Unraveling the Physiological Complexities of Antibiotic Lethality

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Abstract

We face an impending crisis in our ability to treat infectious disease brought about by the emergence of antibiotic-resistant pathogens and a decline in the development of new antibiotics. Urgent action is needed. This review focuses on a less well-understood aspect of antibiotic action: the complex metabolic events that occur subsequent to the interaction of antibiotics with their molecular targets and play roles in antibiotic lethality. Independent lines of evidence from studies of the action of bactericidal antibiotics on diverse bacteria collectively suggest that the initial interactions of drugs with their targets cannot fully account for the antibiotic lethality and that these interactions elicit the production of reactive oxidants including reactive oxygen species that contribute to bacterial cell death. Recent challenges to this concept are considered in the context of the broader literature of this emerging area of research. Possible ways that this new knowledge might be exploited to improve antibiotic therapy are also considered.

INTRODUCTION

The alarming increase in antibiotic-resistant bacterial pathogens, coupled with the concomitant decline in new antibiotics under development, poses an impending threat to global human health (1–8). It is therefore critically important that we expand our conceptual understanding of how antibiotics act and exploit these new insights to enhance our ability to treat bacterial infections. The molecular targets of the major classes of bactericidal antibiotics have been studied intensively: β-lactams interfere with cell wall biosynthesis; fluoroquinolones trap gyrase and topoisomerase in covalent complexes with DNA ends; and aminoglycosides interfere with proper ribosome function, resulting in mistranslation (9). However, the subsequent physiological causes of antibiotic-induced cell death are less well understood (10).

Over the past decade, numerous researchers studying antibiotic effects on a wide range of bacteria have revealed additional complexity, providing evidence that there is a component of antibiotic lethality that cannot be accounted for by considering only their traditionally studied mechanisms of action. Collectively, these studies suggest that this other component of antibiotic killing results from metabolic perturbations (downstream of antibiotic-target interactions) that generate reactive oxidants including reactive oxygen species (ROS), which contribute to cell death. This additional contribution to antibiotic lethality is most prominent at lower lethal antibiotic concentrations and at earlier time points after antibiotic exposure, issues of clinical relevance. In the first comprehensive review of this emerging area of research, we summarize and discuss the body of diverse evidence that has supported the existence of an oxidative component to antibiotic lethality and begun to reveal its complexity. We also consider recent papers (11–13) that have challenged the existence of this oxidative component of bactericidal antibiotic action, placing them in the context of the broader literature on this subject. Finally, we discuss work that has attempted to exploit aspects of the ROS phenotype to enhance the killing efficacy of antibiotics.

EARLY EVIDENCE THAT REACTIVE OXYGEN SPECIES INDUCTION CONTRIBUTES TO ANTIBIOTIC LETHALITY

Analysis of the SoxR regulon led Greenberg et al. (14) in 1990 to hypothesize that the interaction of an antibiotic with its cellular target could generate ROS, but direct evidence supporting this hypothesis did not begin to appear until a decade later. Arriaga-Alba et al. (15), based on their analysis of beta-carotene-mediated protection from fluoroquinolone-induced mutagenesis in *Salmonella typhimurium*, were the first to specifically implicate oxidative stress induction as a component of the mode of action of an antibiotic. In 2002, Becerra & Albesa (16) measured antibiotic-induced intracellular accumulation of reduced nitroblue tetrazolium dye as well as lucigenin and luminol chemiluminescence to test whether oxidative stress is induced by antibiotics and contributes causally to drug lethality. Their observations that fluoroquinolone-sensitive clinical isolates of *Staphylococcus aureus* experienced increased oxidative stress following treatment with ciprofloxacin compared to fluoroquinolone-resistant isolates led the authors to propose that ROS may participate in the action of antibiotics (16).

This hypothesis was supported and broadened by transcriptional profiling studies. Utaida et al. (17) showed that treatment of *S. aureus* with three antibiotics affecting cell wall biosynthesis increased the expression of genes previously identified as markers of oxidative stress (18), leading them to propose that treatment with such antibiotics induces oxidative stress. Waddell et al. (19) observed that each of several antibiotics they tested on *Mycobacterium tuberculosis* induced markers of oxidative stress, and they identified a common set of genes induced by at least three drugs,

including *ahpC* and *recA* as well as the tricarboxylic acid (TCA) cycle metabolism–associated genes *gltAI* and *icl*. Albesa et al. (20) provided additional evidence for a relationship between antibiotic susceptibility and the production of oxidative stress by showing that *S. aureus*, *Escherichia coli*, and *Enterococcus faecalis* sensitive to ciprofloxacin exhibited oxidative stress when they were incubated with the antibiotic, whereas resistant strains did not. They also showed that *Pseudomonas aeruginosa* strains sensitive to ceftazidime and piperacillin similarly exhibited oxidative stress in the presence of these antibiotics, in contrast to resistant strains (20).

Two 2006 studies further advanced the hypothesis that oxidative stress is elicited by antibiotics and contributes to lethality. Becerra et al. (21) reported that guanine nucleotide oxidation and lipid peroxidation induced by ciprofloxacin was enhanced in a *S. aureus* laboratory strain compared to a resistant clinical isolate, whereas Goswami et al. (22) observed that the antioxidants glutathione and ascorbic acid increased the minimum inhibitory concentrations of several fluoroquinolone antibiotics for *E. coli* and reduced ciprofloxacin sensitivity in disc diffusion assays. Genetic evidence that reactive oxidants including ROS contribute causally to lethality elicited by ciprofloxacin was provided by the increased sensitivity of *katG ahpCF* and *katE katG ahpCF* strains to killing by ciprofloxacin (22).

The complexity of the physiological responses elicited by fluoroquinolone antibiotics interacting with their target(s) and their relationship to ROS production was underscored in an important study by Malik et al. (23), who showed for *E. coli* that the lethality of several fluoroquinolones having differing target specificities (gyrase and/or TopoIV) and affinities could be differentially affected by oxygen availability. These findings extended earlier studies by Smith and colleagues that demonstrated the importance of oxygen availability in quinolone lethality (24, 25).

