

Editor's Summary

Antibiotics Affect Mitochondria in Mammalian Cells

Antibiotics hurt only bacteria, right? According to a new study from Kalghatgi and colleagues, certain types of antibiotics may also cause damage to mammalian cells and thus pose problems for patients on long-term antibiotic regimens.

The authors hypothesized that bactericidal—but not bacteriostatic—antibiotics damage mammalian tissues by triggering mitochondrial release of reactive oxygen species (ROS). Indeed, in culture, three representative bactericidal antibiotics—ciprofloxacin (a fluoroquinolone), ampicillin (a β -lactam), and kanamycin (an aminoglycoside)—induced dose- and time-dependent increases in intracellular ROS in various human cell lines. Such increases in ROS led to DNA, protein, and lipid damage *in vitro*. A bacteriostatic antibiotic, tetracycline, had no effect on ROS production. To shed light on the mechanism, Kalghatgi *et al.* showed that bactericidal antibiotics disrupted the mitochondrial electron transport chain, which would lead to a buildup of ROS.

Mice treated with clinically relevant doses of bactericidal antibiotics similarly showed signs of oxidative damage in blood tests, tissue analysis, and gene expression studies. This ROS-mediated damage could be reversed by the powerful antioxidant *N*-acetyl-L-cysteine (NAC) without disrupting the bacteria-killing properties of the antibiotics. These studies by Kalghatgi *et al.* suggest that not only does this damage occur with long-term use of antibiotics, but it can also be prevented by taking antioxidants or by switching to bacteriostatic antibiotics. Nevertheless, it will be important to confirm this antibiotic effect in humans, with a broader range of antibiotics, before any conclusions can be made about oxidative damage to mammalian tissues.

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ANTIBIOTICS

Bactericidal Antibiotics Induce Mitochondrial Dysfunction and Oxidative Damage in Mammalian Cells

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Prolonged antibiotic treatment can lead to detrimental side effects in patients, including ototoxicity, nephrotoxicity, and tendinopathy, yet the mechanisms underlying the effects of antibiotics in mammalian systems remain unclear. It has been suggested that bactericidal antibiotics induce the formation of toxic reactive oxygen species (ROS) in bacteria. We show that clinically relevant doses of bactericidal antibiotics—quinolones, aminoglycosides, and β -lactams—cause mitochondrial dysfunction and ROS overproduction in mammalian cells. We demonstrate that these bactericidal antibiotic-induced effects lead to oxidative damage to DNA, proteins, and membrane lipids. Mice treated with bactericidal antibiotics exhibited elevated oxidative stress markers in the blood, oxidative tissue damage, and up-regulated expression of key genes involved in antioxidant defense mechanisms, which points to the potential physiological relevance of these antibiotic effects. The deleterious effects of bactericidal antibiotics were alleviated in cell culture and in mice by the administration of the antioxidant *N*-acetyl-L-cysteine or prevented by preferential use of bacteriostatic antibiotics. This work highlights the role of antibiotics in the production of oxidative tissue damage in mammalian cells and presents strategies to mitigate or prevent the resulting damage, with the goal of improving the safety of antibiotic treatment in people.

INTRODUCTION

Antibiotics have led to an extraordinary decrease in morbidity and mortality associated with bacterial infections. Yet, despite the great benefits, antibiotic use has been linked to various adverse side effects, including ototoxicity (1), nephrotoxicity (2), and tendinopathy (3). Although antibiotic targets and modes of action have been widely studied and well characterized in bacteria, the mechanistic effects of commonly prescribed antibiotics on mammalian cells remain unclear. Recently, it has been demonstrated that major classes of bactericidal antibiotics, irrespective of their drug-target interactions, induce a common oxidative damage cellular death pathway in bacteria, leading to the production of lethal reactive oxygen species (ROS) (4–12) via disruption of the tricarboxylic acid (TCA) cycle and electron transport chain (ETC) (4, 6). The role of ROS in antibiotic-induced bacterial killing is currently a matter of debate (13, 14) and the subject of intense experimental investigation in our laboratory and other laboratories; however, the techniques critiqued in (13, 14) were not used in the present study, which focuses on mammalian systems.

Bactericidal and bacteriostatic antibiotics have been shown to target mitochondrial components (15–20). In mammalian cells, the mitochondrial ETC is a major source of ROS during normal metabolism because of leakage of electrons (21). Given the proposed bacterial origin of mitochondria (22), we hypothesized that bactericidal antibiot-

ics commonly disrupt mitochondrial function in mammalian cells, leading to oxidative stress and oxidative damage. Previous work has shown that mammalian cells can be damaged by antibiotic treatment, but these results were shown at concentrations considerably higher than those applied clinically. At these high concentrations, select antibiotics inhibited cell growth and metabolic activity, in addition to impairing mitochondrial function *in vitro* (23, 24).

Here, we focused on characterizing the mechanistic effects of clinically relevant levels of bactericidal antibiotics on mammalian cells, both *in vitro* and *in vivo*. We showed that bactericidal antibiotics—quinolones, aminoglycosides, and β -lactams—caused mitochondrial dysfunction and ROS overproduction in mammalian cells, ultimately leading to the accumulation of oxidative tissue damage. We found that these deleterious effects could be alleviated by administration of the Food and Drug Administration (FDA)-approved antioxidant, *N*-acetyl-L-cysteine (NAC), or prevented by preferential use of bacteriostatic antibiotics. These results reflect two therapeutic strategies to combat the adverse side effects of long-term antibiotic treatment.

RESULTS

Bactericidal antibiotics induce oxidative stress and damage in mammalian cells

We first examined whether clinically relevant doses of antibiotics induce the formation of ROS in mammalian cells. Here, clinically relevant doses are defined by peak serum levels (25). We exposed a human mammary epithelial cell line, MCF10A, to representative bactericidal antibiotics from three different classes: ciprofloxacin (a fluoroquinolone), ampicillin (a β -lactam), and kanamycin (an aminoglycoside). All three bactericidal antibiotics induced a dose- and time-dependent increase

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in intracellular ROS production (Fig. 1A and fig. S1), but a bacteriostatic antibiotic (tetracycline) did not lead to a significant increase in ROS production (Fig. 1A). Notably, bactericidal antibiotic-induced ROS was not elevated in mammary epithelial cells as a result of an increased presence of dead or dying cells in vitro (fig. S2). To establish that the observed bactericidal antibiotic-induced oxidative stress was not cell line-specific, we tested several additional cell types, including primary human aortic endothelial cells (PAEC), primary human mammary epithelial cells (HMEC), human gut epithelial cells (CACO-2), and normal human diploid skin fibroblasts (NHDF). In all instances, we found the same pattern of significantly elevated ROS levels induced by bactericidal antibiotics (fig. S3), with little effect seen after bacteriostatic antibiotic treatment.

