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"On Demand" Redox Buffering by H_2S Contributes to Antibiotic Resistance Revealed by a Bacteria-Specific H_2S Donor

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Understanding mechanisms of antimicrobial resistance (AMR) will help launch a counter-offensive against human pathogens that threaten our ability to effectively treat common infections. Here, we report bis(4-nitrobenzyl)sulfanes, which are activated by a bacterial enzyme to produce hydrogen sulfide (H_2S) gas. We find that H_2S helps maintain redox homeostasis and protects bacteria against antibiotic-triggered oxidative stress "on demand" through activation of alternate respiratory oxidases and cellular antioxidants. We discovered, a hitherto unknown role for this gas, that chemical inhibition of H_2S biosynthesis reversed antibiotic resistance in multidrug-resistant (MDR) uropathogenic bacteria of patient origin, whereas exposure to the H_2S donor restored drug tolerance. Together, our study provides greater insight into the dynamic defence mechanisms of this gas, modes of antibiotic action as well as resistance while progressing towards new pharmacological targets to address AMR.

Introduction

Maintenance of redox homeostasis is fundamental to cellular growth and survival. Induction of dysfunctional redox environment is a common mechanism used against pathogens by immune cells.[1] In the past several decades, it has been well established that gases such as hydrogen sulfide (H_2S) and nitric oxide (NO) affect cellular redox balance.[2] Bacteria-derived H_2S through microbiota contribute significantly to repair mechanisms and are vital for the health of the gastrointestinal tract.[3] Bacterial H_2S is also implicated as a cytoprotective agent against antibiotic-induced stress resulting in antibiotic tolerance.[4] Oxidant remediation by bacterial H_2S is operational, but precise mechanisms of protection remain to be completely elucidated.[5] Mapping out these cytoprotective mechanisms will help progress towards new strategies to combat the growing threat of antimicrobial resistance (AMR).[6]

Due to the dwindling arsenal of antibiotics, AMR is possibly the biggest problem that this generation will face in the coming decade. In order to address this complex socioeconomic public health problem, multiple methodologies are necessary including a better understanding of mechanisms of antibiotic action and factors contributing to antibiotic resistance.[7] Here, we systematically investigated the dynamic effects of H_2S in protecting bacteria from antibiotic-induced stress and the role of H_2S in modulating AMR.

Being a gaseous species, reliable detection[8] as well as controlled and site-specific generation of H_2S within cells is fundamental to understanding its biology.[9] Numerous donors of H_2S[3] are in development but none, to our knowledge, distinguish one type of cells over others.[10] Enzymes as metabolic triggers for activation of donors offer distinct advantages as they will facilitate localization of H_2S. A H_2S generating functional group is tethered to a substrate for an enzyme that is normally expressed in cells of interest (Figure 1a). Upon entry into cells, metabolism by the target enzyme frees up the active H_2S generator inside cells thus achieving localized delivery. Recently, two esterase activated H_2S donors were reported with potential for wide applicability in cellular studies (Figure 1b).[11] However, generating H_2S specifically in certain cells over others might be problematic when using esterase as a trigger. We chose *E. coli* nitroreductase (NTR), an oxygen-insensitive bacterial enzyme that is frequently expressed in bacteria but not in mammalian cells.[12] Geminal dithiols are reported to undergo hydrolysis in buffer to produce H_2S;[13] 4-nitroaryl groups are known substrates for NTR. We hence designed **1**, a NTR-activated H_2S donor (Figure 1b).

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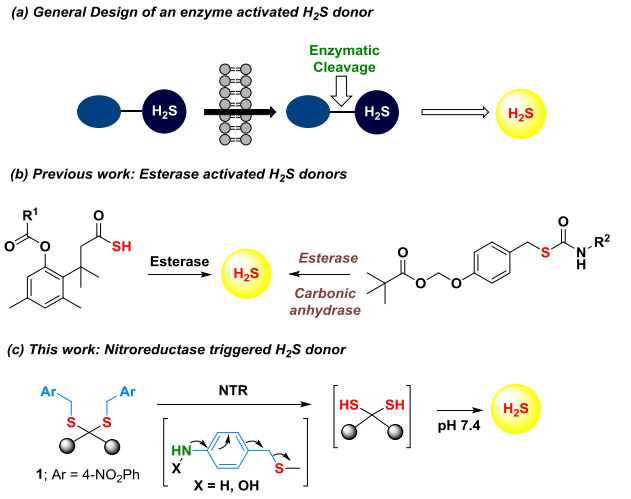
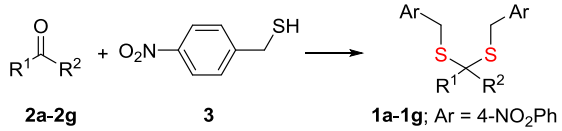