SYSTEMS-LEVEL ANALYSES OF ANTIBIOTIC-INDUCED PHYSIOLOGICAL ALTERATIONS

Systems biology aims to describe how complex behaviors arise from the interactions between biological system components and how such behaviors may be altered by environmental conditions or perturbations (26). An early advocate for the use of systems biology approaches to address the complexities of antibiotic lethality was Hancock, who, along with Brazas (27), reported in 2005 that gene expression signatures of drug treatment included genes specifically associated with drug-target interactions, as well as indirectly affected genes whose expression reflected cellular attempts to bypass or compensate for homeostatic changes induced by antibiotics. They noted that the indirectly affected genes are likely to be commonly activated by diverse drug classes and may ultimately contribute to antibiotic lethality.

In 2007, Dwyer et al. (28) took a systems-level approach toward elucidating the molecular mechanisms contributing to cell death by DNA gyrase inhibition, using microarrays to compare the responses of *E. coli* to treatment with norfloxacin or expression of the peptide toxin CcdB (29), which similarly poisons the gyrase-DNA complex (30). This analysis led to the discovery that gyrase inhibition triggers significant alterations to oxidative stress, iron uptake and utilization, and iron-sulfur cluster synthesis (28). These authors hypothesized that these changes were indicative of iron misregulation, which could promote the formation of hydroxyl radicals. Experiments using the fluorescent reporter dye 3′-(*p*-hydroxyphenyl) fluorescein (HPF), which has high in vitro specificity for hydroxyl radicals (31), showed that an increase in HPF fluorescence accompanied gyrase inhibition (28). HPF fluorescence could be attenuated by addition of the iron chelator o-phenanthroline, first demonstrated by Imlay et al. (32) to reduce the in vivo effects of Fenton chemistry [in which ferrous iron(II) reacts with H_2O_2 to generate hydroxyl radical species] and thus H_2O_2 lethality. The finding that o-phenanthroline reduced norfloxacin- or CcdB-induced

killing led to the conclusion that ROS causatively contribute to gyrase inhibitor lethality (28).

The use of promoter-reporter constructs that indicate the activation of the SoxRS superoxide stress regulon (33), the Fur iron uptake regulon (34), and the *iscRUSA* iron sulfur cluster synthesis operon (35, 36) revealed that norfloxacin or CcdB induced superoxide stress and affected iron homeostasis via Fur and IscR (28). *fur* and *iscS* mutants were killed more slowly and exhibited modestly increased survival, whereas *iscR*, *iscU*, and *iscA* mutants exhibited a delay before killing began. Treatment of *fur* or *iscS* mutants with norfloxacin also resulted in lower HPF fluorescence compared to wild type. To account for these observations, it was proposed that hydroxyl radical production, supported by superoxide-mediated Fe-S cluster damage and iron misregulation, contributes to the lethal effects of gyrase inhibition.

These findings led to the hypothesis that other bactericidal antibiotics, regardless of their diverse, specific targets, may induce ROS production that contributes to drug lethality. To test this hypothesis, Kohanski et al. (37) first used HPF fluorescence as an indicator of oxidative stress and found that bactericidal antibiotics (β-lactams, fluoroquinolones, and aminoglycosides) induced an increase in HPF signal intensity in *E. coli* and *S. aureus*. It was also shown that killing by bactericidal antibiotics could be reduced by addition of the iron chelator 2,2′-dipyridyl [also used by Imlay et al. (32) to reduce the effects of Fenton chemistry in vivo] or the ROS quencher thiourea, or by deletion of *iscS*; these effects were correlated with changes in HPF fluorescence (37). The observation that *recA* mutants also displayed increased sensitivity to the bactericidal antibiotics suggested that the contribution of ROS to drug lethality involves damage to DNA that can be ameliorated by RecA-dependent mechanisms.

Gene expression analyses identified nicotinamide adenine dinucleotide (NADH)-coupled electron transport as the key upregulated pathway among the bactericidal antibiotic treatments (37). Notably, deletion of the gene encoding isocitrate dehydrogenase, *icdA*, which catalyzes the first of three NADH-producing reactions, reduced antibiotic lethality (37), consistent with a decades-old observation that *icdA* mutants exhibit resistance to nalidixic acid (38). Bactericidal antibiotic treatments were also shown to induce a transient alteration in the NADH/NAD^+ ratio (37). Consistent with this result, Akhova & Tkachenko (39) have recently shown that a transient increase in the ratio of adenosine triphosphate to adenosine diphosphate (ATP/ADP), accompanied by heightened oxygen consumption and OxyR and SoxR activation, is induced by bactericidal antibiotics and correlates with lethality. Together, these data support the hypothesis that bactericidal antibiotics induce metabolic changes that alter the cellular redox state.

An important study by Wang & Zhao (40) provided compelling genetic evidence that ROS contribute to the lethality of β-lactams, fluoroquinolones, and aminoglycosides. A *sodA sodB* double mutant was shown to be less sensitive to killing by all three classes of antibiotics, consistent with superoxide dismutases accelerating the conversion of superoxide to hydrogen peroxide, the substrate for Fenton chemistry (40). Moreover, the lethal action of norfloxacin was increased 10–100-fold by a *katG* single mutation or a *katG katE* double mutation, whereas an *ahpC* mutation increased the sensitivity to killing by ampicillin and kanamycin. These findings are consistent with ampicillin and kanamycin causing a lower accumulation of peroxide than norfloxacin, which would result in AhpCF being the primary scavenger for H_2O_2 (40) because of its lower K_m (41). Mosel et al. (42) have recently shown that pretreatment of *E. coli* with subinhibitory concentrations of superoxide-generating redox cycling drugs confers protection against fluoroquinolones, β-lactams, and aminoglycosides, suggesting that a moderate increase in superoxide triggers protective pathways against lethal stress. The authors' conclusion that ROS can have protective as well as lethal effects during antibiotic stress led them to underscore the point that the experimental conditions and choice of antibiotic and organism can critically influence the obtained results.

