Cysteamine, an endogenous aminothiol, and cystamine, the disulfide product of oxidation, increase *Pseudomonas aeruginosa* sensitivity to reactive oxygen and nitrogen species and potentiate therapeutic antibiotics against bacterial infection.

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Cysteamine is an endogenous aminothiol produced in mammalian cells as a consequence of coenzyme A metabolism through the activity of the vanin family of pantetheinase ectoenzymes. It is known to have a biological role in oxidative stress, inflammation and cell migration. There have been several reports demonstrating anti-infective properties targeting viruses, bacteria and even the malarial parasite. We, and others have previously described broad-spectrum antimicrobial and antibiofilm activity of cysteamine. Here we go further to demonstrate REDOX dependent mechanisms of action for this compound and how its antimicrobial effects are at least in part due to undermining bacterial defenses to oxidative and nitrosative challenge. We demonstrate the therapeutic potentiation of antibiotic therapy against P. aeruginosa in mouse models of infection. We also demonstrate potentiation of many different classes of antibiotic against a selection of priority antibiotic-resistant pathogens, including colistin (often considered an antibiotic of last resort), and we discuss how this endogenous antimicrobial component of innate immunity has a role in infectious disease which is beginning to be explored and is not yet fully understood.

Cysteamine (CYS), is produced in the body through the cleavage of pantetheine to form CYS and pantothenate (vitamin B5) as a breakdown product of coenzyme A (1). CYS itself is highly reactive, and it readily oxidises in solution to form the disulfide, cystamine (CTM) in the presence of oxygen, or through Fenton chemistry in the presence of transition metals, releasing free radicals and hydrogen peroxide (2). In a reducing environment in the absence of transition metals, CYS can act as an antioxidant, so activity is highly dependent upon the physiological context. It will
also readily form mixed disulfides with susceptible cysteine sulfhydryl groups in a process called cysteaminylation (3), which is key for many reported biological activities. It may also react with aldehyde groups which has been suggested as the mechanism behind Ehlers-Danlos Syndrome associated side effects of long term systemic clinical use (4). Mammalian cells can express aminothiol dioxygenase (ADO), which can oxidize CYS to hypotaurine, and taurine (5). Bacterial, fungal and many eukaryotic parasites (including Plasmodium sp.) cells do not encode the ADO gene and this may explain selective toxicity. CYS is also a substrate for mammalian vascular adhesion protein-1 (VAP-1, also known as adipocyte copper amine oxidase 3, or AOC3), which can form hydrogen peroxide and ammonia by products (6,7). The breakdown of coenzyme A via CYS to taurine is thought to be an underestimated yet significant proportion of sulfur metabolism in mammals (8), but the short-lived nature of CYS and the difficulty of distinguishing it from other small biological thiols have meant studying this molecule is challenging and its biological role has likely been underappreciated.

CYS has been licenced for use for >25 years for the treatment of nephropathic cystinosis; depleting cystine from human cells (9) to counteract an inability to remove it from the lysosome (10,11,12). It has also been investigated for a wide range of other indications including as a radioprotectant (13), as a treatment for acetaminophen poisoning (14), Huntington’s disease (15), Parkinson’s disease (16) and non-alcoholic fatty liver disease (17). CYS is also known to be a broad spectrum anti-infective (18-20). The oxidized disulfide CTM has been shown to possess anti-retroviral properties against HIV (21), and endogenously produced CYS was recently shown to limit influenza replication in A549 cells (22). It is also being studied as an adjunct to anti-malarial therapy (23,24). We and others have previously...
demonstrated that CYS has antimicrobial properties against bacteria associated with cystic fibrosis (CF) respiratory tract infections, including *Pseudomonas aeruginosa*, *Mycobacterium abscessus* and the *Burkholderia cepacia* complex (BCC). Here we demonstrate that intravenous (i.v.) or dry powder inhalation (d.p.i.) administration of CYS potentiates the activity of both ciprofloxacin and tobramycin in neutropenic thigh and lung mouse models of infection, respectively, against *P. aeruginosa*.

We report herein that exposure to CYS in a pro-oxidative environment or the oxidized disulfide CTM itself, dysregulates bacterial metabolism and reduces the capacity of *P. aeruginosa* to resist oxidative and nitrosative stress elicited by exogenous sources. CYS can also readily react with nitric oxide (NO) donors, to form a new adduct with characteristics suggestive of S-nitrosocysteamine. S-nitrosothiols are a family of compounds with an emerging role in immunity (27), including maintenance of cell barrier integrity against infection and repair (28,29) and cell signalling (30). CYS also impairs bacterial virulence at sub-inhibitory levels, with striking effects on bacterial pigment production including phenazines in *P. aeruginosa* and pyomelanin in BCC.

Colistin is one of the few remaining options for treatment of infections caused by multi-, or extensively (MDR and XDR) strains of Gram-negative pathogens. In the few years there have been reports of resistance to colistin in strains of Enterobacteriaceae mediated by plasmid-borne *mcr* genes encoding phosphoethanolamine transferases, initially in China (31), but subsequently from around the world (32-34). Widespread emergence of transferable colistin resistance in clinical cases would pose a serious public health risk. We demonstrate that CYS can potentiate the activity of colistin, including the reversal of clinically defined resistance in clinical isolates and engineered *Escherichia coli* expressing *mcr-1* and *mcr-4*. 

CYS
against colistin-resistant *Klebsiella pneumoniae* (non-mcr mediated) strains in *in vitro* tests. We have also previously demonstrated CYS potentiation of the macrolide, azithromycin (35). Azithromycin is also a component of the recommended ‘last line’ dual therapy for gonorrhoeae, which treatment option-limiting resistance has recently emerged (36,37). Antibiotic resistance in Gram-positive pathogens also remains a major clinical challenge and we set out to discover if CYS could reverse antibiotic resistance in several MRSA strains. We demonstrate potential utility for CYS to be repurposed as a short-term use antibiotic potentiator with broad spectrum activity in enhancing multiple different classes of antibiotic against a range of Antimicrobial resistant (AMR) pathogens which are of urgent concern to health authorities worldwide.