Figure 1. (a) General design of an enzyme activated H_2S donor. (b) Esterase sensitive H_2S donor; (b) Esterase activated H_2S and COS/H_2S donor; R^1 can be a non-steroidal anti-inflammatory drug (NSAID) while R^2 is an aryl group or benzyl (c) Bacterial enzyme nitroreductase (NTR) activated H_2S donor and inset contains compounds synthesized in this study.

Results and discussion

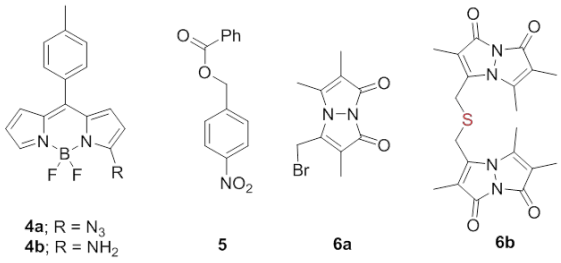
Synthesis of **1** is achieved by the reaction of variety of ketones (**2**) with 4-nitrobenzyl thiol (**3**) (Table 1). A H_2S-sensitive dye BODIPY-azide **4a** was employed to detect H_2S. BODIPY-azide is known to be reduced by H_2S to produce a fluorescent amine **4b**.[14] Compounds **1a**-**1g** were independently exposed to NTR and all compounds were found to generate H_2S under these conditions (Figure 2a). The cyclopentyl derivative **1c** was found to be slightly better than the cohort of donors tested and this compound was used for further studies.

Table 1. Synthesis of **1a**-**1g**



Entry	R^1	R^2	Ketone	Prod	Yield %
1	CH_3	CH_3	**2a**	**1a**	80
2	Et	Et	**2b**	**1b**	78
3	Cyclopentyl		**2c**	**1c**	70
4	Cyclohexyl		**2d**	**1d**	71
5	Ph	CH_3	**2e**	**1e**	60
6	4-FPh	CH_3	**2f**	**1f**	42
7	Thiophene	CH_3	**2g**	**1g**	38

A monobromobimane (mBBr, **6a**) assay (with some modifications) was next used to confirm the production of H_2S.[15] The electrophile mBBr reacts with sulfide anion to produce a thioether which contains two bimane units (**6b**). When **6a** was treated with Na_2S in pH 7.4 buffer, as expected, **6b** was formed (Figure 2b). Under similar conditions, when **1c** was co-incubated with **6a** and NTR, we found evidence for the formation of **6b** again supporting **1c** as a source of H_2S when incubated with NTR (Figure 2b). Next, NBD-Fluorescein, a H_2S sensitive dye was synthesized and was incubated in the presence of **1c** and NTR.[16] Again, we find a distinct increase in fluorescence attributable to H_2S generation (Figure S1, ESI). Thus, the formation of H_2S was validated by several independent assays suggesting that this compound is a reliable donor of H_2S.



The H_2S donor **1c** was able to maintain elevated levels of H_2S over 45 min (Figure 2c). The formation of a hydroxylamino- or amino-aryl derivative, (Figure 1b, Scheme S2, ESI) which self-immolates to generate a geminal dithiol is likely. This geminal dithiol should hydrolyze to produce H_2S and a ketone.[13] Accordingly, when **1e** was incubated in buffer in the presence of Zn and ammonium formate, acetophenone is formed supporting the proposed mechanism (Figure S2, ESI).