Systems-level analyses involving genome-wide screening methods have also been used to study how antibiotics achieve lethality. Girgis et al. (43), for example, screened a transposon mutant library for resistance-conferring mutations under antibiotic selection. Following selection with a range of aminoglycosides, the authors observed that two-thirds (48 of 73) of the transposon insertions beneficial to survival across the drugs tested were in genes associated with the electron transport chain and oxidative phosphorylation, including the iron-sulfur cluster synthesis regulator *iscR*. Such mutations could serve to reduce the respiratory enzyme pool involved in potential ROS-generating reactions or limit NADH consumption and flux through the electron transport chain, thereby constraining ROS production following aminoglycoside treatment. Liu et al. (45) generated sensitivity phenotype profiles for 22 antibiotics by screening the Keio collection (44) of nonessential, single-gene knockouts. They found that a knockout of *recA*, *recB*, or *recC* resulted in increased sensitivity to multiple bactericidal antibiotics (including β-lactams, fluoroquinolones, and aminoglycosides), suggesting that ROS-mediated DNA double-strand breaks (DSBs) contribute to antibiotic lethality.

ANTIBIOTIC LETHALITY AND TWO-COMPONENT SYSTEMS

To identify molecular mechanisms underlying the ROS component of aminoglycoside lethality, Kohanski et al. (46) followed up on their 2007 paper by assessing the factors involved in the cellular response to protein mistranslation that may contribute to oxidative stress. Using gene expression measurements and network analyses, the authors identified the electron transport chain activated by the two-component regulator ArcA and the misfolded protein networks activated by heat shock sigma factor RpoH as the most significantly enriched response pathways. Notably, aminoglycoside lethality was enhanced by deletion of genes involved in the Sec protein translocation system (47), but mutants of the two-component sensor kinases ArcA or CpxA [which respond to envelope stress (48)] were killed markedly more slowly. These data correlated with changes in oxidative stress and membrane potential, as well as measurements of ArcAB- and CpxAR-regulated gene expression (46). Interestingly, deletion of *arcA* or *cpxA* also protected from ampicillin or norfloxacin at low but not high concentrations.

Kohanski et al. (46) further showed that double mutants of Sec translocation system genes and either *arcA* or *cpxA* were killed at a markedly reduced rate. It was therefore proposed that accumulation of mistranslated membrane proteins activates the envelope stress response mediated by CpxAR, in turn activating metabolic changes (and thus oxidative stress) via ArcA and TCA cycle activity because of cross-talk between CpxA and ArcA. Oxidative stress-mediated protein carbonylation has been associated with protein mistranslation (49), and the potential for cross-talk between CpxA and ArcA has been previously proposed (50, 51). As such, these findings suggested that the ROS component of aminoglycoside toxicity arises through envelope stress–mediated activation of central carbon metabolism caused by misfolded protein translocation.

Mahoney & Silhavy (52) concluded that, because the CpxA sensor is a phosphatase as well as a histidine kinase (53), the resistance of a *ΔcpxA* mutant to aminoglycosides is due to increased expression of the Cpx regulon [via unrestrained phosphorylation of the CpxR response regulator by small molecule phosphate donors or heterologous kinases (52)] rather than by its lack of expression, as previously assumed (46). A *ΔcpxA* mutation could still protect cells by interfering with the production of ROS if CpxR upregulates a negative modulator of ROS production or, alternatively, it could upregulate protective functions such as proteases, as suggested by Mahoney & Silhavy. The loss of any individual member of the published Cpx regulon did not affect resistance to aminoglycosides, leading to the suggestion that the functions of multiple genes are needed for protection (52).

However, a subsequent study by Raivio et al. (54) showed that loss of previously unrecognized members of the Cpx regulon affecting aerobic respiration, metal binding, or iron transport did provide protection against aminoglycosides. The resistance of a *ΔcpxA* mutant could also involve some other gene that is potentially regulated by the still incompletely understood Cpx system (55), such as *yibE*, which restricts stress-stimulated ROS accumulation (56). The suggestion that the Cpx regulon must be expressed prior to antibiotic exposure to keep toxic compounds from reaching lethal levels (52) is compatible with the suggestion of Foti et al. (57) that the initial mistranslation caused by aminoglycosides is subsequently amplified by ROS oxidation of ribonucleoside triphosphates. Additionally, the previously reported partial resistance of exponentially growing cells to killing by fluoroquinolones or β-lactams (46, 56) may not have been observed in the Mahoney & Silhavy study (52) because stationary phase cells were plated on medium containing a drug, an assay that does not distinguish between growth inhibition and cell killing.

EVIDENCE FOR A REACTIVE OXYGEN SPECIES–DEPENDENT COMPONENT OF ANTIBIOTIC LETHALITY IN PATHOGENS AND CLINICAL ISOLATES

Since 2007, numerous studies have reported observations supporting a ROS-dependent contribution to the lethal effects of antibiotics in a wide variety of pathogens, offering additional insights into the physiological complexity of the phenomenon. Bacterial genera examined include *Pseudomonas* (58–61), *Mycobacterium* (62–65), *Salmonella* (66, 67), *Listeria* (68), *Staphylococcus* (69, 70), *Streptococcus* (71), and *Acinetobacter* (72–74).

For example, in the case of *P. aeruginosa*, Yeom et al. (58) observed that antibiotics induced the expression of OxyR-regulated genes, which are activated by intracellular H_2O_2, and increased the amount of 8-oxo-guanine in DNA. Antibiotic susceptibility was increased by knockout of *ahpC*, indicating that ROS generation was contributing to drug lethality. The increase in dihydrorhodamine 123 fluorescence upon antibiotic exposure, which is indicative of oxidative stress, could be reduced by addition of an iron chelator that blocks the Fenton reaction. Additional evidence for the involvement of ferrous iron in antibiotic-induced cell death was provided by manipulation of the levels of FprB ferric reductase, which can promote Fenton chemistry by increasing the rate at which oxidized iron is recycled to the reduced ferrous form (58).

