 10 min; this enables oxygenation to a representative arterial partial pressure of oxygen (13 kPa). Whole blood (400 μl) was then incubated with 50 μl of either ATTM, ATTT or NaHS, and 50 μl of PBS. In non-drug treated samples (used both as negative controls and as a reference for total haemoglobin), drug was replaced with PBS. All samples were incubated at 37°C and followed two treatment protocols: a dose-response and a time-course. For the dose-response study, upon dilution in blood, and having accounted for the 4 sulphur groups of the thiometallates, final concentrations of total sulphur were 1-100 mM, with incubation time set at 1 hour. For the time-course study, all groups were set at the highest concentration (100 mM total sulphur) and incubated for 15-360 minutes. After incubation, a 1/20 dilution in PBS was performed, and 100 μl transferred to 96 well microplates. Absorbance values were derived using a microplate reader and BioTek (Gen5) software (Synergy 2, North Star Scientific, Sandy, Beds, UK), with calculation of sulphaemoglobin performed as above.

Cellular uptake studies

We considered that all molecules studied would dissociate into ionic forms (i.e. MoS_4^{2-}, WS_4^{2-}). upon dissolution, and most (85% at physiological pH and temperature) of the free sulphide generated by NaHS, or liberated by the thiometallates, would be present as the hydrosulphide anion. We thus investigated the role of the erythrocyte anion-exchanger 1 (AE-1) as a putative cellular uptake mechanism. Whole human blood (400 μl) was pre-treated for 30 min with either 50 μl of the AE-1 inhibitor, H_2DIDS (4'diisothiocyanato-dihydrostilbene-2,2'-disulfonic Acid; 0.5 mM) or an equivalent volume of PBS. Following pre-treatment, 50 μl of either ATTT, ATTM or NaHS were added (100 mM total sulphur), and samples incubated for either 15 min (NaHS) or 180 min (thiometallates) at 37°C, prior to measurement of sulphaemoglobin.

In separate studies using the two thiometallates (5 mM total sulphur), blood samples (with or without H_2DIDS pre-treatment) were incubated for 180 min then centrifuged (14,000 RCF, 1 min). Excess drug was removed by twice substituting supernatant with an equivalent volume of PBS. Samples were then freeze-thaw lysed and filtered to remove plasma membrane fragments and proteins (10 kDa Nominal Molecular Weight Limit microfilters; MerckMillipore, Watford, Herts, UK). Extracellular (from the initial supernatant) and intracellular (following lysis and filtration) drug levels were assessed by spectrophotometry following a 1-in-20 dilution. Absorbance peaks at 392 nm and 468 nm were used, respectively, for ATTT and ATTM, and

concentrations determined by comparison against standard curves. Studies were not performed with NaHS as it has no distinct colouration. All experiments utilising H_2DIDS were carried out in amber Eppendorf tubes due to the light sensitivity of this molecule.

Pharmacokinetic/pharmacodynamic (PK/PD) studies

To compare the *in vivo* biological activity of the thiometallates, we performed PK/PD studies. These experiments were performed according to local ethics committee and UK Home Office guidelines under the Animals (Scientific Procedures) Act 1986. Male Wistar rats (approximately 300g body weight) were used as mice markedly reduce their metabolism within hours in response to systemic insults, whereas rats more closely mimic human responses (Zolfaghari et al., 2013). As drug responses often vary between species, the use of mice in the context of a significant cardiorespiratory perturbation would likely confound a translational evaluation of metabolism-modifying agents.