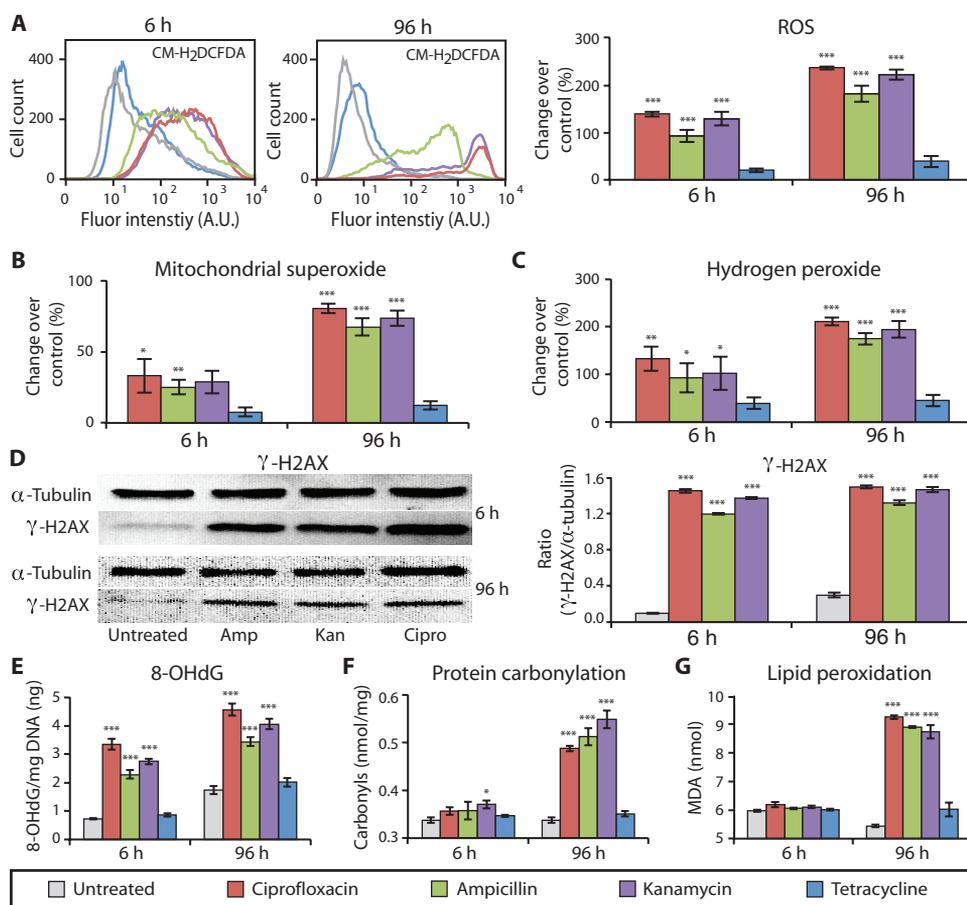


Fig. 1. Bactericidal antibiotics cause oxidative damage to mammalian cells. ROS and oxidative damage were measured in human mammary epithelial cells (MCF10A) after 6 and 96 hours of treatment with bactericidal [ciprofloxacin (10 $\mu\text{g}/\text{ml}$), ampicillin (20 $\mu\text{g}/\text{ml}$) and kanamycin (25 $\mu\text{g}/\text{ml}$)] or bacteriostatic [tetracycline (10 $\mu\text{g}/\text{ml}$)] antibiotics compared to untreated cells. (A) ROS was quantified using CM-H₂DCFDA by flow cytometry (histograms on left) and a microplate spectrophotometer (bar graphs on right). (B) Mitochondrial superoxide was measured using MitoSox Red. (C) Hydrogen peroxide release was quantified by measuring Amplex Red fluorescence. (D) Antibiotic-induced DNA damage was evaluated by Western blot analysis to measure the abundance of the phosphorylated histone protein, γ -H2AX, compared to α -tubulin serving as the loading control (quantification in the bar graph). (E) Additionally, oxidative DNA damage was assessed by measuring the abundance of 8-OHdG. (F) Oxidative protein damage, protein carbonylation, was detected using an enzyme-linked immunosorbent assay (ELISA). (G) Lipid peroxidation was evaluated by measuring MDA. All bar graphs display means \pm SEM ($n \geq 3$). Comparisons between treatments and untreated controls were made using a Student's *t* test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Superoxide, a reactive oxygen by-product generated by leakage of electrons from the ETC to oxygen, is a precursor to many other forms of ROS, one instance being the enzymatic conversion into hydrogen peroxide (H₂O₂) through superoxide dismutase (26). We measured mitochondrial superoxide production and extracellular H₂O₂ release in mammalian (human) MCF10A cells and found that all three bactericidal antibiotics induced a dose- and time-dependent increase in both mitochondrial superoxide (Fig. 1B and fig. S4) and H₂O₂ (Fig. 1C and fig. S5). Bacteriostatic antibiotic treatment did not lead to a significant increase in superoxide (Fig. 1B) or H₂O₂ production (Fig. 1C).

ROS can directly interact with cellular components resulting in DNA, protein, and lipid damage. To characterize DNA damage induced by bactericidal antibiotics in mammalian cells, we used indirect immunofluorescence and Western blot analysis to measure γ -H2AX, a core histone protein that is phosphorylated in response to DNA damage. We observed a persistent and significant increase in γ -H2AX in MCF10A cells exposed to bactericidal antibiotics after 6 and 96 hours of treatment compared with untreated cells (Fig. 1D and fig. S6). Additionally, we quantified the presence of 8-hydroxy-2'-deoxyguanosine (8-OHdG), an oxidized DNA by-product. Similar to γ -H2AX, we found significantly elevated levels of 8-OHdG after 6 and 96 hours of bactericidal antibiotic treatment (Fig. 1E) but negligible change in response to tetracycline compared with untreated controls.

To further investigate the accumulation of cellular oxidative damage, we measured levels of protein carbonyls, a modification of proteins resulting from oxidative damage, and levels of malondialdehyde (MDA), an end product of lipid peroxidation. We found significantly elevated levels of protein carbonylation (Fig. 1F) and lipid peroxidation (Fig. 1G) in MCF10A cells after 96 hours of bactericidal antibiotic treatment. Consistent with the general ROS, mitochondrial superoxide, and H₂O₂ results, bacteriostatic antibiotics had little effect on protein carbonylation or lipid peroxidation (Fig. 1, F and G).

Bactericidal antibiotics induce mitochondrial dysfunction

In bacteria, the common mechanism of killing by bactericidal antibiotics advances the finding that toxic ROS are generated via the disruption of the TCA cycle and ETC (4). Bacteriostatic antibiotics do not stimulate ROS production in bacteria, suggesting that bactericidal antibiotics uniquely affect major sources of ROS. In mammalian cells, mitochondria are major sources of intracellular ROS; therefore, we tested

the hypothesis that bactericidal antibiotics uniquely disrupt the function of the mitochondrial ETC, leading to the observed overproduction of ROS and oxidative damage (Fig. 1).