The ability of antibiotics to elicit ROS production seems to be especially relevant to the treatment of mycobacterial infections. For example, Kim et al. (62) presented evidence that the activation of host cell autophagy by antibiotics is attributable to *M. tuberculosis* ROS production elicited by antibiotics. Pandey & Rodriguez (63) showed that a ferritin mutant of *M. tuberculosis*, in which iron homeostasis is perturbed, is highly susceptible to killing by antibiotics. Particularly exciting is the recent evidence that ROS generation by antibiotics can contribute to the killing of mycobacterial persisters. Grant et al. (64) found that as little as a 20% drop in dissolved oxygen saturation allows persisters to survive in the presence of the bactericidal antibiotics ciprofloxacin and isoniazid. To test the hypothesis that ROS contributed to antibiotic lethality against persisters, the authors demonstrated not only that a new redox-cycling drug, clofazimine, was efficient at killing persister cells under oxygen-limiting conditions, but also that thiourea could reduce persister cell killing by clofazimine, ciprofloxacin, or isoniazid under aerobic conditions (64).

In *Salmonella enterica* serovar Typhimurium, the ferritin-like Dps iron storage protein (66) and the iron-citrate efflux transporter IceT (67), which help to maintain iron homeostasis and thus limit the potential for Fenton chemistry, have been shown to protect cells against antibiotic lethality. The ferritin-like protein Fri has similarly been found to protect *Listeria monocytogenes* against killing

by β-lactams and to contribute to innate resistance to cephalosporins (68). For *S. aureus*, Páez et al. (69) showed that ciprofloxacin stimulated higher production of ROS in sensitive clinical isolates than in the resistant ones, and Liu et al. (70) demonstrated that inhibitors of ROS reduce the lethality of several antibiotics. Recent results have suggested that increased dosage of ROS defenses can protect the pathogen *Acinetobacter baumannii* (75) against antibiotic lethality (72, 73). Interestingly, polymyxin antibiotics have been reported to induce ROS that contribute to lethality (74).

Streptococcus pneumoniae represents an interesting case, as this pathogen lacks genes encoding a complete electron transport chain or the TCA cycle (76, 77). Nevertheless, Ferrándiz & de la Campa (71) provided evidence of a role for ROS-dependent killing of *S. pneumoniae* by the fluoroquinolone levofloxacin. The pyruvate oxidase enzyme SpxB serves as the main source of endogenous H_2O_2 in *S. pneumoniae*, and iron import is upregulated in response to antibiotic treatment, activating the Fenton reaction (71).

Despite a claim to the contrary (12), drug-resistant clinical isolates exhibiting reduced ROS production in response to antibiotics have been reported; several are discussed above (16, 20, 21, 69). Additionally, increased AhpC expression was observed by Webber et al. (78) in a proteomics screen of *S. enterica* serovar Typhimurium isolates exhibiting resistance to the cell wall synthesis inhibitor triclosan, suggesting that increased peroxidase activity is important among multiple factors conferring high-level resistance to a broader set of cytotoxic compounds. Using several fitness-based assays, Chittezham Thomas et al. (79) showed that β-lactam killing of *Staphylococcus epidermidis*, the most common causative agent of indwelling medical device infection, was accompanied by oxidative stress, and ~76% of 126 resistant clinical isolates surveyed exhibited TCA cycle defects that may limit ROS production, consistent with the hypothesis that TCA cycle–dependent ROS may factor in β-lactam lethality.

ANTIBIOTICS ELICIT THE PRODUCTION OF ORGANIC HYDROPEROXIDES

Independent evidence that antibiotics elicit the production of reactive oxidants including ROS was obtained by Hao et al. (80) during their study of the multi-antibiotic resistance regulator MarR. Using a specific sensor for organic hydroperoxides designed by Zhao et al. (81), they demonstrated that treatment of *E. coli* with the bactericidal antibiotics ampicillin or norfloxacin, but not the bacteriostatic antibiotics tetracycline or chloramphenicol, results in the production of organic hydroperoxides. Following up on their evidence that MarR Cys80 oxidation by copper(II) ions prevents DNA binding by generating disulfide bonds between two MarR dimers, they used a highly specific copper(I) probe to show that antibiotics trigger increases in intracellular copper levels (80).

By screening of norfloxacin-treated *E. coli* strains with deletions for genes encoding cell envelope–residing, bound copper–bearing proteins, Hao et al. (80) identified the cytoplasmic membrane proteins Ndh2 (NADH:ubiquinone oxidoreductase II or type IIA NADH dehydrogenase) and CyoB (subunit I of the cytochrome *bo* terminal oxidase) as candidate proteins responsible for the copper release. A possible mechanism for copper release for Ndh2 was suggested by their observation that the organic hydroperoxide *tert*-butylhydroperoxide (82, 83), which impairs the respiratory chain, results in a greater copper release in a wild-type cell than in a *Δndh* strain. The authors suggested that antibiotic-triggered organic hydroperoxide production causes oxidative impairment of cytoplasmic membrane-bound copper proteins such as Ndh2, which leads to the generation of oxidized copper(II) species that can be sensed by MarR.

NITRIC OXIDE AND HYDROGEN SULFIDE PROTECT AGAINST ANTIBIOTIC LETHALITY BY MITIGATING OXIDATIVE STRESS

The recent discoveries that bacterial synthesis of nitric oxide (NO) and hydrogen sulfide (H_2S) protect bacteria against killing by antibiotics underscores the point that the lethality of antibiotics cannot by explained solely by considering their traditional mechanisms of action. They also provide independent evidence that the production of reactive oxidants including ROS contributes to antibiotic lethality.