Animals were purchased from Charles River (Margate, Kent UK) and certified healthy and pathogen-free. One week prior to experimentation, animals were housed in standard cages of four individuals on a 12-h light/dark cycle, with food and water *ad libitum*. All animals were anaesthetised with isoflurane in room air (Abbott, Maidenhead, Berks, UK); 5% for induction and 2-2.5% for surgical procedures. They were placed on a heated mat (Harvard Apparatus, Cambridge, Cambs, UK) to maintain rectal temperature at 37°C. The left common carotid artery and right internal jugular vein were cannulated using 0.96 mm outside diameter PVC tubing catheter (Scientific Commodities Inc., Lake Havasu City, AZ, USA). The arterial line was connected to a pressure transducer (Powerlab; AD Instruments, Chalgrove, Oxon, UK) for continuous monitoring of mean arterial blood pressure, and the venous line used for administration of fluids and drugs. A tracheostomy was sited to secure and suction the airway, and the bladder cannulated through a keyhole laparotomy to measure urine output and renal excretion of thiometallates. Anaesthesia was then switched to intraperitoneal sodium pentobarbitone (Pentoject; Animalcare, York, Yorks, UK), allowing blood-pressure guided intubation with 2.08 mm external diameter polythene tubing (Portex Ltd, Hythe, UK), and subsequent mechanical ventilation using a small animal ventilator (Physiosuite, Kent Scientific, Torrington, CT, USA). Ventilator settings were as follows: fraction of inspired oxygen, 0.21; tidal volume, 10 ml/kg; respiratory rate, 80/min; and positive end-expiratory pressure, 3 cm H_2O. These settings ensured adequate post-surgical gas exchange and consistent minute volumes across all animals used. Perioperative analgesia was provided by buprenorphine, 0.05 mg/kg subcutaneously (Reckitt Benckiser, Slough, Berks, UK). In these non-recovery experiments, euthanasia at experiment-end was performed using IV sodium pentobarbitone.

Following surgery and a 1-hour stabilization period, animals received increasing IV bolus doses of ATTT or ATTM (1-100 mg/kg, n=6 per group). Drugs were dissolved in normal (0.9%) saline and administered (within 2 mins) in a volume of 2 ml/kg over 10 seconds. H_2S in exhaled breath was monitored by connecting the ventilator exhaust to the (above) H_2S detector. Measurements were collected at baseline, then as follows after each dose: blood pressure and exhaled H_2S (peak change or level) within 30 sec, cardiac function (by echocardiography) within 1 min, blood sampling to determine plasma drug concentrations at 2 min, and arterial blood gas analysis (to measure partial pressures of O_2 [PaO_2] and CO_2 [PCO_2], glucose, lactate, and acid/base) at 27 min; doses were escalated every 30 min. After the highest dose, the half-life of both drugs was assessed by sampling at 2 min (for maximal concentration; C_{MAX}) then at regular intervals up to 1-hour post-dose. Urine was collected hourly. Absorbance of plasma or urine samples was assessed using the microplate reader at the wavelength specific to each drug (as above), and concentrations derived by comparison against standard curves. To determine fractional renal excretion, a mass-balance approach was utilised. This was accomplished by calculation of the number of moles of each drug excreted in urine, and expressed as a fraction against the number of moles injected.

Data and Statistics

Data are presented as mean ± standard error or median, quartiles, and range. Group sizes were n=5 for *in vitro* and *ex vivo* studies and n=6 for *in vivo* experiments. Correlations were performed using a non-linear regression, straight line model. Pharmacokinetic data were analysed using a bi-exponential decay curve and least squares fitting method. Parametric data were analysed using repeated measures one- or two-way ANOVA followed by Bonferroni's *post hoc* testing, as appropriate. Nonparametric data were analysed using the Mann-Whitney U test. All statistical analyses were two-tailed and performed using Prism 7.0.1 software (GraphPad Software, San Diego, CA, USA). All data and statistical analyses comply with recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). Multiplicity-adjusted p values <0.05 were considered statistically significant.

Materials

We sourced thiometallates (Sigma-Aldrich, Gillingham, Kent, UK) and the basic sulphur salt, sodium hydrosulphide (NaHS; Alfa Aesar, Heysham, Lancs, UK), from specific suppliers as they provide, respectively, good consistency between batches, and the purest form available commercially (Hughes et al., 2009). H_2DIDS was obtained from Thermofisher (Hemel Hempstead, Herts, UK). All other chemicals and reagents were obtained from Sigma-Aldrich.

Results

In vitro H_2S gas release

We report here the novel finding that ammonium tetrathiotungstate is a new member of the inorganic thiometallate drug class. However, it releases only half the amount of free sulphide compared to ATTM under standard incubation conditions (Fig 3A). This pattern was maintained under different experimental environments. Both thiometallates release sulphide in a concentration-dependent (Fig 3B; $p<0.05$), and linear fashion over time (Fig 3C; $p<0.05$). Like ATTM, ATTT releases sulphide in a temperature ($p<0.05$), thiol ($p<0.05$) and pH-dependent ($p<0.05$) manner (Fig 3D-F). Warmer and more acidic conditions, and the presence of (oxidised or reduced) glutathione, promote liberation of sulphide from these molecules.