We measured the inhibitory effects of bactericidal antibiotics on individual, immunocaptured ETC complexes I to V. Bactericidal antibiotics inhibited complex I activity by 16 to 25% and complex III activity by 30 to 40% compared to untreated samples (Fig. 2A), whereas bacteriostatic antibiotics and negative controls exhibited only 5 to 10% inhibition (fig. S7). In addition, we observed a varied degree of inhibition of complexes II and IV across the tested bactericidal antibiotics, with complex V showing less change in activity compared to complexes I to IV. These data indicate that bactericidal antibiotics inhibit mitochondrial ETC complexes, in particular complexes I and III, which have been identified as major sources of ROS formation (27).

Because mitochondrial energy metabolism is tightly linked to organelle function, disruption of the ETC should lead to a decrease in mitochondrial membrane potential ($\Delta\Psi_m$), adenosine triphosphate (ATP) levels, and overall metabolic activity. Indeed, bactericidal antibiotic treatment led to a corresponding and significant decrease in all three metabolic functions after 96 hours of treatment (Fig. 2, B to D), further supporting the hypothesis that bactericidal antibiotics induce mitochondrial dysfunction.

If bactericidal antibiotics induce ROS by impairing the function of the ETC, then cells devoid of a functional ETC should show little ROS formation after antibiotic treatment. Mammalian cells devoid of mitochondrial DNA (mtDNA), but retaining their nuclear genomes, lack functional ETC complexes; these cells are referred to as ρ_0 cells. By selectively eliminating mtDNA and providing nutrient supplementation, we generated an MCF10A ρ_0 cell line (fig. S8A). Bactericidal antibiotic treatments of ρ_0 cells showed no difference in ROS production when compared to untreated cells (fig. S8B). This was in sharp contrast to the large, significant increase in ROS production stimulated by bactericidal antibiotic treatment in normal MCF10A cells (Fig. 1 and fig. S8B). Furthermore, we found relatively high levels of nuclear DNA damage (γ -H2AX) in normal cells after bactericidal antibiotic treatments, whereas the level of DNA damage in ρ_0 cells was similar to untreated controls (Fig. 2E). These results suggest that the mitochondrial ETC is a major source of bactericidal antibiotic-induced intracellular ROS.

The homeostatic balance between mitochondrial fission and fusion can be altered in response to oxidative stress (28). Disruption of this balance can lead to changes in mitochondrial morphology and function, resulting in the generation of ROS (28). Using live-cell imaging of primary human mammary epithelial cells, we measured the morphological changes in mitochondria and found short, swollen, fragmented mitochondria (smaller aspect ratio) with highly reduced branching (smaller form factor) in bactericidal antibiotic-treated cells compared to long, tubular (larger aspect ratio), and extensively branched (larger form factor) mitochondria in untreated cells (Fig. 3). These results suggest that bactericidal antibiotics shift the balance toward a profission state. Consistent with our data in Fig. 2, a profission state can lead to a loss of membrane potential, loss of metabolic activity, and an overall increase in oxidative stress (28).

Thus far, we have characterized isolated processes involved in mitochondrial respiration. To directly test the mitochondrial respiratory capacity of antibiotic-treated cells, we measured changes in the oxygen consumption rate (OCR) of intact MCF10A cells using the Seahorse XF24 flux analyzer. Compared to untreated cells, bactericidal

antibiotic-treated cells exhibited a significant reduction in both basal respiration and maximal respiratory capacity after 6 hours of treatment (Fig. 4A), which was further reduced after 96 hours of treatment (Fig. 4B). In contrast, cells treated for 6 and 96 hours with tetracycline showed little change in respiratory capacity compared to untreated cells (Fig. 4). These data demonstrate that bactericidal, but not bacteriostatic, antibiotics impair the function of mitochondria and, consequently, the overall respiratory capacity of the cell.

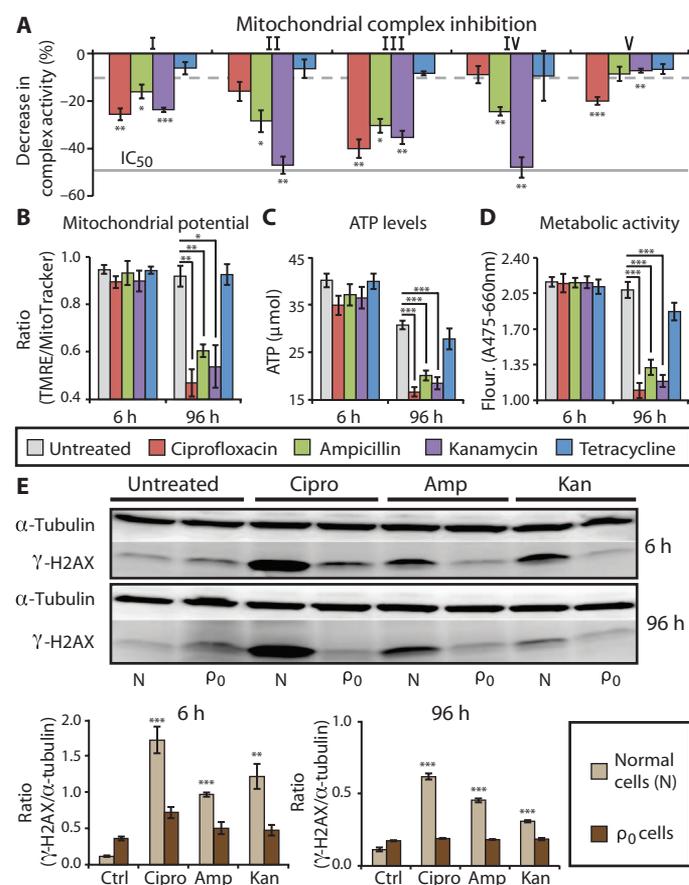


Fig. 2. Bactericidal antibiotics induce mitochondrial dysfunction. (A) The effects of bactericidal [ciprofloxacin (10 μ g/ml), ampicillin (20 μ g/ml), and kanamycin (25 μ g/ml)] or bacteriostatic [tetracycline (10 μ g/ml)] antibiotics on the function of individual, isolated ETC protein complexes were measured. Bars represent the change in activity of individual antibiotic-treated complexes compared to untreated complexes. The dashed gray line represents the maximum inhibition (10%) seen across several independent negative controls. The solid gray line represents the IC_{50} (median inhibitory concentration) of positive control drugs shown to inhibit specific target complexes (see fig. S7 for all positive and negative control results). (B) Mitochondrial membrane potential was quantified using tetramethylrhodamine ester (TMRE) and MitoTracker Green. (C) ATP levels were measured using a luciferin/luciferase assay. (D) Metabolic activity was measured using a colorimetric tetrazolium dye (XTT). (E) DNA damage in normal (N) and mtDNA-depleted (ρ_0) MCF10A cells was measured by Western blot to evaluate the ratio of γ -H2AX compared to the loading control, α -tubulin. Quantification is shown below the blots. All bar graphs display means \pm SEM ($n \geq 3$). Comparisons between treatments and untreated controls were made using a Student's t test (* $P < 0.5$, ** $P < 0.01$, *** $P < 0.001$).