Some gram-positive bacteria express nitric oxide synthases (NOSs) that allow them to generate NO intracellularly (84). Gusarov & Nudler (84) showed that within 5 s of administration, too fast for changes in gene expression, exogenous NO protects *Bacillus subtilis* and *S. aureus* against killing by H_2O_2 and that endogenously produced NO could protect from H_2O_2 killing as well. Two mechanisms were implicated: (*a*) reactivation of catalase and (*b*) transient suppression of the Fenton reaction by interfering with the reduction of oxidized cysteine, thereby preventing the reduction of Fe^{+3} to Fe^{+2} necessary for continuing cycles of the Fenton reaction. Bacterial NO production is important for *Bacillus anthracis* to survive macrophage oxidative attack (85). Following up on these observations, Gusarov et al. (86) demonstrated that endogenously produced NO could reduce the lethality of an array of antibiotics, including quinolones, aminoglycosides, β-lactams, and cephalosporins, to gram-positive bacteria and presented evidence that the protection arises in part through alleviation of the oxidative stress imposed by the antibiotics. Notably, loss of the ability to synthesize endogenous NO sensitized methicillin-resistant *Staphylococcus aureus* to ROS and certain antibiotics (87). The recent identification of bacterial NOS inhibitors (88) that enhance the sensitivity of *B. subtilis* to H_2O_2 and the antimicrobial agent acriflavine, which has a ROS-dependent component to its lethality (86), raises the possibility that such inhibitors could serve as therapeutic adjuvants.

Building on the findings that endogenous NO protects certain gram-positive bacteria against antibiotics and oxidative stress, Shatalin et al. (89) provided evidence that endogenously synthesized H_2S serves as a universal defense against antibiotics by mitigating oxidative stress. Almost all bacterial genomes have genes encoding one of two enzymatic pathways for H_2S synthesis (cystathionine β-synthase/cystathionine γ-lyase or 3-mercaptopyruvate sulfur transferase). Genetic inactivation of H_2S synthesis in *B. anthracis*, *P. aeruginosa*, *S. aureus*, and *E. coli* rendered these pathogens highly sensitive to a multitude of antibiotics, an effect suppressed by exogenous H_2S. Various experimental data support the authors' conclusion that H_2S increases bacterial resistance to oxidative stress and antibiotics by two mechanisms: (*a*) suppressing the DNA-damaging Fenton reaction by sequestering Fe^{+2} and (*b*) stimulating the major antioxidant enzymes catalase and superoxide dismutase. In bacteria that can produce both NO and H_2S, the two gases were shown to act synergistically in protecting cells against killing by antibiotics (89). Interestingly, plasmid-borne elements enhancing basal H_2S production were identified in multidrug-resistant patient isolates of *E. coli* nearly 40 years ago (90, 91).

EVIDENCE THAT OXIDIZED NUCLEOTIDE TRIPHOSPHATES CONTRIBUTE TO ANTIBIOTIC LETHALITY

Efforts to understand why overexpression of the *E. coli* Y Family DNA polymerase DinB (DNA pol IV) is lethal unexpectedly led to an independent line of evidence that bactericidal antibiotics elicit the production of ROS and furthermore suggested a mechanistic basis for cell death (57). Suppression of cell killing by anaerobic conditions and other observations indicated that elevated levels of DinB might be lethal because of increased incorporation of oxidized deoxynucleotides

into DNA. Guanine is particularly easily oxidized because of its low redox potential, with 7,8-dihydro-8-oxoguanine (8-oxo-guanine) being a major product (92). DinB can readily use 8-oxo-deoxyguanosine triphosphate (8-oxo-dGTP) as the incoming nucleotide, pairing it with either deoxycytidine or deoxyadenosine (dC or dA), with a preference for dA (57, 93). The hypothesis that incorporation of 8-oxo-dG into nascent DNA might underlie lethality (57) was supported by the demonstration that co-overproduction of MutT, a nucleotide sanitizer that hydrolyzes 8-oxo-dGTP to 8-oxo-deoxyguanosine monophosphate (8-oxo-dGMP) (94), eliminated the cell killing caused by DinB overproduction. Incorporation of 8-oxo-dG nucleotides into the nascent DNA at the replication fork could result in closely spaced 8-oxo-dG nucleotides, which could lead to potentially lethal DSBs (95) because the proximity of individual DNA lesions can alter the cell's ability to repair damage (96, 97). Genetic experiments supported the hypothesis that lethal DSBs are generated by incomplete base excision repair when the MutM and MutY DNA glycosylases act at closely spaced dC:8-oxo-dG and dA:8-oxo-dG pairs (57).

These unanticipated findings raised the possibility that the lethality of bactericidal antibiotics could result, in part, from DNA polymerases using 8-oxo-dGTP and generating closely spaced 8-oxo-dG lesions that can be converted to lethal DSBs by incomplete DNA repair (57). Observations supporting this hypothesis include (*a*) strikingly reduced killing by ampicillin, norfloxacin, and kanamycin when the 8-oxo-dGTP nucleotide sanitizers MutT and RibA were overproduced or when *E. coli*'s DNA polymerase content was genetically altered to reduce 8-oxo-dG incorporation (*dnaE911 ΔdinB ΔumuDC* triple mutant); (*b*) increased resistance of a *ΔmutM ΔmutY* strain to killing by bactericidal antibiotics and increased sensitivity of strains defective in DSB repair (*ΔrecA* and *ΔrecB*); and (*c*) the observation of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining preceding cell death in ampicillin-treated cells and its reduction in a *ΔmutM ΔmutY* strain (57). The subsequent observation that overproduction of the mismatch repair protein MutS, which can recognize A:8-oxo-dG base pairs (98), reduces antibiotic lethality further supported the hypothesis that incorporation of oxidized nucleotides into nascent DNA contributes to antibiotic lethality (99). Because MutT can also sanitize the ribonucleotide 8-oxo-guanosine triphosphate (8-oxo-GTP) (100), incorporation of 8-oxoguanosine into mRNA, rRNA, and tRNA by RNA polymerase (101) may also contribute to antibiotic lethality by reducing the fidelity of protein synthesis, especially in the case of aminoglycosides (57). 8-oxo-GTP might also contribute to lethality by interfering with essential guanosine triphosphatases (GTPases) (57).