Sulphaemoglobin formation and cellular uptake

We assessed (intracellular) sulphaemoglobin formation as an indicator of plasma membrane transport to examine cellular uptake mechanisms. All three drugs generated sulphaemoglobin in a concentration-dependent manner (Fig 4A). Sulphaemoglobin formation by the thiometallates was also dependent on time, with ATTM exhibiting significantly more bioactivity than ATTT between 60-360 min (Fig 4B). In contrast to both ATTT and ATTM, NaHS produced sulphaemoglobin at significantly higher levels in the dose response study, and significantly quicker in the time course experiment, (Fig 4A-B). Pre-treatment of blood with the AE-1 inhibitor H_2DIDS significantly reduced sulphaemoglobin formation by both ATTT (Fig 4C; $p<0.05$) and ATTM (Fig 4D; $p<0.05$). AE-1 inhibition had no effect on sulphaemoglobin formation in NaHS-treated samples (Fig 4E; $p=0.29$).

The distinct spectral signatures of the thiometallates (Fig 5A) allows for their quantification using basic absorbance assays. To confirm use of the AE-1 transporter by thiometallates using an alternative approach, we assessed disappearance from the plasma of blood treated with these drugs *ex vivo* and, in the same samples, the appearance of drug inside erythrocytes. A similar pattern was observed for both thiometallates, whereby preincubation with H_2DIDS reduced both disappearance from plasma (Fig 5B, ATTT; Fig 5C, ATTM; both $p<0.05$) and detection of their intracellular presence (Fig 5D, ATTT, Fig 5E, ATTM; both $p<0.05$). The measurement most sensitive to H_2DIDS treatment was the intracellular appearance of the drugs, with AE-1 inhibition reducing ATTT and ATTM concentrations by 73% and 65%, respectively.

PK/PD studies

Eleven of the 12 studied animals survived until experiment end. One (ATTM-treated) animal died from an accidental air embolus. Final group sizes were six for ATTT and five for ATTM.

Both thiometallates caused a dose-dependent metabolic acidaemia, evidenced by falls in arterial pH (Fig 6A) and base excess (Fig 6B). No changes in the respiratory component, arterial PCO_2, were observed in these mechanically ventilated animals (Fig 6C). The magnitude of these metabolic changes was significantly ($p<0.05$) pronounced in ATTM-treated animals; this was mirrored by a dose-dependent rise in blood lactate (Fig 6D; $p<0.05$) and fall in blood glucose ($p<0.05$), indicative of a compensatory increase in glycolysis (anaerobic respiration) (Fig 6E). ATTT-treated animals only showed a rise in arterial lactate at the highest dose (Fig 6D) and this was not accompanied by any change in blood sugar (Fig 6E). A dose-dependent fall in blood pressure (Fig 6F) was observed at supra-pharmacological doses (≥30 mg/kg). However, this reduction was transient (typically <2 min), did not differ significantly between drugs ($p=0.34$), and did not impact upon cardiac output at any dose level (Fig 6G; $p=0.12$). No exhaled H_2S was detected over the dose-range studied.

In order to determine if the pharmacodynamic differences between thiometallates (i,e, greater potency with ATTM) were due to differences in drug handling, we performed pharmacokinetic analyses (Fig 7). Colouration of the thiometallates allowed for their quantification in blood and urine. Plasma concentrations of both drugs followed a direct linear correlation with the quantity administered in the dose range 1-100 mg/kg (Fig 7A). Assessment of the half-life following administration of the highest dose revealed, as discovered previously for ATTM, a bi-exponential decay in plasma concentrations for both thiometallates (Fig 7B). Half-lives were calculated for the fast (distribution) and slow (elimination) phases of these decay curves. These were, respectively, 1.28 and 23.4 min for ATTT, compared with 1.36 and 21.5 min for ATTM. An assessment of urine volume (Fig 7C; $p=0.93$) and the number of moles in urine (data not shown) showed comparable results for ATTT and ATTM. Accordingly, percentage renal excretion was similar for both compounds (ATTT, 0.59±0.11%; ATTM, 0.67±0.23%; $p=0.75$. Using acidification of the blood as a biomarker of mitochondrial inhibition, PK/PD analysis (Fig 7D) demonstrated that ATTM was twice as potent as ATTM; the PK/PD slope, i.e the relationship of plasma drug concentration to blood acidification (reduction in pH), was 0.14±0.02 for ATTM and 0.07±0.01 for ATTT ($p<0.05$).