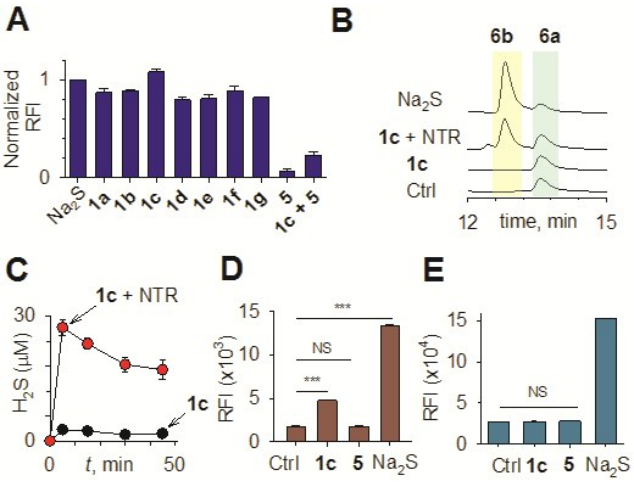


Figure 2. (a) Hydrogen sulfide generated during incubation of **1a**-**1g** (50 μM) with NTR in HEPES buffer pH 7.4 was estimated using a BODIPY-based sensor. (b) A monobromobimane (**6a**) method for estimation of hydrogen sulfide. The formation of thioether **6b** supports the intermediacy of H_2S. (c) Time course of H_2S generation during incubation of **1c** in pH 7.4 buffer alone and in the presence of NTR was assessed by BODIPY-based sensor **4a**. (d) Flow cytometry analysis of intrabacterial H_2S generation in *E. coli* using the probe **4a**. **1c** was incubated for 20 minutes*** *p*-value=0.0001; NS = not significant. (e) Flow cytometry analysis of intracellular H_2S generation in THP-1 cells. **1c** was incubated for 20 minutes (100 μM) and **4a** was used for H_2S detection NS = not significant.

Having established that **1c** generated H_2S in cell-free conditions in the presence of a bacterial enzyme, the ability of this compound to permeate cells to be metabolized by NTR to generate H_2S was evaluated. First, a HPLC-based method was used: *E. coli* cells were incubated with the H_2S-sensitive dye **4a** and **1c**. Cells were lysed and HPLC analysis of the lysate revealed the formation of **4b** supporting H_2S generation (Figure S3, ESI); a similar result was recorded for a variety of bacteria supporting the broad utility of this donor. Next, flow

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cytometry analysis revealed the generation of H_2S inside intact bacterial cells when treated with **1c** supporting the ability of this donor to enhance H_2S levels in live bacterial cells (Figure 2d). 4-Nitrobenzylbenzoate **5** (a likely competitive inhibitor) was next synthesized using a reported method.[17] This compound was by itself incapable of generating H_2S in the presence of NTR and also inhibited H_2S generation from **1c** (Figure 2a). The negative control **5** was did not generate H_2S within bacteria suggesting that the metabolism of the nitro group does not contribute to H_2S production (Figure 2d). The H_2S donor **1c** was ineffective in generating H_2S in *E. coli* strains lacking NTR (Figure S4, SI), confirming NTR-specificity *in vivo*. As NTR is predominantly produced in bacteria but not in mammalian cells, the H_2S donor **1c** must selectively enhance H_2S in bacteria. Human monocytic cells (THP-1) were treated with **1c** and H_2S levels were assessed by flow cytometry. Here, we find that while Na_2S was capable of enhancing H_2S levels within THP-1 cells, **1c** remained completely ineffective (Figure 2e). Thus, **1c** was selective in its ability to enhance H_2S in bacteria over mammalian cells (Figure S5, ESI). To our knowledge, this is the first example of a H_2S donor with species selectivity. Thus, this study lays the foundation for novel methodologies for site-specific enhancement of H_2S using this class of H_2S donors. For example, selectively enhancing H_2S in microbiota to study effects of this gas on colorectal cancer and other similar pathophysiologies is possible.[2]

To begin understanding the mechanisms of H_2S-mediated oxidation remediation, we used a non-invasive redox biosensor (roGFP2) and assessed dynamic changes in the cytoplasmic redox potential of *E. coli* in response to oxidative stress.[18] An increase in 405/488 nm excitation ratio of roGFP2 indicates oxidative stress, while reverse suggests reductive changes.[18b] We first exposed *E. coli* expressing roGFP2 to **1c** and measured the roGFP2 biosensor response and no significant changes in 405/488 ratio was observed (Figure 3a). Hence, H_2S alone does not affect ambient redox-potential of *E. coli.* In contrast, oxidative challenge with H_2O_2, a reactive oxygen species (ROS), rapidly increased the 405/488 ratio, and pre-treatment with **1c** significantly reversed this response (Figure 3a). However, pre-treatment with either Na_2S or **5** had no influence on H_2O_2-induced oxidative changes in biosensor response (Figure S6, ESI).