These various observations cannot readily be explained by the traditional mechanisms of antibiotic killing and instead support the involvement of ROS production in antibiotic lethality. It is worth noting that nucleotides and DNA are not damaged directly by superoxide or hydrogen peroxide, but rather require Fe^{+2}-mediated Fenton chemistry (102). The pools of deoxyribonucleotide triphosphates and ribonucleoside triphosphates (dNTPs and NTPs) may be at particular risk of oxidation because chelation of Fe^{+2} by their triphosphates, which can be biologically significant (103), favors the localized production of hydroxyl radicals by Fenton chemistry (104). The increase in the rate of the Fenton reaction caused by chelation of Fe^{+2} by an NTP is comparable to that caused by chelation of Fe^{+2} by ethylenediaminetetraacetic acid (EDTA) or nitrilotriacetate (105). Furthermore, the C8 position of purine nucleotides is particularly close to the complexed Fe^{+2} (106) and hence would be favorably disposed to react with a newly generated hydroxyl radical (102) or with a ferryl-oxo radical (107). Interestingly, Tkachenko et al. (108) suggested that the mechanisms by which polyamines reduce oxidative stress in cells exposed to bactericidal antibiotics include competing with Fe^{+2} for binding to DNA. Oxidation events that impart a gain of function to the target can be significant even if they are minor (109), and Pursell et al. (110) have shown that trace amounts of 8-oxo-dGTP in dNTP pools can have significant biological effects. Intriguingly, recent results suggest that an apparently related mechanism of cell death caused by

oxidized dNTPs is widespread in eukaryotes, including humans. For example, cancer cells, which have altered redox regulation that results in ROS production, require the human ortholog of *E. coli* MutT, MTH1, to be viable (111, 112). The effects of overexpressing MTH1 range from suppressing RAS-induced DNA damage and the attendant premature cellular senescence (113, 114) to suppressing neurodegeneration (115, 116) and death of photoreceptor cells in retinitis pigmentosa (117).

EVIDENCE THAT MUTAGENESIS CAUSED BY SUBLETHAL DOSES OF ANTIBIOTICS IS DUE TO OXIDATIVE STRESS

Another independent line of evidence indicating that antibiotics elicit the production of reactive oxidants including ROS grew out of the discovery that exposure of bacteria to subinhibitory doses of antibiotics increases mutagenesis and can lead to multidrug resistance (118). ROS involvement in this mutagenesis was suggested, in part, by the discovery that anaerobic growth reduces the mutation rate to near-normal levels, as does the addition of the potent radical scavenger thiourea (118).

Mutagenesis caused by exposure to subinhibitory levels of antibiotics is *recA*$^+$-dependent (119). Furthermore, in the intensively studied case of exposure of *E. coli* to subinhibitory concentrations of ampicillin, the vast majority of antibiotic mutagenesis was shown to be dependent on DinB (120). These observations together indicate that antibiotic-induced mutagenesis does not result from an increase in simple replicative errors. As discussed above, DinB is capable of using oxidized dNTPs as substrates (57, 93), and it can also extend a DNA strand after the incorporation of an oxidized nucleotide by the replicative DNA polymerase (121). The additional dependence of subinhibitory ampicillin mutagenesis on RpoS was explained by the demonstration that the key mismatch repair enzyme MutS must be depleted, a process that is mediated by the RpoS-dependent induction of SdsR, a small regulatory RNA (120).

The resulting hypothesis proposed by Gutierrez et al. (120)—that incorporation of oxidized nucleotides into nascent DNA underlies the mutagenesis caused by subinhibitory levels of antibiotics—shares a strong mechanistic commonality with the hypothesis proposed by Foti et al. (57) that incorporation of 8-oxo-dGTP into nascent DNA underlies much of the ROS-dependent component of killing caused by exposure to lethal levels of antibiotics. The fact that increasing the levels of the mismatch repair protein MutS can suppress both phenomena (99, 120) additionally supports a mechanistic relationship between the two biological outcomes. In nature, bacteria are most likely to be exposed to sublethal doses of antibiotics (122, 123); thus, the evolution of a system that increases mutagenesis under the stress of low levels of antibiotics would have been beneficial (120). Exposure to lethal doses of antibiotics would be much rarer in nature, so the ROS-dependent component of antibiotic killing could simply be a deleterious consequence of a strategy that is normally beneficial for adaptation under less stressful conditions (99).

CHALLENGES TO THE HYPOTHESIS THAT A COMPONENT OF ANTIBIOTIC LETHALITY IS DUE TO OXIDATIVE STRESS

Despite this substantial body of literature, two recent papers (12, 13) have challenged the very notion that ROS-dependent mechanisms are a factor in the bactericidal effects of antibiotics. Notably, each study focused almost exclusively on testing particular features of the model proposed in 2007 by Kohanski et al. (37) and reached their conclusions without accounting for, or offering alternate explanations for, the large body of evidence summarized in the present review. Unfortunate confusion has also arisen from a misconception introduced by Keren et al.

(12), who incorrectly claimed that the proposed ROS-dependent model is an alternative to the traditionally studied action of antibiotics. Kohanski et al. (37) instead originally proposed that ROS production contributes to the killing efficacy of antibiotics, a more modest hypothesis that is completely consistent with antibiotics killing bacteria by their traditional mechanisms of action. A more extensive discussion of this issue can be found in Dwyer et al. (99).

In experiments that were central to their conclusions that ROS are not involved in antibiotic lethality, Liu & Imlay (13) and Keren et al. (12) reported that they did not observe differences in killing by norfloxacin and ampicillin under aerobic and anaerobic conditions. These findings are inconsistent with the significant protection against fluoroquinolone killing provided by anaerobic conditions previously published by Malik et al. (23) and Morrissey & Smith (25), work that was not acknowledged nor accounted for by either group. The protection against killing by norfloxacin that is afforded by the use of strict anaerobic conditions has subsequently been confirmed by Dwyer et al. (99) and extended to ampicillin.