Discussion

The putative therapeutic utility of thiometallates has been investigated for more than 40 years. The most frequently used drug from this class, ATTM, has had preclinical efficacy demonstrated in >10 diverse disease pathologies that range from its initial indication, Wilson's disease, to Alzheimer's disease, pain and cancer (Brewer, 2009, 2014). These studies assumed the mechanism of action to be via copper chelation, with examples including the depletion of

injurious levels of copper in Wilson's disease, to reduction of copper-dependent angiogenesis by solid tumours. However, in addition to its modulation of copper status, we recently reported that ATTM is a slow-release sulphide donor (Dyson et al., 2017).

Interest in sulphide as a potential therapeutic surged following publication in 2005 of a landmark study in which mice breathing H_2S entered a profound and reversible 'suspended animation-like state' (Blackstone et al., 2005). However, unsuccessful clinical trials using basic sulphur salts encouraged the notion that delivery of sulphide needs to better targeted. This drove the design of more sophisticated sulphide-releasing molecules (Szabo and Papapetropoulos, 2017; Yang et al., 2017) that, once activated, aim to release sulphide in a more controlled manner. The combination of chemically and biologically relevant factors promoting sulphide release by ATTM are unique (Dyson et al., 2017). These characteristics help to ensure that sulphide release is better targeted to its intended intracellular site of action. Coupled with the fact that this class of drugs has been used historically over many decades in humans, led us to investigate its utility in modifying reperfusion injury in a clinically relevant setting.

Given this therapeutic potential of ATTM, we explored other molecules within this drug class and, as remains largely unknown for other sulphide donors, potential mechanisms of cellular uptake. We examined the tungsten analogue of ATTM and, in line with our hypothesis, confirmed that ATTT also acts as a sulphide donor. In accordance with similarities in chemical composition, and the proximity of molybdenum and tungsten in the periodic table, several parallels were seen with regard to their sulphide-release profile. ATTT releases sulphide in a controlled fashion over time when dissolved in aqueous solution; like ATTM, this is accelerated by heat, acidity, and the presence of thiols. However, across this wide range of environmental conditions, ATTT releases around half the quantity of sulphur as sulphide compared to ATTM. These results imply that ATTT is better able to resist scission of its sulphur atoms by hydrolysis. Lee et al (2007) demonstrated rates of hydrolysis of ATTT at various pH levels were significantly slower than those of ATTM. The mechanisms of hydrolysis are likely complex and can yield, particularly in acidic solutions, a vast array of polynuclear thiometallates. For example, following dilution of ATTM with equimolar HCl, the most abundant species (>40%) was the polynuclear anion, $Mo_3S_9^{2-}$, and this trimeric formation liberates free sulphide (Quagraine et al., 2009). The mechanism(s) of enhanced sulphide release by thiols remain incompletely understood for this class of compounds. While not the primary focus of our current study, we determined that the oxidation status of thiols did not impact on the promotion of sulphide release by the thiometallates. Further work is required to elucidate this precise mechanism.

To our knowledge, only one direct pharmacological comparison of ATTM and ATTT has been performed *in* vivo, assessing the preclinical efficacy of both drugs as anti-tumour and anti-inflammatory agents (Hou et al., 2007). Perhaps surprisingly, the authors reported that more ATTT (compared to ATTM) was required to have the same decoppering effect, and was related to less stability. This finding is at odds with those reported here, and hydrolysis rates reported elsewhere (Lee et al., 2007). It should be noted that Hou et al administered thiometallates in drinking water, where ongoing sulphide release from these dissolved compounds would be expected. An alternative explanation for the superior anti-tumour and anti-inflammatory effects reported for ATTM could be due, at least in part, to a combination of copper chelation and sulphide-dependent pharmacology. To this end, other studies have shown that sulphide donors, in particular those with a slow release profile, are effective as both anti-cancer agents (Lee et al., 2014) and modulators of inflammation (Whiteman and Winyard, 2011).