**MATERIALS AND METHODS**

Unless otherwise stated chemicals, reagents and media were purchased from Sigma-Aldrich (MO, USA).

**Bacterial strains and culture conditions**

The commercially available *E. coli* NEB® Express strain was chosen for transformation with *mcr-1* as this laboratory strain was shown to be susceptible to colistin and is designed to maximize the transformation efficiency and express recombinant proteins. The clinical MDR *E. coli* RH14000226 strain was confirmed as colistin-resistant. Two MDR *K. pneumoniae* strains (NB01216 and NB02216) were also chosen for checkerboard analysis alongside CYS due to their susceptibility profile to colistin and clinical significance. For the colistin antimicrobial susceptibility experiments all strains were screened for the carriage of *mcr-1* gene, using the
Eleven strains of *S. aureus* were examined including 6 defined as MRSA by selective isolation on mannitol salt agar with oxacillin, and susceptibility profiling to oxacillin. Nine strains were clinical isolates and 2 were MRSA type strains. Seven out of 11 strains were shown to be resistant to azithromycin using Etest® screening and CLSI standardized MIC broth microdilution experiments (see below).

Two MDR strains of *Neisseria gonorrhoea* (NB03916 and NB04916) were chosen for experimentation based upon clinical importance and susceptibility profiling.

*P. aeruginosa* PAO1 well-characterized type strain was used for *in vitro* antimicrobial mechanism of action studies. The MDR LES431 strain of *P. aeruginosa* was chosen for the mouse thigh model of infection and *P. aeruginosa* ATCC27853 is the established strain used in the mouse acute lung model of infection. A selection of CF isolates was also tested for phenazine production as were pigmented type and clinical strains of *Burkholderia cenocepacia*.

All bacteria were grown in Mueller Hinton broth (with cation modification) except for *N. gonorrhoeae* strains which were grown on GC agar plus Vitox supplement (Oxoid, Thermo Scientific, UK). Strains were maintained with the addition of the appropriate antimicrobial agents where required, although these were withdrawn prior to overnight subculture for antimicrobial susceptibility testing with no detectable loss in plasmid or cloned insert.

MIC broth microdilution experiments (see below) were also conducted in M9 minimal media supplemented with different carbon sources at 0.4% w/v and corrected to pH 7.0, to examine the impact of different carbon sources on the antimicrobial activity of
cystamine. M9 minimal media consisted of 50 mM Na$_2$HPO$_4$$\cdot$7H$_2$O, 22 mM KH$_2$PO$_4$, 18.7 mM NH$_4$Cl, 8.5 mM NaCl, 2mM MgSO$_4$, and 0.1 mM CaCl$_2$. Carbon sources including glucose, oxaloacetic acid (oxaloacetate), sodium succinate dibasic hexahydrate (succinate), sodium citrate dihydrate (citrate), were added to 0.4% w/v from filter-sterilised stock solutions.

**Determination of thiol content using dithionitrobenzoic acid (DTNB)**

*P. aeruginosa* PAO1 was grown overnight 37°C on Mueller-Hinton agar prior to inoculation of 19 ml pre-warmed glucose minimal medium with 1 ml, 2 x $10^{12}$ cfu suspended in phosphate-buffered saline (PBS) in a sterile flask and incubated with shaking at 150 rpm for 4 h to reach logarithmic growth phase. Ten minutes prior to exposure to CTM, selected cultures were treated with 10 µM carbonyl cyanide m-chlorophenyl hydrazine (CCCP) to collapse the proton gradient. Background thiol content of the supernatants were also determined by removing 1 ml culture and pelleting bacteria via centrifugation at 2,350 x g for 5 min. DTNB is added to clarified supernatant to a final working concentration of 0.1 mM per 0.1 ml volumes (from a 20 x stock containing 2 mM DTNB and 50 mM sodium acetate) in a 96-well microtitre plate and incubated for 10 min at room temperature prior to reading at 410 nm on Biotek plate readers. Reduced solutions of cysteine were used as standards for concentration determination. Background controls for CCCP, CTM and media alone were also determined. Cellular thiol contents were determined by mechanical disruption using ZR BashingBead™ lysis tubes for 5 min prior to DTNB assay.

**Detection of ROS using 2′,7′-Dichlorofluorescin diacetate (H2DCFDA)**

H2DCFDA is a cell-permeable non-fluorescent compound which reacts with reactive oxygen species to form the highly fluorescent 2′,7′-dichlorofluorescein. *P.
aeruginosa PAO1 at 5 x 10^5 cfu/ml were challenged with increasing concentrations of ciprofloxacin and CTM dihydrochloride as described in a checkerboard format (38) in 100 µl volumes on a microtitre plate with the addition of H2DCFDA at 1 µM working concentration and fluorescence was detected over time incubated at 37°C at Ex/Em 485/20,528/20 nm.

NADP/NADPH ratio determination

P. aeruginosa PAO1 cells were grown in defined Roswell Park Memorial Institute (RPMI) media with or without glucose at 2 g/l for 20 h at 37°C and treated with or without cystamine dihydrochloride as described and NADP/NADPH ratio was determined colorimetrically at 450 nm using a microtiter assay kit as per manufacturer’s instructions (Sigma-Aldrich) including the removal of enzymes which may utilize NADPH by passage of clarified lysate through 10 kDa-cutoff filters (Amicon, Merck, NJ, USA).