There is widespread agreement that anaerobic conditions provide protection against killing by aminoglycosides (12, 13, 99). This has been attributed to a well-studied phenomenon of the decreased drug uptake caused by a reduced proton motor force (PMF) associated with anaerobic conditions (12, 13). However, the conclusion that aminoglycosides do not induce ROS under aerobic conditions (12, 13), based in part or largely on anaerobic-aerobic comparisons, does not account for the previously reported evidence that (*a*) kanamycin induces the SOS response in an *E. coli mutM mutY mutT* mutant that is defective in processing 8-oxo-dG, (*b*) overproduction of MutY prevents kanamycin-induced SOS induction in *Vibrio cholerae* (124), (*c*) kanamycin causes damage to DNA bases (125), and (*d*) aminoglycoside-induced protein aggregation can be prevented by a H_2O_2 scavenger (126).

Central to Liu & Imlay's (13) conclusion that antibiotics do not exert their lethal actions through the known mechanisms of oxidative stress was their failure to detect an increase in H_2O_2 production after antibiotic treatment. However, in a subsequent study using a direct assay for intracellular H_2O_2 levels, Dwyer et al. (99) observed an increase in H_2O_2 production with all three classes of bactericidal antibiotics.

In a subsequent paper, Ezraty et al. (11) discounted a role for ROS in the killing effects of aminoglycosides and β-lactams largely on the basis of observing no differences in killing between the wild type and a *sodA sodB* double mutant or an *oxyR* mutant (they reported a slight protective effect for the *oxyR* mutant against β-lactams 4.5 h post-treatment). The results with the *sodA sodB* double mutant are inconsistent with the seminal work by Wang & Zhao (40) described earlier, which was not acknowledged (11). Gene knockouts can induce the activity of gene expression pathways that ultimately obscure the phenotype of interest, underscoring the difficulty that physiological compensations (99, 127) can introduce when interpreting the effects of genetic perturbations. Interestingly, Ezraty et al. (11) provide evidence that the electron transport chain and Fe-S clusters are important for aminoglycoside lethality; the authors hypothesize that these elements interact primarily to generate PMF, enabling drug uptake. Although Ezraty et al. (11) did not discuss it in their paper, the SUF Fe-S cluster repair system implicated by these authors for preservation of PMF during aminoglycoside treatment is activated by OxyR and is indispensable for Fe-S cluster protein maintenance under conditions of oxidative stress to compensate for ROS-mediated inactivation of the ISC Fe-S cluster repair system (128–130). These data nonetheless support the general notion that aminoglycosides, downstream of their drug-target interaction, induce changes to cellular metabolism and respiration that could alter the cellular redox state. The recent discovery that IscS plays a role in a tRNA thiolation pathway that modulates intracellular redox stress (131) suggests an additional interpretation for the effects of an *iscS* mutation in these experiments (11, 37).

Evidence from multiple labs summarized in this review has provided several independent lines of evidence that antibiotics elicit the production of reactive oxidants including ROS that contribute to their lethality. Although the 2007 Kohanski et al. (37) model suggested a possible explanation for ROS generation elicited by antibiotic treatment that was consistent with the data available at the time, the complexity of the additional evidence published since then has made it clear that the actual situation is considerably more complex than originally suggested. Models to explain this phenomenology will require ongoing revision. These new data also indicate that the molecular events leading to ROS generation in response to a particular antibiotic are not identical in all bacteria. In another intriguing example of physiological complexity, the degree to which a ROS-dependent component contributes to overall antibiotic lethality can vary strikingly between antibiotics within the same class and can also be influenced by new protein synthesis (132).

HARNESSING OXIDATIVE STRESS TO ENHANCE THE KILLING EFFICACY OF ANTIBIOTICS

Although our mechanistic understanding of the oxidative component of bactericidal antibiotic action remains incomplete, bioengineering and chemical biology approaches can nonetheless be used to harness certain features of the ROS phenotype as a means to boost the killing efficacy of antibiotics. From a biological perspective, experiments demonstrating that adjuvants that enhance the effects of oxidative stress increase the sensitivity of bacteria to bactericidal antibiotics provide compelling evidence for the expanded view of drug lethality involving ROS. Likewise, from a clinical translational perspective, the development of therapeutic approaches that exploit the ROS component of lethality to improve antibiotic efficacy validate the role of drug-induced oxidative stress in the lethal mechanisms of antibiotics.

As an early example of this approach, Lu & Collins (133) engineered nonlytic bacteriophage to overexpress proteins that target gene networks, enhancing bacterial killing by antibiotics. As noted above, antibiotic-induced oxidative stress leads to DNA damage, and bacterial killing by bactericidal antibiotics can be enhanced by knocking out *recA* and disabling the SOS response. Lu & Collins took an alternative approach and engineered bacteriophage to overexpress *lexA3*, a repressor of the SOS response. The engineered bacteriophage enhanced antibiotic-mediated killing of bacteria by several orders of magnitude and significantly increased survival of infected mice. The engineered bacteriophage could also be used to restore antibiotic susceptibility to resistant strains and readily modified to target different gene networks. This work established a novel bioengineering platform for the rapid translation of identified targets into effective antibiotic potentiators (133).

Recent work by Brynildsen et al. (134) showed that genome-scale metabolic modeling of ROS production in *E. coli* can be used to rationally and accurately predict genetic targets for adjuvant therapies that enhance conventional antibiotics by amplifying basal ROS production. Rather than identifying ways to suppress ROS defense systems, Brynildsen et al. sought instead to boost oxidative stress to substimulatory levels, thereby lowering the threshold for antibiotic-induced ROS to contribute to drug lethality. This subtle notion—that a marginal increase in basal ROS production can increase the severity of oxidative stress promoted by bactericidal antibiotics—was supported by genetic and chemical enhancement of β-lactam and fluoroquinolone antibiotics (134).