In view of the increased appreciation of the need for cell-targeted sulphide donors, there is a surprisingly small literature on cellular uptake mechanisms. Although there are considerable biological differences between water and gaseous sulphide, their similarity in structure prompted exploration of the ability of H_2S to use plasma membrane aquaporins (Mathai et al., 2009). The authors found that no facilitator was required and, in accordance with the known lipid solubility of sulphide, concluded that simple diffusion occurs without enablement by membrane channels. However, physiologically-relevent free sulphide is maintained in equilibrium in two forms; $H_2S \leftrightarrow HS^-$ with a pKa value of 6.76 (Liu et al., 2012). This dictates that the majority of sulphide (around 85%) is present as the hydrosulphide anion under physiological conditions. As such and, notably, in addition to free diffusion, a role for the most ubiquitous ion transporter in human erythrocytes, the anion exchanger-1, has been suggested (Jennings, 2013).

The mechanism of cellular uptake by thiometallates has not been previously explored. Further to the rationale for studying transfer of sulphide by ion exchange, the ionic nature of ATTT and ATTM following dissolution suggested an anion exchanger would be a strong candidate. We initially used formation of intracellular sulphaemoglobin in intact human erythrocytes as a surrogate of plasma membrane transport. Accordingly, pre-incubation of human blood with the AE-1 inhibitor, H_2DIDS prevented, at least in part, the formation of sulphaemoglobin. Not surprisingly, native sulphide (NaHS) did not exclusively use this channel as diffusion alone can permit rapid cellular entry (Jennings, 2013). To confirm the role of the AE-1 exchanger in cellular thiometallate uptake, we measured intra- and extracellular concentrations of these drugs using a basic absorbance assay. H_2DIDS treatment reduced both extracellular disappearance and intracellular detection of both thiometallates. Given that the formation of sulphaemoglobin could not be fully prevented, it is conceivable that some free extracellular

sulphide cleaved from these molecules may gain access by diffusion and contribute to its formation. Other ion channels and/or additional unstudied mechanisms may also be involved. However, given the substantial inhibition (65-73%) of intracellular cellular detection, we consider the AE-1 exchanger is an important mechanism governing the targeted intracellular delivery of this class of drugs. Although we focussed our attention on the human erythrocyte AE-1 exchanger, this channel is present in various forms elsewhere (Walsh and Stewart, 2010; Redzic, 2011), including the α-intercalated cells of the kidney, endothelial cells of the blood brain barrier and a truncated variant in rat heart. This suggests that the AE-1 exchanger uptake mechanism may be relevant for other target tissues.

As we have previously assessed the utility of thiometallates for the putative treatment of reperfusion injury, we examined the pharmacology of ATTT and ATTM *in vivo*, with a particular focus on haemodynamics and biomarkers of inhibition of aerobic respiration. The known hypotensive effect of sulphide-releasing drugs, particularly at high concentration, was observed here. This is attributed to free circulating sulphide that occurs via multiple putative mechanisms described in detail elsewhere (Liu et al., 2012). However, this hypotensive effect was less marked with ATTT, was transient in nature (<2 min), was only present with supra-pharmacological dosing, and occurred in the absence of detectable exhaled H_2S which is indicative of high quantities of free circulating sulphide.

We monitored blood acid/base status and lactate levels as biomarkers of mitochondrial electron transport chain inhibition. By inhibiting oxidative phosphorylation, cells augment both net ATP hydrolysis and glycolysis. Both these processes release H^+ ions that contribute to (metabolic) acidaemia, and the latter to an excess of glycolysis-derived pyruvate that, unable to enter the mitochondrion, undergoes dehydrogenation to lactate. Given the enhanced stability profile of ATTT, these effects were less pronounced than ATTM. We confirmed that the metabolic disparities observed were not due to differences in drug handling. There was a remarkably similar pharmacokinetic profile for both drugs; on comparison of the PK/PD relationship, we conclude that ATTM is twice as potent as a modulator of oxidative respiration.