Importantly, protective influence of **1c** on intrabacterial redox potential translated into significantly higher resistance displayed by *E. coli* against bactericidal concentrations of H_2O_2 (Figure 3b). Interestingly, H_2S itself did not have any significant effect on growth of *E. coli*. Thus, intervention by H_2S occurs when other endogenous oxidant-remediation systems are overwhelmed. This property is consistent with the lower reduction potential of H_2S when compared with major cellular thiols such as glutathione[19] and thus affords H_2S a unique role in cellular redox chemistry. Furthermore, in contrast with other routinely used antioxidants in redox biology such as thiourea and bipyridyl, **1c** does not significantly affect bacterial growth (Figure 3b) suggesting that this tool would be appropriate for studying H_2S-mediated response to dynamic redox alterations during antibiotic-induced stress and lethality.

The emerging model for antibiotic lethality involves the induction of complex redox and metabolic alterations as a consequence of drugs' interaction with their specific targets.[5, 20] Thus, it is important to understand the dynamic effects of H_2S in mitigating antibiotic-induced redox stress.[21] To do this, we exposed *E. coli* to clinically relevant concentrations of bactericidal antibiotic ampicillin (Amp; cell wall targeting) and an oxidative shift was recorded (Figure 3c).[21a, 22] More importantly, pre-treatment with **1c** reduced the degree of oxidative shift induced by Amp, resulting in significant tolerance to antibiotics (Figure 3d). Amp-mediated increase in roGFP2 ratios emerged earlier than the time points at which significant killing was observed, indicating that oxidative stress precedes death, and **1c**-derived H_2S protects bacteria by maintaining cytoplasmic redox potential.