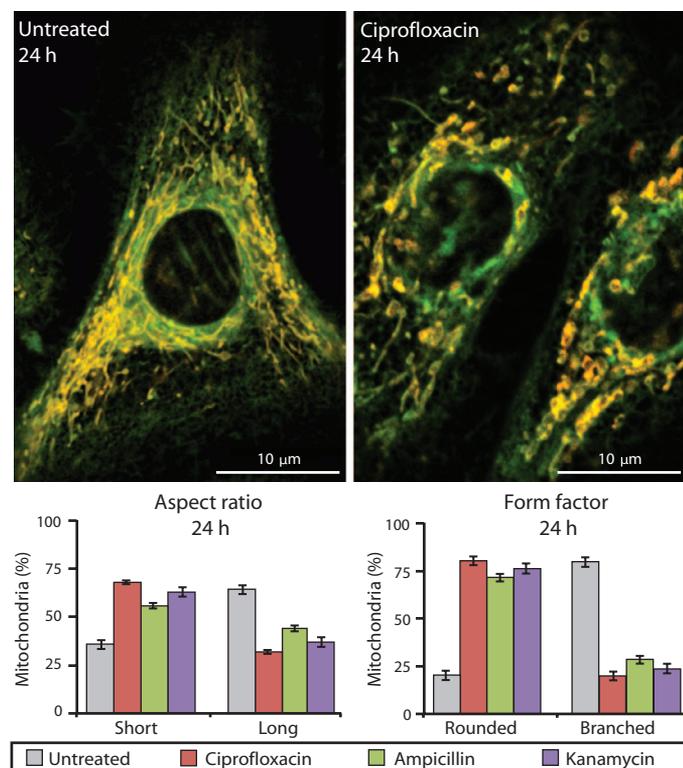


Fig. 3. Mitochondria in bactericidal antibiotic-treated cells show an abnormal, profission state. Mitochondrial morphology was measured in primary human mammary epithelial cells using TMRE and MitoTracker Green. Untreated samples (left image) show mitochondria with normal morphology, which are long and highly branched. Ciprofloxacin-treated cells (10 $\mu\text{g/ml}$) (right image) had abnormally short and truncated mitochondria. The percentages of mitochondria with a short versus long aspect ratio and rounded versus branched form factor were measured for untreated and bactericidal antibiotic-treated cells. Data are means \pm SEM ($n \geq 5$).

Antibiotic-induced oxidative damage is rescued by an antioxidant

Characterization of bactericidal antibiotic effects on mammalian cells points to widespread cellular oxidative damage. Therefore, we explored the possibility of using an antioxidant to alleviate these deleterious effects. We chose to test the ability of NAC to alleviate bactericidal antibiotic-induced oxidative damage in mammalian cells. NAC was selected because it is an FDA-approved antioxidant that is well tolerated by patients and commonly used to buffer extraneous intracellular ROS in mammalian systems (29, 30). MCF10A cells were pretreated with NAC for 2 hours, followed by bactericidal antibiotic treatment for 6 and 96 hours. NAC pretreatment reduced bactericidal antibiotic-induced ROS levels (Fig. 5A and fig. S9) and restored mitochondrial membrane potential to levels seen in untreated cells after 96 hours of antibiotic treatment (Fig. 5B). Furthermore, NAC restored basal respiration and maximal respiratory capacity of bactericidal antibiotic-treated cells to near-normal levels (Fig. 5C and figs. S10 and S11) and alleviated bactericidal antibiotic-induced DNA damage (γ -H2AX) (fig. S12), 8-OHdG formation (Fig. 5D), protein carbonylation (Fig. 5E), and lipid peroxidation (Fig. 5F) after only 6 hours of treatment. Cells treated with NAC alone showed no significant effects from untreated cells (Student's t test).

Combining an antioxidant with a bactericidal antibiotic carries the potential risk of reducing the bacterial killing efficacy of the antibiotic,

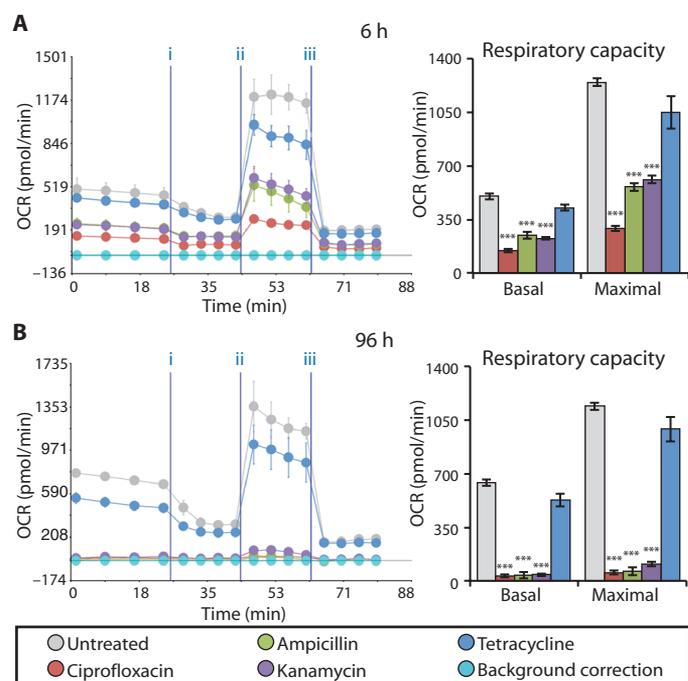


Fig. 4. Bactericidal antibiotics decrease mitochondrial basal respiration and maximal respiratory capacity. (A and B) OCR was measured in MCF10A cells after 6 hours (A) and 96 hours (B) of bactericidal [ciprofloxacin (10 $\mu\text{g/ml}$), ampicillin (20 $\mu\text{g/ml}$), and kanamycin (25 $\mu\text{g/ml}$) or bacteriostatic [tetracycline (10 $\mu\text{g/ml}$)] antibiotic treatment. Cells were treated with antibiotics followed by the Seahorse OCR protocol including treatment with three mitochondrial ETC complex inhibitors: (i) oligomycin, (ii) carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, and (iii) antimycin A. OCR measured before (i) represents basal respiration, whereas OCR measured between (ii) and (iii) represents the maximal respiratory capacity. Representative Seahorse OCR plots are shown, and the bar graphs are means \pm SD for $n = 3$. Comparisons between treatments and untreated controls were made using a Student's t test ($***P < 0.001$).