The proposed mechanism of copper chelation by the thiometallates is formation of a tripartite complex between the thiometallate anion, a metal (mainly copper, but to a lesser extent, iron, cobalt and other metal ions) and a protein (e.g albumin) (Hou et al., 2007). Although some copper chelation can occur in di- or trithiomolybdates (i.e. $MoO_xS_{4-x}^{2-}$ where x=1-2) (Lannon and Mason, 1986; Laurie, 2000), it is generally accepted an intact 'tetrathio' anion in the form MoS_4^{2-} (or WS_4^{2-}) is required (Brewer, 2009). This is supported by the absence in nature of hydrolysis-derived di- or trithiomolybdates, as the intact MoS_4^{2-} ion degrades directly to MoO_4^{2-} (Laurie,

2000). Hydrolysis rates of these molecules are determined by the nature of the cation (Lee et al., 2007). In a previous study we showed that ATN-224, the bis-choline salt of tetrathiomolybdate, released less sulphide *in vitro* (Dyson et al., 2017). As a result, it was less effective than ATTM as a metabolic modulator *in vivo.* This is considered desirable for ATN-224 as it is undergoing clinical development as a copper-sequestering agent for Wilson's disease and shows some utility for cancer (Lowndes et al., 2008; Brewer, 2009, 2014). Our results here, and the demonstration elsewhere that tungstate is a better chelator than molybdate (Lee et al., 2007), suggest that ATTT falls into the same category as ATN-224, being better suited as a copper chelator over a sulphide donor. While this could potentially lessen its utility in ischaemia/reperfusion injury, hypoxia and circulatory shock states, it could provide additional benefit in pathologies sensitive to depletion of copper. We speculate that this ability to chelate copper could be further strengthened if the tungsten core of the molecule were coupled with a cation and/or other protecting moieties that confer greater resistance against hydrolysis.

With respect to ATTM, we have identified a safe and effective lead candidate for the treatment of ischaemia/reperfusion injury. Its sulphide release profile is better suited to this indication over other molecules (ATTT, bis-choline tetrathiomolybdate) within this class. Importantly, given the appreciated need for sulphide drugs that are better targeted, we have demonstrated for the first time that thiometallates use non-selective ion transporters to permit cellular access. Moreover, the sulphide release profile of these drugs (sensitive to thiols and low pH) dictates that more sulphide be released intracellularly, particularly in ischaemic cells that exhibit lower pH levels and have a greater need for treatment.

Conflict of interest

AD and MS are developing thiometallates for the treatment of reperfusion injury

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Legend for figures

Fig 1. H_2S release test. Comparison of (A) H_2S values over time and (B) the area under curve (AUC) during washout. Inset picture in (A) shows the apparatus for H_2S detection. Values recorded are in parts per million (ppm).

Fig 2. Sulphaemoglobin assay. (A) spectrophotometry-derived absorbance spectrum of oxyhaemoglobin and sulphaemoglobin. (B) shows equations for the calculation of absorbance for (1) one, and (2) an infinite number (n) of dissolved chromophores. (C) Extinction coefficients for deoxyhaemoglobin, oxyhaemoglobin, and sulphaemoglobin (Zwart et al., 1981). Coefficients required for calculation of sulphaemoglobin are shown in shaded cells. λ, wavelength.

Fig 3. In vitro H_2S release of from ATTM and ATTT under different environmental conditions. In (A), standard conditions indicate that drugs (100 mM total sulphur) were incubated for 1h at physiological pH (7.4) and temperature (37°C). (B) shows concentration dependence of drugs incubated for 1h. Effects of time, temperature, the presence of thiols and variations in pH are shown, respectively, in (C-F). The dotted lines reflect typical H_2S gas levels (4 ppm) obtained from ATTM (100 mM total sulphur) following 1h incubation at normal physiological pH and temperature. GSH and GSSG represent reduced and oxidised glutathione, respectively. *p<0.05, 2-way ANOVA followed by Bonferroni's test. Stated p-values are the result of overall ANOVA.