Molecular Biology

The open reading frame sequence for the gene mcr-1, a putative phosphoethanolamine transferase (accession number A0A0R6L508) was synthesized using the GeneArt gene synthesis service (Thermo Fisher Scientific, MA, USA). This sequence was amplified using the polymerase chain reaction (PCR) with flanking cloning primers (clon_for: ATTCATATGATGCAGCATCTTTCTGTGTGGTACCG clon_rev: TGTAATCGAGGGATGAATGCGGTG) to introduce restriction enzyme sites (underlined). These were digested with appropriate restriction enzymes (Ndel and Xhol) and ligated, in-frame, into the multiple cloning site of plasmid pET29b. Plasmids with, and without, mcr-1 insert were transformed into E. coli NEB® Express 8
laboratory strain of *E. coli*. Internal detection primers (described above) were used
to confirm presence or absence of the *mcr-1* insert in the transformed cells and
expression of the *mcr-1* gene was confirmed due to phenotypic change in the MIC of
this strain to colistin (using the method as described below).

**Antimicrobial susceptibility MIC and checkerboard experiments**

The MIC (minimum inhibitory concentration at which 100% of bacteria were killed)
and minimal bactericidal concentrations (MBC) was determined versus CYS and
other antibiotics as described using the CLSI broth microdilution procedure M07-A10
(64). Checkerboard assays of CYS, CTM, and antibiotics were conducted (38) to
assess the combinations of CYS and CTM with ROS, and NO generating
compounds. Definitions of susceptibility or resistance were determined using
EUCAST clinical breakpoints for bacteria

(http://www.eucast.org/clinical_breakpoints/).

**Etest® MIC determination of *S. aureus* on cation-adjusted Mueller Hinton agar**

(CA MHA) and *Neisseria gonorrhoeae* strains on GC agar plates

Azithromycin impregnated Etest® strips were placed upon CA MHA or GC agar (plus
Vitox supplement) containing different concentrations (0-512 μg/ml) of CYS as
indicated, inoculated with suspensions of *S. aureus* or *N. gonorrhoeae* respectively
according to manufacturer’s instructions (BioMérieux, France) and incubated for 24
hours at 37°C for *S. aureus* strains, and 48 h at 37°C in 5% CO₂ atmosphere for *N.
gonorrhoeae* strains.

**Procedures**
Animal studies were conducted by Evotec (UK) Ltd. (Mouse acute lung model) and Eurofins Panlabs, Taipei, Taiwan (Mouse Thigh Model). All procedures performed in accordance with appropriate regulatory licenses held by the entities/personnel who performed the following studies.

**Mouse acute lung model of infection**

Male CD1 mice were immunosuppressed/pre-conditioned with 200 mg/kg and 150 mg/kg cyclophosphamide at 4 and 1 days prior to study respectively. CYS and tobramycin were prepared for inhalation formulation in a mannitol vehicle respectively. An infection was established with *P. aeruginosa* ATCC27853, with an inoculum of $5 \times 10^6$ cfu/ml, administered intranasally in a volume of 40 µl following anaesthetisation with a ketamine/xylazine anaesthetic cocktail for 15 minutes. The treatments were administered approximately 10 minutes after infection. All treatments, and mannitol vehicle control, were administered using a Penn Century device. The lung tissue burden of each animal, at the clinical end-point of 24 h post-infection, was determined. The lungs were homogenized in 2 ml PBS, serially diluted in PBS and plated onto *Pseudomonas*-selective agar before quantification after 24-48 h at 37°C.

**Mouse thigh model of infection**

Mice were rendered neutropoenic with cyclophosphamide prior to infection as described above for the acute lung model of infection. They were separated into groups of 5 animals per treatment. Inoculation (confirmed by culture) was with 1.52 x $10^6$ cfu/ml of *P. aeruginosa* strain LES431 and conducted 1 hour prior to treatment which was i.v. for saline vehicle controls, ciprofloxacin, and CYS treated mice, and subcutaneous for colistin (used as a positive control). Animals were sacrificed 25 h
post infection (24 h post-treatment) and thigh weights and cfu/g of tissue were calculated and recorded.

RESULTS

Cysteamine and cystamine sensitize *P. aeruginosa* to killing by reactive oxygen and nitrogen species

We have previously demonstrated that CYS can potentiate the activity of the fluoroquinolone ciprofloxacin against resistant BCC strains (19). In table 1 we demonstrate that CYS and CTM consistently, though modestly potentiate the activity of ciprofloxacin against sensitive *P. aeruginosa* type strain PAO1, but that they also (particularly CTM) potentiated the antimicrobial activity of ROS generating chemicals paraquat and hydrogen peroxide to which *P. aeruginosa* is usually resilient. CYS and CTM also potentiated the antimicrobial activity of NO donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP). CTM had more pronounced potentiation of MIC than CYS (Table 1), but CYS formed an adduct species with SNAP and NaNO₂ which was bacteriostatic in nature. This was evident from a colour change (to pink) in microtitre plates used in checkerboard experiments at high concentrations for determining the relative effects on MIC, and peaks in UV spectroscopy at 333 nm and 545 nm, characteristic of the S-nitrosothiol compound S-nitrosocysteamine, although the identity was not confirmed (39). Like other biological S-nitrosothiol compounds the reaction between NO donor and thiol was optimal in slightly acidic pH conditions (39) but within physiological parameters (61) for inflamed tissues (Fig. 1). The reaction was thiol specific as no change in absorbance above background was seen across the same pH range when using the disulfide CTM.
Cystamine disrupts *P. aeruginosa* metabolism, reducing power and defense against ROS

We determined that *P. aeruginosa* PAO1 rapidly exports free thiol in response to treatment with the disulfide CTM by a process that wasn’t inhibited by the ionophore CCCP (Fig. 1), and found that more thiol accumulates in bacterial cells treated with CTM than CYS. The dysregulation of cellular thiols caused by entry of CTM also disrupts *P. aeruginosa* PAO1 metabolism, as demonstrated by reduced MIC of this strain when grown in M9 minimal media with selected TCA cycle carbon sources in the presence of CTM in comparison to media containing glucose (Table 2). The MIC in minimal media with glucose as a carbon source was higher than in standard conditions using Mueller Hinton broth, at 1024 μg/ml, whereas when grown on oxaloacetate the MIC drops to 2 μg/ml. The availability of different carbon substrates has a big effect on cystamine toxicity.