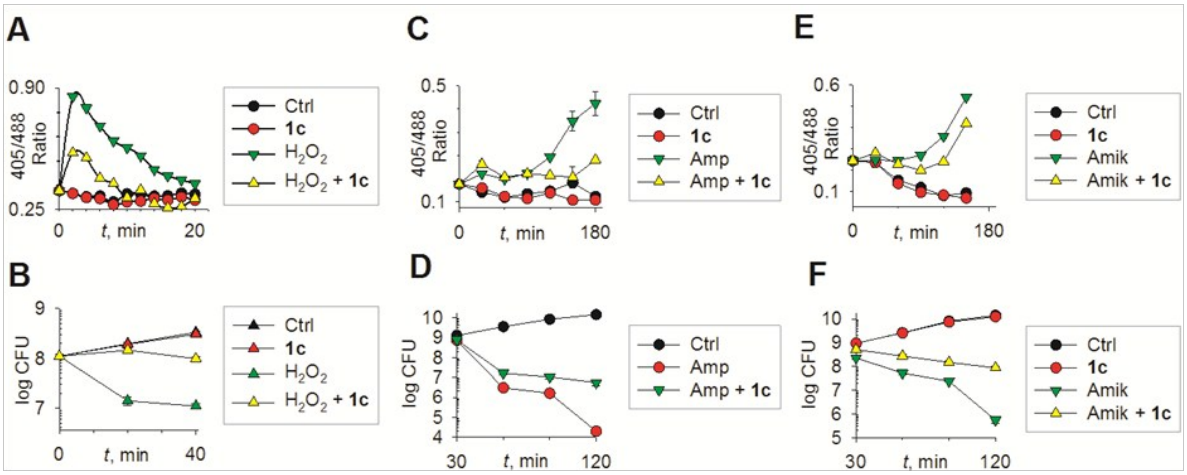

Figure 3. (a) Reduction-oxidation sensitive GFP (roGFP2) was used to measure dynamic changes in cytoplasmic redox potential of *E. coli* upon exposure to: H_2O_2, 1 mM; **1c**, 100 μM; (b) Time-kill analysis of *E. coli* treated with hydrogen peroxide (1 mM) and **1c** (100 μM) during 40 min. (c) Dynamic changes in cytoplasmic redox potential of *E. coli* upon exposure to: Amp, 5 μg/mL; **1c**, 100 μM; (d) Time-kill analysis of *E. coli* treated with Amp (5 μg/mL) and **1c** (100 μM) during 120 min. (e) Dynamic changes in cytoplasmic redox potential of *E. coli* upon exposure to: Amik, 20 μg/mL; **1c**, 100 μM; (f) Time-kill analysis of *E. coli* treated with Amik (20 μg/mL) and **1c** (100 μM) during 120 min.

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Similar results were recorded for Amikacin (Figure 3e-3f), a translation inhibitor, and ciprofloxacin, a replication inhibitor (Figure S7, ESI). Altogether, data demonstrate that elevating endogenous H_2S levels can arrest antibiotics-triggered redox stress and killing.

Mechanisms of H_2S-mediated protection from antibiotic-induced lethality are poorly understood. It has been shown that H_2S elevates cellular antioxidant capacity and suppresses iron load to mitigate antibiotic-linked ROS production.[23] Since sulfide is a potent ligand of copper and heme moieties, H_2S efficiently inhibits aerobic respiration by targeting copper-heme containing cytochrome *bo* oxidase (CyoA).[24] Under these conditions respiration becomes primarily dependent upon less energy efficient cytochrome bd oxidase (CydB).[24] Interestingly, modulation of cytochrome oxidases expression is known to influence antibiotic toxicity.[21b] Therefore, we assessed whether terminal oxidases are important contributory factors in H_2S-mediated antibiotic tolerance. First, quantitative reverse transcription-PCR (qRT-PCR) analysis of *E. coli* cells in the absence or presence of **1c** was conducted (see ESI). A significant down-regulation of the genes encoding CyoA was observed with **1c**-treated bacteria (Figure 4a). The expression of alternate oxidases was however either maintained (cytochrome *bd* oxidase I [*cydB*]) or highly induced (cytochrome *bd* oxidase II [*appY*]) by H_2S (Figure 4a). During growth under low-O_2 tension, *E. coli* down-regulates *cyo* operon and upregulates *cyd* and *app* operons, indicating that H_2S triggers genetic and physiological changes comparable to O_2-limitation.[25] Amp treatment reversed the influence of H_2S on the expression of cytochrome oxidases as *cyoA* and *cydB* transcripts were significantly induced and repressed, respectively, as compared to untreated cells (Figure 4a). The *appY* transcript remained down-regulated in response to Amp. Data suggest that Amp treatment promotes respiration via energetically efficient CyoA, which is consistent with a recent study demonstrating acceleration in aerobic respiration in response to bactericidal antibiotics.[21b]

Having observed divergent effects of H_2S and Amp on cytochrome oxidases gene expression, we next examined the outcome of H_2S and Amp combination on transcription. qRT-PCR analysis of *E. coli* pre-treated with **1c** followed by exposure to Amp showed severely down-regulated expression *cyoA*, whereas expression of *cydB* and *appY* was robustly maintained (Figure 4a) as compared to Amp alone. Thus, maintenance of respiratory flux through cytochrome bd oxidase I/II in response to H_2S treatment may be a key trait that permits adaptation upon subsequent exposure to antibiotics. To examine this possibility, we assessed cell-killing in respiratory mutants lacking either *cyoA* or *cydB*. While **1c**-pretreatment resulted in a significant attenuation of Amp lethality in the case of CyoA mutant (like WT strain), it was completely ineffective in protecting *cydB* mutant (Figure 4b). In LB medium, WT, *cyoA*, and *cydB* strains showed comparable growth profiles in the absence or presence of **1c**, indicating the differences in Amp susceptibility is not a consequence of reduced growth rates. Sustenance of *cydB* expression in response to H_2S-Amp combination, coupled with maintenance of H_2S-mediated antibiotic tolerance in *cyo* mutant (where aerobic respiration is mainly permitted by CydB) but not in *cydB* mutant, suggest that H_2S effect is likely dependent upon *cydB*.[24] Along with its role in respiration, CydB from *E. coli* has been shown to reduce H_2O_2 by displaying catalase and quinol peroxidase activities.[26] Therefore, maintenance of *cydB* expression by H_2S can potentiate antibiotic tolerance by bolstering the bacterial antioxidant capacity. Agreeing to this, *cydB* mutant displayed heightened sensitivity to H_2O_2 as compared to *cyoA* and WT strains (Figure S8, ESI).