Oxidative stress has also been implicated in the synergistic effects of bactericidal antibiotics in combination with silver (an ancient antibacterial) and tellurite. For example, Hwang et al. (135) showed that silver nanoparticles enhance the lethality of ampicillin or kanamycin, in part by enhancing oxidative phosphorylation and promoting ROS. Additional studies by Hwang et al. (136) and Choi & Lee (137, 138) extended these findings to several antimicrobial peptides that have

been shown to possess a ROS component of lethality and the ability to enhance antibiotic killing. More recently, Morones-Ramirez et al. (139) demonstrated that silver disrupts multiple cellular processes in bacteria, leading to the production of ROS and increased membrane permeability. In a series of in vitro experiments and in vivo animal studies, they showed that these mechanistic effects could be harnessed to enhance the killing efficacy of existing antibiotics against gram-negative

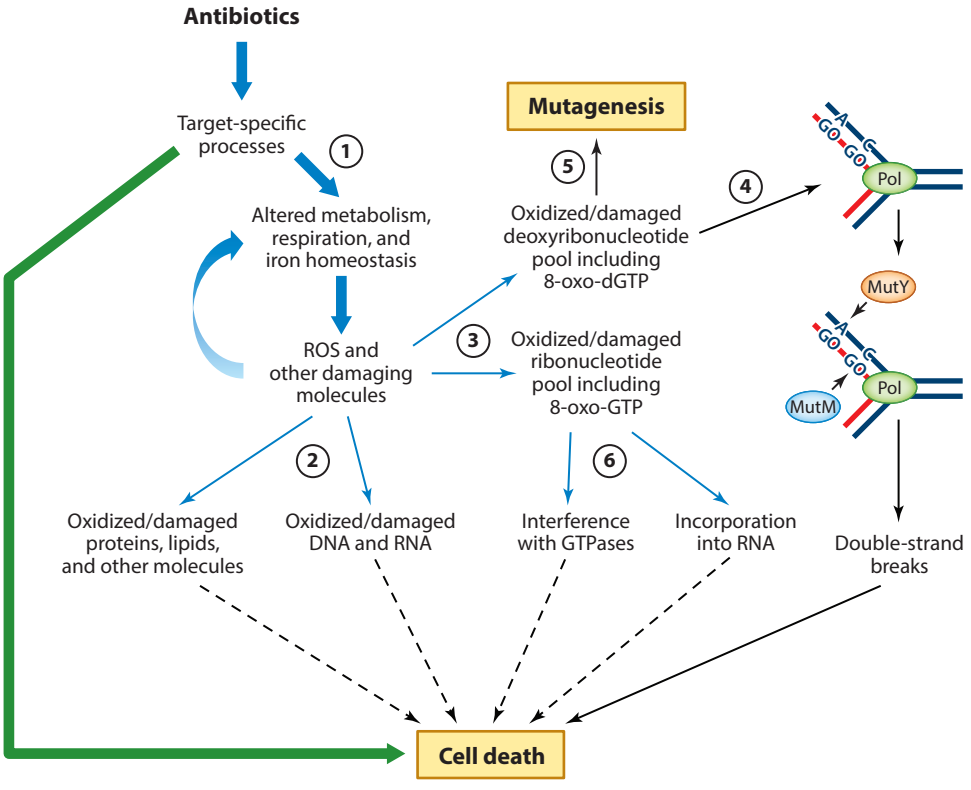

Figure 1

The physiological complexity of antibiotic lethality. Evidence summarized in this review suggests that the interaction of antibiotics with their primary cellular targets results in metabolic perturbations that contribute to cell death in addition to that caused directly by the interactions of antibiotics with their targets (*green arrow*). ① These perturbations include alterations of metabolism, respiration, and iron homeostasis that result in the production of ROS and other damaging molecules. ② These in turn can cause oxidative and other forms of damage to proteins, lipids, nucleic acids, and other molecules that can in principle contribute to cell death. ③ Evidence suggests that oxidation of the nucleotide pool plays a particularly important role. ④ If oxidized dNTPs—for example, 8-oxo-dGTP, which can pair with either dC or dA—are used as substrates for DNA synthesis by DNA polymerases, then incomplete base excision repair at nearby oxidized deoxynucleotides in nascent DNA (for example, by MutM and MutY) can give rise to lethal double-strand breaks. ⑤ These oxidized dNTPs also contribute to the mutagenesis observed when aerobically grown cells are exposed to sublethal doses of antibiotics. ⑥ Oxidized ribonucleotide triphosphates—for example, 8-oxo-GTP—may also contribute to lethality by interfering with essential GTPases or by being incorporated into RNA, causing a cascade of protein mistranslation. Abbreviations: dA, deoxyadenosine; dC, deoxycytidine; dNTP, deoxyribonucleotide triphosphate; GO, 8-oxo-deoxyguanosine; GTPase, guanosine triphosphatase; NTP, ribonucleoside triphosphate; Pol, DNA polymerase; ROS, reactive oxygen species; 8-oxo-dGTP, 8-oxo-deoxyguanosine triphosphate; 8-oxo-GTP, 8-oxo-guanosine triphosphate. Components of the figure have been adapted from Dwyer et al. (99) and Foti et al. (57).

bacteria, including resistant strains, persisters, and biofilms. With respect to tellurite, two recent studies by Molina-Quiroz et al. (140, 141) found that nonlethal levels of this tellurium oxyanion can enhance the lethality of several antibiotics, including ampicillin and gentamicin. They demonstrated that a ROS component is involved in the lethal synergy of cefotaxime (a cephalosporin β-lactam) with tellurite by showing that the combination treatment results in oxidative damage to DNA and proteins (141).

FUTURE RESEARCH

The extensive body of evidence reviewed here collectively indicates that metabolic perturbations resulting from the interactions of antibiotics with their targets contribute to antibiotic lethality through mechanisms that involve the generation of ROS and other damaging molecules (**Figure 1**). These reactive species can damage many important cellular components, but oxidation of the nucleotide pool appears to be particularly significant. However, this body of evidence also underscores the physiological complexity of antibiotic action and the numerous gaps in our knowledge. Given the growing crisis resulting from the emergence of resistant organisms and our diminishing abilities to treat bacterial infections, it is critical that we work together to gain further basic insights into the mechanisms targeted and triggered by antibiotics that can guide our efforts to combat infectious disease. These efforts will require new perspectives and approaches.

DISCLOSURE STATEMENT

D.J.D and G.C.W. are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review. J.J.C. is scientific founder and SAB chair of Enbiotix, a start-up company focused on antibiotic development.

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