Glucose can be utilised to replace pools of NADPH which can be utilised for generating energy, but is also required for restoring REDOX balance in the cell. We discovered that the ratio of NADP:NADPH in *P. aeruginosa* PAO1 is also altered by CTM. When grown in RPMI with glucose and a sub inhibitory concentration of 256 μg/ml of CTM for 20 h at 37°C, the ratio of NADP:NADPH was 1.49:1 compared with 1.19:1 for cells treated with vehicle (water) alone. When grown without glucose the ratio of NADP:NADPH was 0.66:1 when treated with CTM at 256 μg/ml compared with 0.56:1 with vehicle only (n=3), demonstrating that CTM treatment reduces the cellular NADPH pool.

Unlike CYS (data not shown), the addition of CTM to Mueller Hinton broth growth media does not raise the level of ROS above those of controls in uninoculated media.
as detected by H2DCFDA fluorescence. However, when inoculated with *P. aeruginosa* PAO1 (at standardized 5 x 10⁵ cfu/ml the same as used in MIC determination) the addition of CTM raises the ROS produced relative to uninoculated and untreated controls (Fig. 2). Ciprofloxacin itself is known to induce ROS production, secondary to its activity against DNA gyrase and topoisomerase IV, and this is detectable over time when used in this sensitive strain at concentrations far above the MIC (42) but this was not significant at the concentrations shown using this inoculum size. Interestingly CTM dose-dependently significantly induces ROS formation by *P. aeruginosa* PAO1 at 512 µg/ml, one doubling dilution below the MIC from 70 min after exposure onwards. Fluorescence relative to background peaks for most treatments at 2 h (Fig S1), and at this time point the combination of CTM and ciprofloxacin induces significantly greater ROS than either treatment alone at the concentrations shown.

**Cysteamine potentiates antibiotic therapy in mouse models of infection**

Here we demonstrate (Fig. 3) that CYS could potentiate the activity of the fluoroquinolone, ciprofloxacin in the neutropenic mouse thigh model of infection against the MDR *P. aeruginosa* Liverpool epidemic strain 431 (LES431), which unlike PAO1 has a resistant MIC of 4 µg/ml (43) as defined by CLSI and EUCAST clinical breakpoints. Combined i.v. therapy with CYS at 1.25 mg/kg and ciprofloxacin at 15 mg/kg significantly reduces microbial burden in the thigh compared with either treatment alone, with a 4.6 log₁₀ reduction in cfu/g. There was no significant difference when compared with a mean 5.02 log₁₀ reduction in cfu/g for colistin at 5 mg/kg (used as a positive control). Individually ciprofloxacin achieved 2.02 log₁₀ reduction and CYS alone elicited a mean reduction of 0.74 log₁₀ reduction, although this was not statistically significant.
Tobramycin is also known to induce ROS formation in BCC (42) and is commonly used clinically as a maintenance therapy in adults and children above 6 years old with cystic fibrosis who are colonized by *P. aeruginosa*. Therefore, we examined (Fig. 3) the potentiation of tobramycin by inhaled CYS in a neutropenic acute lung *P. aeruginosa* infection mouse model.

**Cysteamine and cystamine inhibit pigment virulence factors**

The effective dose for potentiating antibiotic activity *in vivo* is below that seen *in vitro* and achievable therapeutically, so additional factors may be at play. CYS has known immunomodulatory activity (25,26) which should be considered in an immunocompetent model or host, but it has also been shown to have anti-virulence activity (18). Whilst relatively high concentrations of CYS were required for antimicrobial activity *in vitro*, it was noted that sub-inhibitory concentrations markedly inhibited phenazine production in *P. aeruginosa* strains (Fig S2). The phenazine, pyocyanin is a REDOX active virulence factor with multiple host targets that is implicated in the establishment of lung infection (44,45). Similarly, pigment secretion in *Burkholderia cenocepacia* strains was also inhibited. It appears that for *Burkholderia cenocepacia*, CYS may inhibit the polymerization of the secreted homogentisate precursor and inhibit the secretion of pyomelanin from the cell, as the pigment can be released from lysed pellets (Fig S3). Pyomelanin pigment, detected by absorbance at 480 nm (63), was significantly inhibited by CYS at concentrations above 8 μg/ml. The inhibition of these virulence determinants at sub-MIC concentrations may have further benefit therapeutically to enhance co-therapies and assist the innate and adaptive immune response to infection.

**Potentiation of colistin antimicrobial activity**
We have also explored whether CYS has efficacy for potentiating antibiotics against pathogens beyond the context of cystic fibrosis. We demonstrate that the transformation of a laboratory strain of *E. coli* (NEB® Express) with a plasmid-encoded *mcr-1* gene confers CLSI-defined resistance to colistin. Our transformant was resistant to colistin (MIC = 8 µg/ml), compared with the strain containing the empty vector, which was susceptible to colistin (MIC = 2 µg/ml). We show (Table 3) an 8-fold reduction in the MIC of colistin when tested in combination with CYS against the transformant in checkerboard studies, with the MIC reduced from 8 µg/ml (defined as resistant) to 1 µg/ml (susceptible). The MIC for the strain harbouring the empty vector was also consistently reduced slightly by two-fold, from 2 µg/ml to 1 µg/ml, which confirms that the effects of CYS are not specific to the *mcr-1* mediated resistance mechanism.