given that bactericidal antibiotic-induced ROS formation facilitates bacterial killing (4). Tested in bacterial cultures, we found that the concentration of NAC used to rescue antibiotic-induced oxidative damage in mammalian cells did not decrease the bacterial killing efficacy of bactericidal antibiotics (fig. S13). We extended this study to test the antimicrobial efficacy of the antibiotic-NAC combination in a mouse urinary tract infection (UTI) model. *Escherichia coli* were transurethrally introduced into the bladder of mice. Twenty-four hours after establishing infection, mice were treated with ciprofloxacin (50 $\mu\text{g/ml}$), NAC (10 mM), and ciprofloxacin + NAC or untreated (vehicle only). Ciprofloxacin + NAC and ciprofloxacin-only treatments showed similar levels of bacterial killing (fig. S14), suggesting that NAC does not interfere with the antimicrobial activity of clinically relevant doses of bactericidal antibiotics. Given that other antioxidants, such as glutathione, have been shown to reduce the bacterial killing efficacy of bactericidal antibiotics (8), further work is needed to characterize the effects of NAC on both Gram-negative and Gram-positive bacteria.

Oxidative damage in vivo caused by bactericidal antibiotics can be rescued

To determine the in vivo relevance of bactericidal antibiotic-induced ROS production and mitochondrial dysfunction, we investigated oxidative

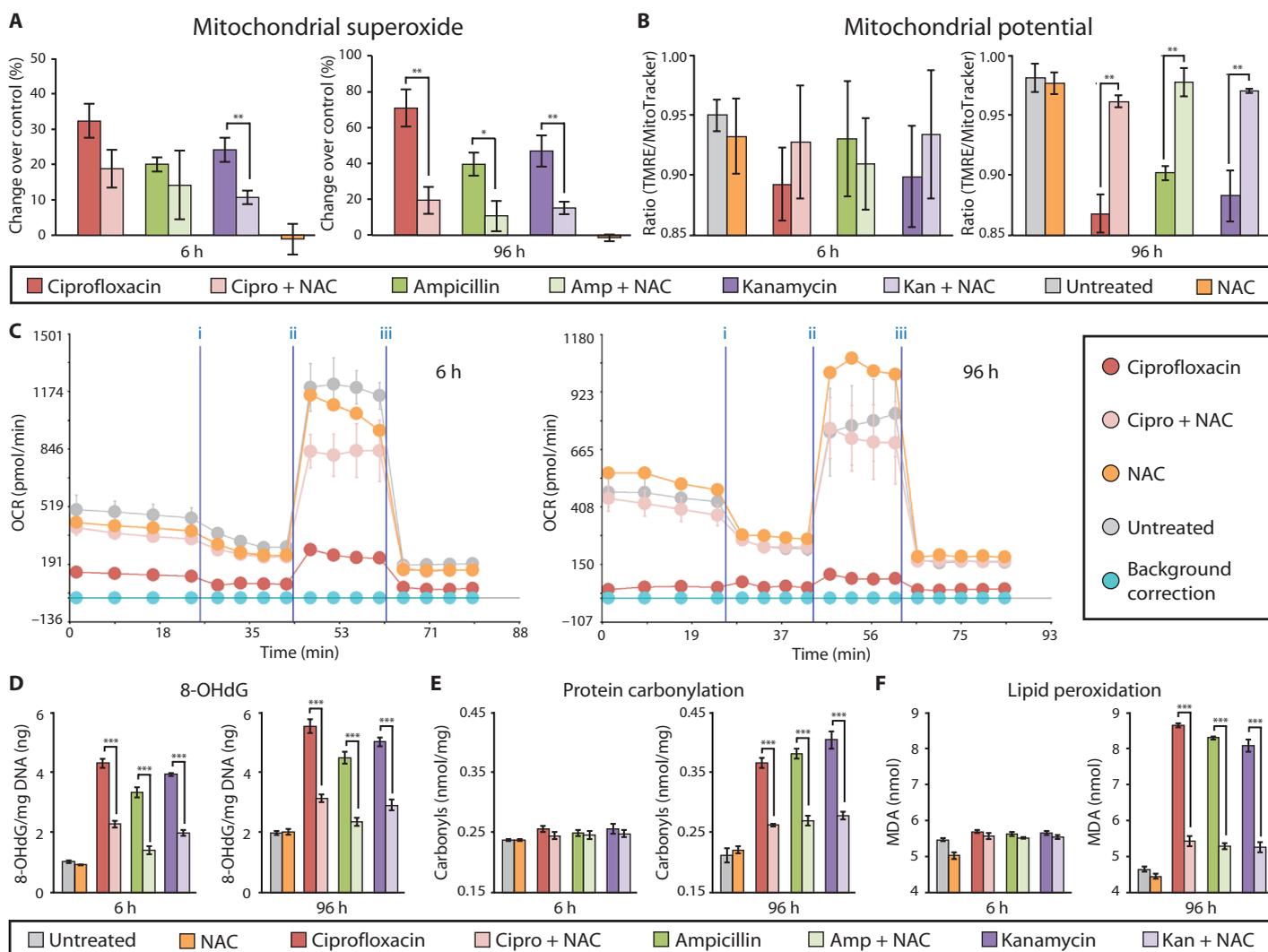


Fig. 5. NAC rescues bactericidal antibiotic-induced oxidative damage in vitro. MCF10A cells were incubated with and without NAC (10 mM) for 2 hours, followed by treatment with ciprofloxacin (10 μ g/ml), ampicillin (20 μ g/ml), kanamycin (25 μ g/ml), or tetracycline (10 μ g/ml) for 6 and 96 hours. (A) Mitochondrial superoxide was measured using MitoSox Red. (B) Mitochondrial membrane potential was quantified using TMRE and MitoTracker Green. (C) OCR was measured using the Seahorse XF24

flux analyzer, and a representative diagram testing the ciprofloxacin + NAC is shown (see figs. S10 and S11 for ampicillin and kanamycin). (D) Oxidative DNA damage was assessed by quantification of 8-OHdG. (E) Protein carbonyls were quantified to evaluate oxidative protein damage. (F) Lipid peroxidation was measured by quantifying MDA. Data are means \pm SEM ($n \geq 3$). Comparisons between treatment + NAC and treatments were made using a Student's t test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

stress markers and accumulation of oxidative damage in tissues of mice treated with antibiotics. Mice received clinically relevant doses of antibiotics [ciprofloxacin (12.5 mg/kg per day), ampicillin (28.5 mg/kg per day), and kanamycin (15 mg/kg per day)] in their drinking water ($n \geq 3$ animals per treatment group). At 2 and 16 weeks, peripheral blood was drawn from the same animals to measure intracellular ROS, lipid peroxidation, and glutathione levels. A reduction in glutathione is a proxy measure for ROS production because it is an intracellular scavenger of ROS and a key component of the enzymatic antioxidant system (31).