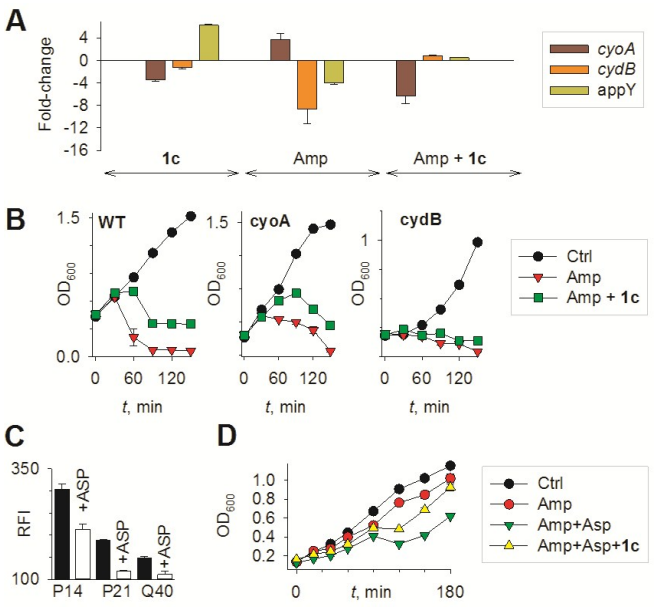

Figure 4. (a) qRT-PCR analysis of *E. coli* cells treated with **1c** alone, Amp alone. The fold-change is with respect to untreated control; **1c** + Amp where fold-change is with respect to Amp-treated cells. Fold change for each transcript was calculated by normalizing expression with the housekeeping 16SrRNA transcript. A 2-fold ($p<0.05$) increase or decrease in expression was considered significant in all cases. (b) Growth curves of *E. coli* treated with Amp (5 μg/mL) with and without **1c** (100 μM); Ctrl is untreated bacteria: Wild-type (WT); cyoA mutant; cydB mutant. (c) H_2S levels measured using the H_2S-sensitive dye **4a** in the presence of ASP, a biosynthesis inhibitor of H_2S generation [d] Bacterial growth measurements: Amp (100 μg/mL); Asp (4 mM) and **1c** (100 μM) were used in this study

In addition, consistent with previous results,[4d] we observed that H_2S is ineffective in protecting cells lacking cytoplasmic catalase and peroxidase (KatA, KatE, ahpCF; HPX-) from Amp-

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induced lethality (Figure S9, ESI). Altogether, data implicate a central role for cytochrome bd oxidase and oxidant-remediation mechanisms in diminishing the effectiveness of antibiotic by H_2S (Figure 5).[27]

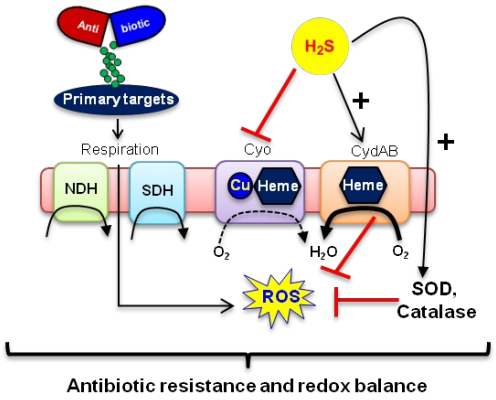

Figure 5. Evolving model for H_2S mediated antibiotic tolerance. Bactericidal antibiotics alters respiration and metabolism to elevate the production of endogenous ROS. Based on our findings, we propose that bacterial H_2S provides tolerance to antibiotics by two mechanisms; (i) down-regulation of energy-efficient cytochrome bo oxidase (Cyo) and induction of less-energy efficient cytochrome bd oxidase I/II (CydAB/AppBC) to maintain respiratory flux and redox balance, and (ii) augmentation of antioxidant capacity by elevating catalase and superoxide dismutase (SOD) activities. NDH: NADH dehydrogenase and SDH: Succinate Dehydrogenase.