We have also demonstrated resistance breaking by CYS in a clinical strain of *E. coli* RH14000226 harbouring the *mcr-1* gene (confirmed by PCR). *E. coli* RH14000226 is a sequence type (ST) 457 isolate harbouring the extended-spectrum beta-lactamase CTX-M-27 and the colistin resistance gene *mcr-1*, the latter of which is located on an IncHI2 plasmid15 (Table 3).

CYS potentiated the activity of colistin against two resistant *K. pneumoniae* strains that do not carry *mcr-1*. Isolate NB02216 belongs to ST258 and harbours the KPC-2 carbapenemase, whilst NB01216 belongs to ST14 and harbours the NDM-1 carbapenemase. In both isolates resistance to colistin was due to inactivation of *mgrB*: in NB02216 via insertion of an IS5-like transposase at nucleotide position 70 of *mgrB* and in NB01216 due to an A→T nucleotide substitution at nucleotide position 7 of *mgrB*, resulting in an early stop codon. In the ST258 strain we
demonstrated full restoration of clinical susceptibility to colistin (a 32-fold reduction in MIC to 1 \( \mu \)g/ml) in the presence of CYS; although the ST14 strain remained colistin-resistant, CYS reduced its colistin MIC by four-fold (Table 3).

**Potentiation of macrolide antimicrobial activity**

We demonstrated considerable synergy between CYS and the macrolide azithromycin against *Staphylococcus aureus*. The MICs for five azithromycin-resistant isolates (four MRSA and one MSSA strain) reduced 16 to 1024-fold, in each case to below the CLSI and EUCAST clinical susceptibility breakpoints (Table 3); by contrast, the MICs for five azithromycin-susceptible *S. aureus* (one MRSA and four MSSA) were reduced only 2-fold in the presence of CYS.

When azithromycin Etest strips were used to determine MIC, it was noted that the appearance of a resistant sub-population of colonies which grew close to the Etest strip in resistant strains was abolished by the incorporation of CTM into the CA MHA plates (Fig. 4).

CYS also potentiated the activity of azithromycin against an MDR strain of *N. gonorrhoeae* strain NB04916 (Fig. 4), reducing its MIC from 32 \( \mu \)g/ml to 4 \( \mu \)g/ml (Table 3). Whilst this did not restore clinical susceptibility as defined by EUCAST (MIC <0.5 \( \mu \)g/ml), the 87.5% reduction may still be of clinical value and will be investigated further. This isolate has the -1A deletion in the 13pb inverted repeat region between -10 and -35 of the mtrR promoter, as well as D79N, T86A and H105Y mutations. In contrast, the addition of CYS did not alter demonstrably the MIC for an *N. gonorrhoeae* strain in which high-level azithromycin resistance is mediated through the mutation A2059G in all 23S rRNA alleles.
CYS did not potentiate beta-lactam containing antibiotics against all bacteria we have tested so far in the in vitro systems which we employed (data not shown). Indeed, there is concentration-specific antagonism with all classes of antibiotic containing the beta-lactam ring that we have tested to date. Suspecting that these findings (which contradicted previous CF clinical observations) were specific to beta-lactam/CYS chemistry within the limitations of in vitro systems, we investigated if CYS reacts with nitrocefin in bacterial growth media. Hydrolysis of this beta lactam produces a red product which is rapidly detectable when combined with CYS (data not shown). CTM itself doesn’t react.

DISCUSSION

The antimicrobial activity of CYS is mediated by a complex interplay of the REDOX environment, conversion to CTM and access to the cell membrane or cytoplasm (which is itself likely dependent upon microbial substrate availability), and the chemistry of the site, such as the presence of transition metals, nitrosating species or other compounds which may react with CYS (Fig 5.). The rate of enzymatic, or chemical, removal of CYS and CTM from the bacteria or host environment is likely to be another factor which has not been fully explored here. This complexity means that conventional (64) in vitro antimicrobial susceptibility test (AST) methods cannot fully capture the utility (or otherwise) that this compound may have as an anti-infective or as an adjunct potentiator of antibiotics. Although direct, broad spectrum, antimicrobial activity for CYS has been demonstrated by ourselves and others (18-20), the concentrations required for microbicidal activity in standardized tests are much higher than those required to achieve antimicrobial/potential activity in vivo. The effects upon host cellular immunity cannot be ruled out. Studies (25, 26) have demonstrated that CYS enhanced the microbicidal clearance of P. aeruginosa and
BCC from experimentally infected macrophages bearing the F508del CFTR mutation, attributed to reduced proteostasis and enhanced autophagy. Clearly these effects are not relevant to neutropenic wild-type animal studies, but we do not yet have a full understanding of the impact of CYS on the immune response to infection.

Modification of conventional antibiotic susceptibility tests can bridge this MIC gap. For example, we have demonstrated repeated low dosing (at physiologically achievable levels) \textit{ex vivo} in sputum is antimicrobial (35) against the bacterial load in CF sputum. Substrate availability is important to bacterial sensitivity to CYS and we are also investigating the role of growth phase and aeration upon the MIC of CYS and CTM. Our results suggest CYS might disrupt the TCA cycle in \textit{P. aeruginosa} where we achieved particularly low MIC values when grown in oxaloacetate M9 minimal media. Some of these factors appear to explain the disparity between the relatively high \textit{in vitro} MIC and the lower effective doses in animal studies and human trials. The context of the infection might be critical to the impact of CYS/CTM upon it. Interactions and access to immune mediators will also vary from infection to infection.