Bactericidal antibiotic-treated mice showed elevated lipid peroxidation in their peripheral blood; these increases were statistically significant after 16 weeks of treatment (Fig. 6). The treated mice also exhibited reduced glutathione levels after 2 weeks of antibiotic treat-

ment, which was significantly decreased after 16 weeks of treatment (Fig. 6). However, only ciprofloxacin led to a significant increase in ROS in treated animals after 16 weeks.

To further characterize the in vivo oxidative stress response, we evaluated the transcriptional changes in key genes involved in antioxidant defense mechanisms. Mammary glands were extracted from mice treated with bactericidal antibiotics in the presence and absence of NAC or the bacteriostatic antibiotic tetracycline. From these tissues, the expression of the antioxidant defense genes—*Sod1*, *Sod2*, *Gpx1*, and *Foxo3a*—was measured by quantitative polymerase chain reaction (qPCR). After 2 weeks of treatment, these genes showed more than a 2-fold increase in expression in tissues extracted from bactericidal antibiotic-treated mice, which were further elevated to ~10-fold change after 16 weeks of treatment (fig. S15A). These genes were not up-regulated

in the tissues of tetracycline-treated mice. Tissues from mice treated with a combination of bactericidal antibiotic + NAC or NAC alone showed decreased expression of the tested genes compared to antibiotic treatment (fig. S15B). These data support our *in vitro* results, demonstrating that NAC rescued bactericidal antibiotic-induced oxidative damage in MCF10A human epithelial cells (Fig. 5).

To evaluate bactericidal antibiotic-induced oxidative stress at the tissue level, we measured the accumulation of oxidative tissue damage directly in mammary glands. Quantification of protein carbonylation (Fig. 7A) and lipid peroxidation (Fig. 7B) revealed that bactericidal antibiotic-induced oxidative damage was significantly mitigated by co-administration of NAC. We also found no significant difference between tetracycline-treated and untreated mice. To further characterize these effects, we measured oxidative damage of proteins (nitration) caused by peroxynitrite, the product of the reaction between superoxide and nitric oxide. After 16 weeks of bactericidal antibiotic treatment, with and without NAC, as well as the bacteriostatic antibiotic tetracycline, we evaluated the number and size of anti-nitrotyrosine-stained foci in mouse mammary glands. Animals treated with bactericidal antibiotics showed increased protein nitration compared to untreated mice, whereas mice treated with tetracycline showed no change (Fig. 7C). The observed increase in protein damage caused by bactericidal antibiotic-treated mice was rescued by co-administration of NAC. Immunohistochemical analysis revealed that the damaged proteins in bactericidal antibiotic-treated mice tended to localize to the cytoplasm of mammary ductal epithelial cells (Fig. 7D). However, damaged proteins in NAC-treated mice, in the presence or absence of bactericidal antibiotics, were more

likely to be found in the connective tissue, localizing to the nuclei of adipocytes and stromal cells (Fig. 7D).

DISCUSSION

The emergence of drug-resistant bacterial strains has been one unintended consequence of the ubiquitous and frequent use of antibiotics in medicine and food production. With the belief that antibiotics specifically target bacteria, the consequences of how they interact with mammalian cells have largely been overlooked, despite instances of known adverse effects, including ototoxicity (1), nephrotoxicity (2), and tendinopathy (3). Gaining a deeper mechanistic understanding of the effects of commonly prescribed antibiotics in mammalian systems, particularly for extended periods of treatment, is critical for achieving a clear safety profile for these drugs.

According to the endosymbiotic theory, mitochondria originated from free-living, aerobic bacteria (22). It is likely then that antibiotics target mitochondria and mitochondrial components, similar to their action in bacteria. Indeed, previous studies in mammalian systems have revealed parallel antibiotic-target interactions in mitochondria (15–19, 32). It has been shown, for instance, that aminoglycosides target both bacterial (33) and mitochondrial ribosomes (16), quinolones target bacterial gyrases (34) and mtDNA topoisomerases (15), and β -lactams inhibit bacterial cell wall synthesis (35) and mitochondrial carnitine/acylcarnitine transporters (19). The work presented here advances our understanding of antibiotic action in mammalian systems in two distinct and important ways. First, we show that, regardless of their molecular targets, three major classes of bactericidal antibiotics—quinolones, aminoglycosides, and β -lactams—induce ROS production in mammalian cells, leading to DNA, protein, and lipid damage. Second, we demonstrate that these deleterious effects are produced by clinically relevant doses of bactericidal antibiotics, both in cell culture and in mice. These findings are analogous to our previous work in bacteria, in which we showed that clinically relevant doses of bactericidal antibiotics induce a common oxidative damage pathway (4).

Identifying bactericidal antibiotics as a cause of ROS overproduction and mitochondrial dysfunction in mammalian cells provides a basis for developing therapeutic strategies that could help alleviate adverse side effects associated with antibiotics. For instance, by co-administering an intracellular antioxidant, in this case NAC, we showed that ROS levels and oxidative damage induced by bactericidal antibiotics could be abrogated while having little effect on the bacterial killing efficacy of the antibiotics. Additionally, we showed that bacteriostatic antibiotics, such as tetracycline, did not contribute to the overproduction of harmful ROS in mammalian cells. When appropriate for patient health, substituting a bacteriostatic antibiotic for a bactericidal antibiotic could be a simple treatment strategy aimed at preventing cellular oxidative damage.

To establish the link between bactericidal antibiotics and ROS production, we have limited this study to antibiotic-treated human cell lines and mice. It will be important to extend our investigation to human subjects to confirm our findings, prove their relevance to humans, and maximize the translational value of our work. Epidemiologic studies could provide valuable insight into the clinical implications of antibiotic-induced oxidative damage and define some of the risks associated with bactericidal antibiotic exposure. Another limitation to consider is the set of antibiotics tested. Although we present results on