Fig 4. Ex vivo formation of sulphaemoglobin in human erythrocytes. A dose response (incubation for 1h) and timecourse (all drugs at 100 mM total sulphur) are shown in A and B, respectively. The sensitivity of sulphaemoglobin formation to (30 mins) pretreatment with the anion exhanger-1 inhibitior (by H_2DIDS; 4'diisothiocyanatodihydrostilbene-2,2'-disulfonic Acid; 0.5 mM) is shown in panels (C-E). Total sulphur for each drug was 100 mM. Incubation time was 3h for the thiometallates (in C and D) and 15 mins for NaHS (E). *p<0.05, unpaired T-test. NS; not significant.

Fig 5. Extra- and intracellular concentrations of thiometallates. Absorbance spectrum of ATTT and ATTM is shown in (A) with λ_{MAX} values of 392 and 448 nm, respectively. Drug concentrations detected extracellularly are shown in panels B and C. Corresponding intracellular measurements are shown in D and E. H_2DIDS; 4'diisothiocyanatodihydrostilbene-2,2'-disulfonic Acid; 0.5 mM). *p<0.05, unpaired T-test.

Fig 6. In vivo pharmacology of thiometallates. Panels (A-C) concern acid/base interactions with (A) arterial pH, (B) base excess (the metabolic component of acid/base status) and (C) arterial partial pressure of carbon dioxide (PCO_2; respiratory component). Blood lactate and glucose levels are shown in (D) and (E), respectively. (F) shows maximal changes in mean arterial blood pressure while (G) depicts cardiac output, measured by echocardiography. n=6 per group. *p<0.05; two-way repeated measures ANOVA followed by Bonferroni's test. Stated p-values are the result of overall ANOVA.

Fig 7. Pharmacokinetics. (A) shows the dependence of plasma concentration on IV dose level and (B) the decay in drug levels after the highest dose (100 mg/kg). (C) shows urine output. NB, the number of moles in urine and fractional renal excretion was the same for both compounds (not shown). (D) depicts the relationship between drug plasma levels (measured at 2 mins post-administration) and subsequent (25 min later) changes in arterial pH. The slope in (E) is used as a surrogate for drug potency, calculated as 0.10 and 0.15 for ATTT and ATTM, respectively. n=6 per group. P-value; unpaired T-test.

Supporting information - In vitro H_2S gas release (expanded)

Free (biologically active) sulphide constitutes H_2S gas and two anions, HS^- and S^{2-}. The assay, developed in-house and described in detail herein, relies on detection of free H_2S gas that is measured using a commercially available H_2S detector (Z900XP, Environmental sensors, Boca Raton, FL, USA). The default protocol that we have established (elaborated upon in supporting information) is as follows:

1) Dissolve compound (ATTM or ATTT) in room temperature phosphate-buffered saline; ([PBS], pH 7.4) to 10x stock solutions in Eppendorf tubes (1 ml volume). The typical 10x stock concentration is 250 mM for the thiometallates (1 M total Sulphur). For reference, 3 mM stocks of simple salts e.g. NaHS, generate approximately the same quantity of H_2S under these conditions (Dyson et al., 2017).
2) Vortex for 30-40 seconds.
3) Dilute 1/10 (0.5 into 4.5 ml) rapidly into airtight Falcon tubes (50 ml; Corning Science Mexico, Reynosa, Mexico) containing PBS. The PBS is typically pre-warmed to 37°C but can be adjusted as necessary (e.g. to different temperatures, pH levels, or to contain thiols, other adjuvants or alternative matrices). The liquid and gas (headspace) phases constitute 5 and 45 ml, respectively.
4) Replace the Falcon tube cap, tighten and further seal the lid with Parafilm (Bemis, Neenah, WI, USA).
5) Typically incubate in a water bath for one hour at 37°C.
6) Remove from the water bath and puncture one side of the Falcon tube lid with an orange (25 gauge) needle (Terumo, Egham, Surrey, UK). Puncture the other side of the Falcon tube lid with a blue (23-gauge needle) attached to a 5 ml syringe. Withdraw the 5 ml of headspace gas over 10 seconds.

Pass the gas sample through the detector using a 3-way tap (closed to room air) to accommodate the syringe; this is attached to the detector inlet, as shown inset in Fig 1A. Once all of the gas has been drawn from the syringe, *remove* to allow room air to wash out the system. Failure to remove the syringe causes a build-up of H_2S gas around the sensor leading to erroneously high readings.