The potential interplay of CYS and other innate immune effector molecules such as NO is fascinating. S-nitrosocysteamine has been considered as a potential NO carrier before (39) but it was thought to be too short-lived a molecule for use therapeutically. The identity of this adduct needs to be confirmed with NMR spectroscopy. Whether this species does form endogenously during infection or inflammation needs to be investigated further, though we can detect its formation at physiological pH (though acid pH promotes formation) and are investigating the ability of cysteine, via transnitrosation, to accelerate the production and removal of this species.
There is widespread tissue distribution of vanin-1, including gut, lung and liver etc, and differing REDOX environments, including pH and pO$_2$, at different body sites. It is therefore possible that expression of the same biological thiol might have different impacts on physiology (including innate immunity) in the lung, for example, compared with the gut. The same might be said for inflamed and healthy tissue. It would also be very interesting to examine the effect of a pro-oxidative insult upon the usually anaerobic environment of the gut (for example, caused by injury) and any role CYS might play in inflammation, immunity or repair, as vanin-1 is highly expressed here. The $vnn$-1 knockout mouse is known to be protected from intestinal inflammation (47,48), but so far, the only study relating to this mouse knock out and immunity to bacterial infection (to our knowledge) was an investigation into the role of $vnn$-1 in granuloma formation and macrophage activation in response to Coxiella burnetii (49). For this pathogen, there was no difference in the ability of the mutant to eliminate infection, but there were impairments in leukocyte recruitment and macrophage-driven antimicrobial activity. Given the potential interaction with the CYS product of vanin-1 and nitric oxide shown in this study, it is interesting that the $vnn$-1 knockout had impaired iNOS expression in response to $C.\ burnetii$ infection. Studies examining vanin-1 discuss the potential interaction of CYS with the GSH pool of the host (47,50). Indeed $vnn$-1 mice themselves have been demonstrated to be less susceptible to oxidative injury by paraquat than wt (47), which has been attributed to inhibition of gamma-glutamylcysteine synthetase (γGCS) by CTM, which is responsible for de novo synthesis of GSH. Studies examining the toxicity of CYS against human leukocyte cell lines also demonstrated inhibition of mammalian glutathione peroxidase (2), and glutathione transferases with peroxidase activity are widely expressed in aerobic bacterial pathogens (51), though it is not yet known if
this activity is also inhibited by CYS. Although synthesis of GSH has not been studied here, the activity of *E. coli* \( \gamma \)GCS has been shown to be resistant to CTM (52). It is known that the disulfide cystine, like what is seen here with CTM, sensitises bacteria to oxidative stress (41) and that mammalian and bacterial cells respond differently to this disulfide (53). Cystine import systems have been described in *E. coli* which are widely conserved in other bacteria, including *P. aeruginosa*, and have been described as part of the cysteine/cystine futile cycle, a vulnerability in bacterial metabolism which leads to over-import of cystine and GSH dependent reduction back to cysteine, much of which is then exported and then (in a sufficiently oxidative environment) oxidized to cystine again completing a cycle (41,54). We demonstrate that thiol export in response to CTM is not dependent upon proton motive force, but whether the import/export mechanisms for CTM/CYS are the same as cystine/cysteine is not yet known. Early observations in *Streptococcus mutans* (40) also showed treatment with the disulfide also led to an accumulation of CYS in the culture media. We suggest that CYS enters the cell as CTM in a Trojan horse-like manner before being reduced back to CYS or perhaps other thiols in the cytoplasm whereupon it is rapidly excreted in a manner similar to that described for cystine. There is then a window of opportunity for CYS to gain access to cytoplasmic cysteine (thiol) targets, including the small thiol pool (such as glutathione (GSH) or bacilithiol in Gram positive species) or iron-sulfur clusters which might be sensitive to oxidation, or cause damage via Fenton chemistry with intracellular transition metal ion stores (2), and that this window may be wider depending upon the energy status, and substrate availability to the cell. This certainly concurs with studies conducted in our lab comparing glucose containing and glucose-free media, which demonstrated lower MICs in the absence of glucose.
The ability of CYS to sensitise bacteria to oxidative stress by depleting cellular reductants is one mechanism which can explain the broad-spectrum potentiation of different classes of antibiotic which are known to induce ROS formation in bacterial cells, indeed the addition of exogenous catalase raises the MIC of CYS marginally (data not shown), however we believe it is not the only mechanism at work. We can potentiate azithromycin in both resistant Gram positive *S. aureus* and Gram negative *N. gonorrhoeae* where resistance is mediated by very different mechanisms, and azithromycin is a known antioxidant (55) which did not induce ROS formation in our assays. The azithromycin-potentiating activity of CYS against *N. gonorrhoeae* strain NB04916 where mutations in *mtrR* gene and promoters is likely to mediate efflux-driven resistance (56) contrasts with the lack of effect in NB03196, which suggests an ability to inhibit efflux of this antibiotic. Similarly, the antimicrobial action of colistin does not involve ROS formation (57). Multiple effects on metabolism, virulence and biofilm formation also suggest this is not the only mechanism, and a number of potential targets are being investigated.

The antibiofilm formation effects of CYS have been reported previously (18), but here we demonstrate for the first time the inhibition of phenazine production in various type and clinical strains of *P. aeruginosa*. Phenazines, such as pyocyanin, are REDOX active antimicrobial virulence factors which have been demonstrated to have a detrimental effect on host immune defenses (44,45). A previous study examining the function of the *Pseudomonas* quinolone signal (PQS) biosynthesis pathway has identified cysteamine phosphate as a substrate for PqsE, a protein which plays an incompletely defined role in the PQS quorum sensing regulation of a range of virulence factors, including phenazine production (58). It is not known at this stage if CYS modification of this target, or via competitive inhibition, is responsible...
for the reduction in pigment production seen after sub MIC exposure but it is being investigated. It is interesting that CYS also inhibited pyomelanin synthesis and/or secretion in *B. cenocepacia* (Fig. S2). Pigment production in *B. cenocepacia* is also implicated in resistance to host mediated oxidative defense and intracellular survival (46). The synthesis of this pigment is distinct from phenazine synthesis in *P. aeruginosa* though some *P. aeruginosa* strains do produce it, as well as a range of other pathogens, and it is thought to contribute to persistence in CF airways. This pigment is exported from the cell via the ABC-transporter, HatABCDE (62), and retention of pyomelanin within the *B. cenocepacia* pellet again points to inhibition of ATP-driven export.