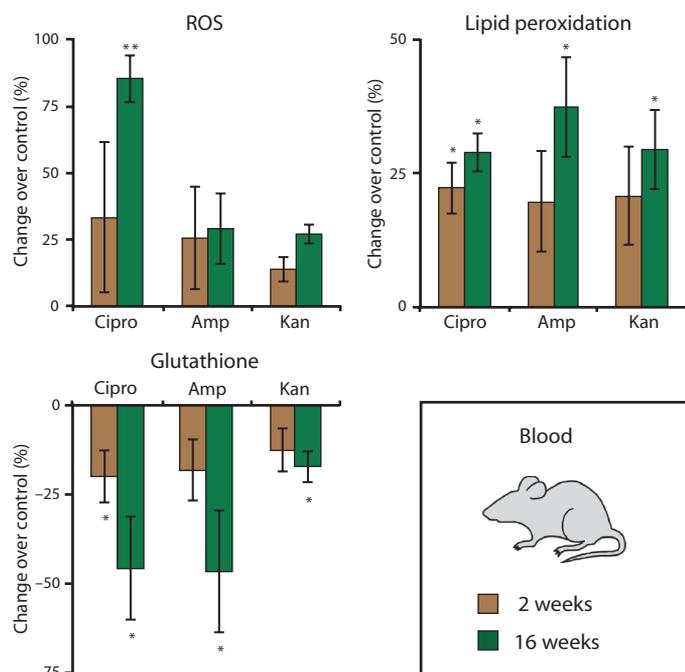


Fig. 6. Bactericidal antibiotics induce oxidative damage in mice. Oxidative stress markers were measured in blood drawn from wild-type mice treated with ciprofloxacin (12.5 mg/kg per day), ampicillin (28.5 mg/kg per day), or kanamycin (15 mg/kg per day) for 2 or 16 weeks. Data are means \pm SEM ($n \geq 3$ animals per treatment group). Comparisons between treatments and untreated controls were made using a Student's *t* test (* $P < 0.05$, ** $P < 0.01$).

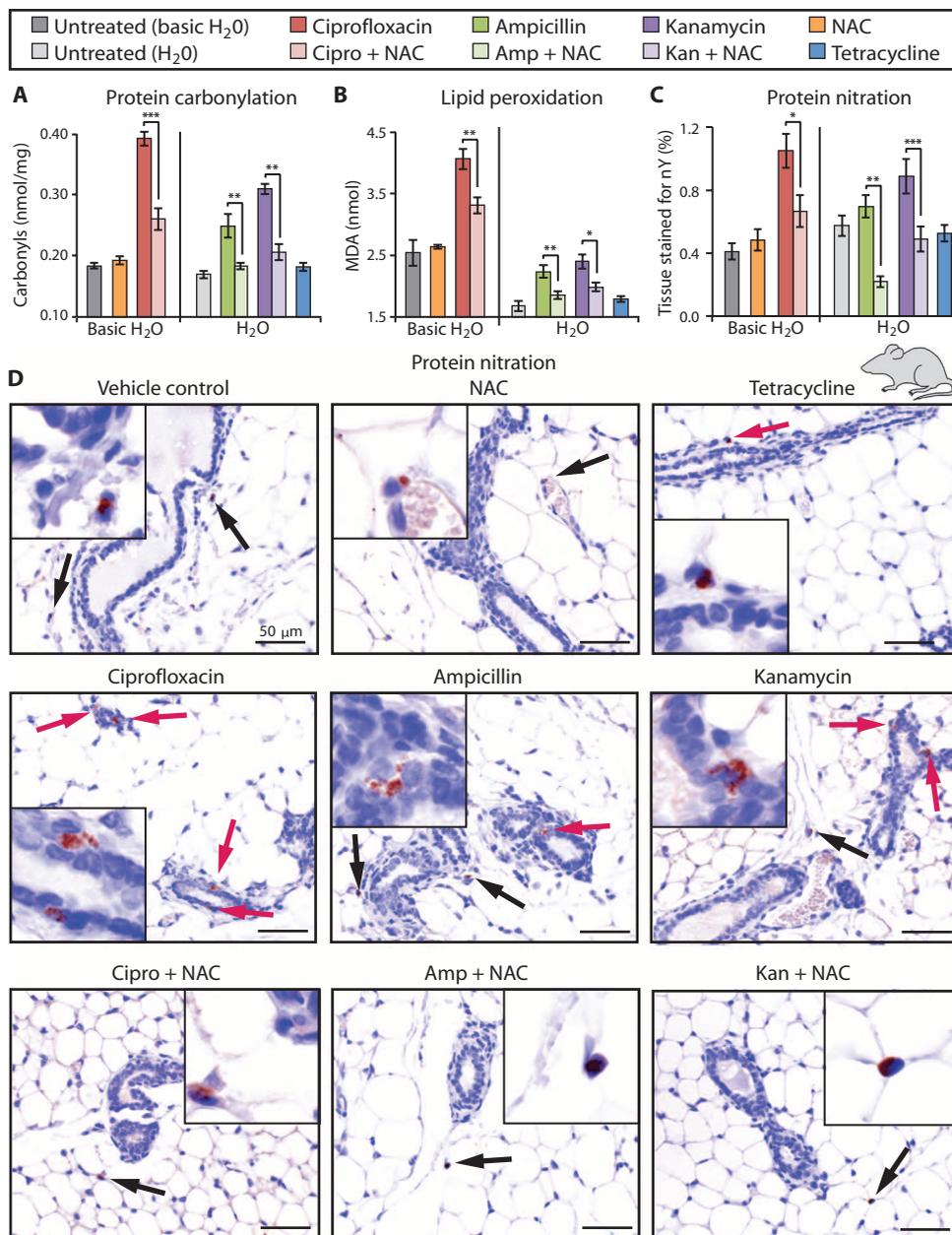


Fig. 7. Oxidative damage induced in mouse mammary gland tissue by bactericidal antibiotics is rescued by an antioxidant. Mouse mammary glands were harvested 16 weeks after being treated with bactericidal antibiotics [ciprofloxacin (12.5 mg/kg per day), ampicillin (28.5 mg/kg per day), and kanamycin (15 mg/kg per day)], with and without NAC (1.5 g/kg per day), or a bacteriostatic antibiotic [tetracycline (27 mg/kg per day)]. Ciprofloxacin requires basic water (pH 8.0) to dissolve; thus, the antibiotic treatments in each plot have been grouped according to their control treatments: basic H₂O (pH 8) for ciprofloxacin, and H₂O for ampicillin, kanamycin, and tetracycline. **(A and B)** Protein carbonylation and lipid peroxidation were measured in mouse mammary gland tissue collected from treated mice. **(C)** Mammary tissue was stained with an anti-nitrotyrosine (nY) antibody to measure oxidative protein damage (nitration). Quantification of the protein nitration was defined as the percentage of total tissue area that was stained with the anti-nitrotyrosine antibody. Data are means \pm SEM ($n \geq 3$ animals per treatment group). Comparisons between treatment + NAC and treatments were made using a Student's *t* test (* $P < 0.5$, ** $P < 0.01$, *** $P < 0.001$). **(D)** Representative immunohistochemical images that were quantified in **(C)**. Red arrows indicate protein damage foci (nitration) to ductal epithelial cells; black arrows point to protein damage to connective tissue cells (adipocytes and stromal cells).

three major classes of bactericidal antibiotics, interrogation across a broader range of antibiotic classes is warranted before conclusions can be drawn about the general category of bactericidal antibiotics. Last, it has been shown that both bactericidal and bacteriostatic antibiotics target mitochondrial components (17–19, 23, 24), yet we observed a marked differential effect between bactericidal and bacteriostatic antibiotic treatments on cellular oxidative damage. Disentangling these differences in antibiotic action will be essential to gain a full understanding of how antibiotics interact with mammalian systems.