Lastly, in order to examine if elevated endogenous H_2S levels are associated with drug resistance in the physiological context of human infections, we measured the intracellular H_2S levels of several multidrug-resistant (MDR) *E. coli* strains isolated from patients (Table S2, ESI for resistance profile) suffering from urinary tract infections (UTI). The endogenous H_2S levels were considerably higher than WT indicating a possible functional role for H_2S in antibiotic resistance (Figure S10, SI).[28] In the presence of a well-established 3-mercaptopyrvuate sulfurtransferase (3-MST) inhibitor (aspartate, Asp), we find that H_2S levels were significantly diminished (Figure 4c).[4d] To understand the functional relevance of endogenous H_2S levels in drug resistance, we monitored P14 strain's resistance to Amp. We find that pre-treatment with Asp efficiently inhibits the growth in response to Amp (Figure 4d). More-importantly, co-treatment with Asp and **1c**, significantly restored resistance to Amp in the P14 strain (Figure 4d). Altogether, these findings revealed a previously unknown contribution of H_2S in cooperating with genetic mechanisms of antibiotic resistance (Figure 5). Further work is needed to examine H_2S-mediated mechanisms contributing to the emergence of drug-resistance in clinical strains.[5, 28] Among the major infectious diseases, UTI affects millions and is further complicated by conditions such as diabetes. *E. coli* has now become resistant to most major classes of antibiotics and therefore there is an urgent need to develop new therapeutics. Recently, Berkowitz and co-workers have developed a CBS inhibitor that helps prevent the deleterious effects of enhanced H_2S such as neuronal cell death during episodes of stroke.[29] The inhibitors developed in their study showed a marked diminution in neuronal cell death when compared with untreated control. It is likely that inhibitors of 3-MST may find similar use in sensitizing resistant pathogens.[30] Our results provide a sound pharmacological basis for design of inhibitors of biosynthesis of H_2S as a possible adjuvant.[29-31] Furthermore, we identified critical aspects of bacterial physiology that could be exploited as part of new potentiation strategies. For examples, targeting antioxidant enzymes and alternate respiratory complexes (Cyd/App) is likely to enhance the killing potential of antibiotics. A combination of molecules/drugs targeting H_2S, antioxidants, and respiration could have a remarkable impact on drug-resistance and clinical outcomes.

Conclusions

In summary, we report a new H_2S donor that reliably and selectively enhances H_2S within bacteria. An application of our new tool clearly revealed that H_2S is a key player in the maintenance of intracellular redox balance of bacteria to counteract lethal degree of oxidative stress induced by antibiotics and the critical role that H_2S played in modulating drug resistance. Antibiotic resistance is emerging as possibly the biggest global health challenge for this generation. Therefore understanding pathogen defence mechanisms and their consequences in drug resistance is critical. From the evolutionary perspective, H_2S generating enzymes are prevalent in most sequenced bacterial genomes including environmental bacteria, indicating a naturally conserved role of H_2S in ensuring survival. It is likely that H_2S producing capability is under the selective pressure in diverse environmental bacteria owing to antimicrobials secreted by other bacteria and fungi inhabiting the same niche. Our study presents significant advances towards a complete understanding of antibiotic-induced stress and cytoprotective mechanisms of H_2S.

Acknowledgements

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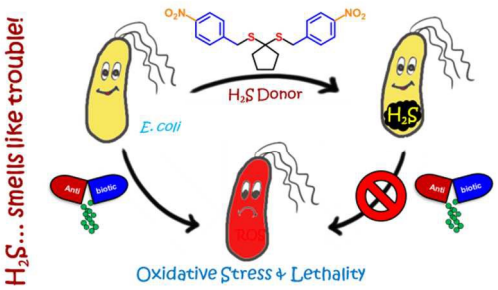

Selective enhancement of hydrogen sulfide in bacteria reveals a key role for this gas in mediating antibiotic resistance