Although there is still a great deal of work to be done to fully unravel and calibrate the role of CYS in immunity to infection *in vivo*, it is important for us now to consider CYS as an endogenous active sulfur species alongside the much better known reactive oxygen and nitrogen species. Our present study highlights the potential for CYS to enhance the antimicrobial activity of therapeutic antibiotics against pathogens for which the emergence of resistance is growing concern and limits current clinical options (59,60). Understanding how the physiological context impacts upon CYS activity will help to inform the choice of target infection in any future development for repurposing CYS as an adjunct for antimicrobial therapy, but our data thus far clearly demonstrates that it has possible benefits for the treatment of pulmonary infections in cystic fibrosis and far beyond.

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|          | CYS (μg/ml) | ciprofloxacin (µg/ml) | CTM (μg/ml) | paraquat (µg/ml) | H$_2$O$_2$ (mM) | SNAP (mM) |
|----------|-------------|------------------------|-------------|------------------|----------------|------------|
| **CYS vs. ciprofloxacin** |             |                        |             |                  |                |            |
| MIC alone | 256         | 0.0625                 |             |                  |                |            |
| MIC in combination | 128         | 0.0325                 |             |                  |                |            |
| **CTM vs. ciprofloxacin** |             |                        |             |                  |                |            |
| MIC alone | 1024        | 0.0625                 |             |                  |                |            |
| MIC in combination | 64          | 0.3                    |             |                  |                |            |
| **CYS vs. paraquat** |             |                        |             |                  |                |            |
| MIC alone | 256         | >154.3                 |             |                  |                |            |
| MIC in combination | ≤16         | 77.1                   |             |                  |                |            |
| **CTM vs. paraquat** |             |                        |             |                  |                |            |
| MIC alone | 1024        | >154.3                 |             |                  |                |            |
| MIC in combination | 64          | 0.3                    |             |                  |                |            |
| **CYS vs. H$_2$O$_2$** |             |                        |             |                  |                |            |
| MIC alone | 256         | 2.443                  |             |                  |                |            |
| MIC in combination | 256         | 1.22                   |             |                  |                |            |
| **CTM vs. H$_2$O$_2$** |             |                        |             |                  |                |            |
| MIC alone | 1024        | 2.443                  |             |                  |                |            |
| MIC in combination | 64          | 0.305                  |             |                  |                |            |
| **CYS vs. SNAP** |             |                        |             |                  |                |            |
| MIC alone | 256         | >6                     |             |                  |                |            |
Table 1. CYS and CTM potentiate the antimicrobial activity of ciprofloxacin, reactive oxygen and reactive nitrogen species. The MIC of *P. aeruginosa* PAO1 versus ciprofloxacin, paraquat, H$_2$O$_2$ and SNAP is shown above.

| Minimal media carbon source (5mM) | Median CTM MIC against *P. aeruginosa* PAO1 (µg/ml) at 20 h |
|----------------------------------|--------------------------------------------------|
| Glucose                         | 1024                                             |
| Oxaloacetate                    | 2                                                |
| Succinate                       | 256                                              |
| Citrate                         | 128                                              |

MIC alone

| MIC alone | 1024 | >5 |
| CTM vs. SNAP | CTM (µg/ml) | SNAP (mM) |
|------------|----------|-----------|
| Mic in combination | 128 | 5 |

MIC in combination

| MIC in combination | 64 | 0.3125 |

The MIC of *P. aeruginosa* PAO1 versus ciprofloxacin, paraquat, H$_2$O$_2$ and SNAP is shown above.
Table 2. Antimicrobial activity of CTM against *P. aeruginosa* PAO1 is greatly affected by carbon source. The MIC of CTM against *P. aeruginosa* PAO1 is shown when grown in minimal media using different carbon sources including glucose and selected TCA cycle intermediates.
| Antibiotic | Bacterial species, strain and genotype or phenotype | Median MIC (μg/ml) without CYS (S/I/R) | Median MIC (μg/ml) with CYS (S/I/R) | Percentage reduction in effective dose (%) |
|------------|--------------------------------------------------|---------------------------------------|-------------------------------------|------------------------------------------|
| Colistin   | E. coli NEB® pET-29B                              | 2 (S)                                 | 1 (S)                               | 50                                       |
|           | E. coli NEB® pET-29B mcr-1                        | 8 (R)                                 | 1 (S)                               | 87.5                                     |
|           | E. coli RH14000226 mcr-1                          | 4 (R)                                 | 2 (S)                               | 50                                       |
|           | K. pneumoniae NB01216 bla<sub>NDM-1</sub>, mcr<sup>-1</sup> (70ins IS<sub>5</sub>-like) | 64 (R)                                 | 16 (R)                              | 75                                       |
|           | K. pneumoniae NB02218 bla<sub>KPC-2</sub>, mcr<sup>-1</sup> (7A>T) | 32 (R)                                 | 1 (S)                               | 96.9                                     |
| Azithromycin | S. aureus SACF636 (MRSA)                        | 0.5 (S)                               | 0.25 (S)                            | 50                                       |
|           | S. aureus SACF652 (MRSA)                        | >256 (R)                              | 0.25 (S)                            | >99.8                                    |
|           | S. aureus SACF662 (MRSA)                        | >256 (R)                              | 0.5 (S)                             | >99.8                                    |
|           | S. aureus SACF667 (MRSA)                        | >256 (R)                              | 0.5 (S)                             | >99.8                                    |
|           | S. aureus SACF660 (MSSA)                        | 0.5 (S)                               | 0.25 (S)                            | 50                                       |
|           | S. aureus SACF661 (MSSA)                        | 1 (S)                                 | 0.5 (S)                             | 50                                       |
|           | S. aureus SACF663 (MSSA)                        | 128 (R)                               | 0.25 (S)                            | 99.8                                     |
|           | S. aureus SACF665 (MSSA)                        | 0.5 (S)                               | 0.25 (S)                            | 50                                       |
Table 3. CYS can potentiate colistin and azithromycin *in vitro*. The MIC of colistin and azithromycin with and without CYS against a selection of pathogens is shown above, including the interpretive criteria where possible when used alone and in combination.

| Pathogen                  | MIC (S) | CYS MIC (S) | CYS MIC (%) |
|---------------------------|---------|-------------|-------------|
| S. aureus SACF666 (MSSA) | 1       | 0.5         | 50          |
| S. aureus DSM 11729 (MRSA)| 16      | 1           | 93.8        |
| S. aureus BAA-1717 (MRSA) | 8       | 4           | 50          |
| N. gonorrhoeae NB04916     | 32      | 4           | 87.5        |
| N. gonorrhoeae NB03916     | >256    | >256        | 0           |
Figure 1. CYS and NaNO₂ react to form a new product with absorbance peaks at 333 and 545 nm typical of the S-nitrosothiol, S-nitrosocysteamine. The reaction favours acidic conditions as shown from differences in absorbance at 333 nm above background where (A) 1 mg/ml CYS is reacted with 1 mg/ml NaNO₂ at 37°C in the dark for 1 h in phosphate buffered saline across 6-7.4 pH range. The disulfide CTM did not react with NaNO₂ under the same conditions at the same concentrations. CTM (B) is rapidly converted into thiol by P. aeruginosa PAO1 when 1 x 10⁹ cfu/ml were challenged with 3 mM and grown in glucose minimal media over time as detected in the culture media by DTNB. The addition of 10 µM CCCP did not prevent conversion of CTM to thiol, or export from the cell.
Figure 2. ROS production in *P. aeruginosa* PAO1 as detected by H2DCFDA fluorescence over time in response to CTM (A) and after 2 h challenge with: (B) CTM only; (C) ciprofloxacin only or; (D) selected combinations of both. Columns 1-3 shows CTM only at 64, 256 and 512 μg/ml. Columns 4-6 shows cipro only at 0.5, 1 and 2 μg/ml. Columns 7-9 show CTM at 64 μg/ml with ciprofloxacin at 0.5, 1 and 2 μg/ml. Columns 10-12 show CTM at 256 μg/ml with ciprofloxacin at 0.5, 1 and 2 μg/ml. Columns 13-15 show CTM at 512 μg/ml with ciprofloxacin at 0.5, 1, and 2 μg/ml (n=3). One-way ANOVA with Tukey’s post hoc analysis (ns = not significant, * = p<0.05 ** = p<0.01 *** = p<0.001)
Figure 3. The effects of ciprofloxacin and CYS on microbial load expressed as log10 cfu/g of tissue from (A) neutropenic mouse thigh model experimentally infected with *P. aeruginosa* LES431 and treated with 1. vehicle control; 2. colistin (5 mg/kg); 3. CYS (1.25 mg/kg); 4. ciprofloxacin (15 mg/kg) and; 5. ciprofloxacin + CYS (n=5 animals per treatment group). The effects of d.p.i. administered tobramycin and CYS (B) expressed as log10 cfu/g tissue in the neutropenic acute lung model of infection with *P. aeruginosa* ATCC 27853 treated with 1. vehicle only (3 mg mannitol); 2. 3 mg of 5% CYS; 3. 3 mg 10% CYS; 4. 0.188 mg tobramycin in lactose (4.5 mg total); 5. 5% CYS + tobramycin and; 6. 10% CYS + tobramycin (n = 8 animals per treatment group). One-way ANOVA with Tukey's post hoc analysis (ns = not significant, * = p<0.05 ** = p<0.01 *** = p<0.001).
Figure 4. The incorporation of 512 µg/ml CTM in CA MHA (A) removes a sub-population of resistant cells of *S. aureus* DSM 11729 cultured with azithromycin Etest strips (control plate on left). (B) Increased zone of clearance of *N. gonorrhoeae* strain NB04916 can be observed surrounding azithromycin Etest strips on plates with increasing concentrations of cysteamine (1. 0 µg/ml; 2. 128 µg/ml; 3. 256 µg/ml; 4. 512 µg/ml) incorporated into GC (+ Vitox supplement) agar, whereas there is no apparent difference for strain NB03916.
Figure 5. The REDOX dependent anti-infective mechanisms of action for cysteamine/cystamine. 1. Cysteamine can be supplied therapeutically or produced endogenously through the action of vanin-1 pantetheinase. Cysteamine itself doesn’t pass the bacterial cytoplasmic membrane of rapidly dividing cells (2.), but is reported to have impacts upon host immunity to infection and autophagy (3.). Cysteamine can react (reversibly) with susceptible cysteine residues in a process termed cysteaminylation (4.), and we demonstrate it can form adducts with reactive nitrogen species, possibly forming S-nitrosocysteamine in mildly acidic environments (5.). Cysteamine will readily form the disulfide cystamine in the presence of oxygen (6.) in a temperature-dependent manner, and at millimolar concentrations will rapidly generate ROS in the presence of transition metal ions (7.). Cystamine itself may interact with unknown periplasmic targets in Gram negative cells and can enter the bacterial cell (8.) via an unknown mechanism, where it generates ROS, and interacts with susceptible intracellular targets leading to dysregulation of small thiol pools, and metabolism (9.), disrupting pigment production or export. Reduced thiols, probably including cysteamine are exported via an unknown mechanism (10.).