In humans, it is likely that the oxidative stress and related oxidative cellular damage induced by bactericidal antibiotics underlie many of the adverse side effects associated with these antibiotics (1–3). In particular, patients with compromised antioxidant defense systems or those genetically disposed to developing a mitochondrial dysfunction disease (36) might be at greater risk from bactericidal antibiotic treatments. Thus, rapid detection (for example, via measurements from peripheral blood) and mitigation of these adverse effects could have important implications for patient care. Our data suggest that oxidative damage markers can be measured in the blood, but further work is needed to determine the efficacy of such a test in humans. Once detected, mitigation strategies such as co-administration with an antioxidant or treatment with bacteriostatic antibiotics could be used. It will be intriguing to explore these possibilities with appropriate clinical trials, with the goal of developing effective antibacterial therapies with minimal adverse side effects.

MATERIALS AND METHODS

Study design

The objective of our work was to investigate the effects of clinically relevant doses of bactericidal and bacteriostatic antibiotics on mammalian systems in vitro and in vivo. A human mammary epithelial cell line (MCF10A) served as our in vitro model, whereas 8-week-old female wild-type mice (FVB) were used to study the effect of antibiotics in vivo. To understand the putative role of antibiotic-induced ROS in mammalian cells and accumulation of oxidative damage, we used an antioxidant

(NAC) alone or in combination with the bactericidal antibiotics. In vitro, ROS was measured with fluorescent indicators [CM-H₂DCFDA, Amplex Red and horseradish peroxidase (HRP), MitoSox Red], and oxidative damage to DNA (8-OHdG), proteins (carbonylation), and lipids (peroxidation) was quantified, whereas the functional implications were assessed by evaluation of mitochondrial respiration (oxygen consumption). In vivo, oxidative stress was evaluated in the peripheral blood of treated mice with fluorescent indicators (H₂DCFDA, fluor-DHPE, DCF), whereas oxidative damage to mammary gland tissue (protein carbonylation, lipid peroxidation, protein nitration) was assessed by immunohistochemistry and qPCR quantification of key antioxidant genes. Mice were purchased from the vendor and randomly assigned to treatment groups. Antibiotics were prepared and administered in a nonblinded fashion, both in vitro and in vivo. Experiments included three [Figs. 1 (C, D, F, and G), 2 (A, B, and E), 4, 5B, and 6 and figs. S3, S5 to S7, S9 to S11, S13, and S15], four [Fig. 1 (A and B), 5 (A and D to F), and 8 (A to C) and figs. S1, S2, S4, and S14], five (Figs. 1E and 3), six (Fig. 2, C and D), or eight (fig. S8) replicates per group.

Cell culture and antibiotic treatments

Human mammary epithelial cells, MCF10A [American Type Culture Collection (ATCC)], were maintained in Dulbecco's modified Eagle's medium and Ham's F12 50/50 Mix (Cellgro, Mediatech) supplemented with 5% horse serum, epidermal growth factor (100 µg/ml), hydrocortisone (1 mg/ml), cholera toxin (1 mg/ml), insulin (10 mg/ml), and 5 ml of penicillin (10,000 U/ml) and streptomycin (10,000 µg/ml). Hereafter, this medium will be referred to as "complete medium." For antibiotic treatments, complete medium was used without the addition of penicillin/streptomycin. We refer to this medium as "treatment medium." All supplements were purchased from Sigma-Aldrich.

Before antibiotic treatments, cells were washed with phosphate-buffered saline (PBS), detached with 0.25% trypsin-EDTA (Life Technologies), and seeded in 6-, 12-, 24-, or 96-well plates (Corning) in treatment medium. After 24 hours, peak serum levels (25) of antibiotics were added: ciprofloxacin (10 µg/ml), ampicillin (20 µg/ml), kanamycin (25 µg/ml), tetracycline (10 µg/ml), and spectinomycin (100 µg/ml) (Fisher Scientific). Cells were plated at roughly equal numbers (near confluence) at each measurement time point. NAC (10 mM; Sigma-Aldrich) was used as an intracellular ROS scavenger. For antibiotic treatments in conjunction with NAC, cells were incubated in treatment medium plus NAC (pH adjusted to 7.4) for 2 hours and then washed with PBS before the addition of antibiotics. The growth conditions for the additional cell lines tested are described in the Supplementary Materials and Methods.

MCF10A ρ_0 cell line

MCF10A rho-zero (ρ_0) cells are MCF10A cells that have been cultured to eliminate mtDNA. Following the protocol from King and Attardi (37), MCF10A cells were incubated in complete medium supplemented with D-glucose (4.5 g/liter), ethidium bromide (50 ng/ml), uridine (50 µg/ml), and pyruvate (1 mM) for 6 weeks. After complete elimination of mtDNA (as verified through PCR described below), cells were maintained in complete medium supplemented with D-glucose (4.5 g/liter), uridine (50 µg/ml), and pyruvate (1 mM). A set of MCF10A control cells was grown alongside the MCF10A ρ_0 cells maintained in complete medium supplemented with D-glucose (4.5 g/liter), uridine (50 µg/ml), and pyruvate (1 mM). The verification of ρ_0 is described in the Supplementary Materials and Methods.

ROS, H₂O₂, and mitochondrial superoxide

To detect ROS, cells plated in 6- or 96-well tissue culture plates were washed twice with prewarmed PBS and then incubated with 10 µM CM-H₂DCFDA (Life Technologies) at 37°C for 45 min. Cells were washed with PBS to remove excess dye and allowed to recover in treatment medium at 37°C for 15 min in the dark. Imaging was carried out on a fluorescence-enabled inverted microscope, and quantification was done with a BD FACSAria II flow cytometer (Becton Dickinson) or SpectraMax M5 microplate reader (Molecular Devices) at an excitation/emission of 495/525 nm. This protocol was repeated for all other tested cell lines.

To detect extracellular hydrogen peroxide, we adapted a protocol from Panopoulos *et al.* (38). Cells plated in 96-well tissue culture plates were washed once with Krebs-Ringer phosphate buffer (KRPG). KRPG containing 0.1% horse serum (KRPG 0.1%) and clinical levels of antibiotics were added to the cells in a total volume of 50 µl. Next, 50 µl of prewarmed KRPG containing 100 µM Amplex Red reagent and horseradish peroxidase (0.2 U/ml) (reaction mixture) was added to each well. Absorbance was measured at 560 nm after the addition of reaction buffer with a SpectraMax M5 precision microplate reader (Molecular Devices).

To detect mitochondrial superoxide, cells plated in 6- or 96-well tissue culture plates were washed twice with prewarmed PBS and then incubated with 5 µM MitoSox Red at 37°C for 10 min in the dark. After incubation, cells were washed and resuspended in prewarmed Hanks' balanced salt solution (Fisher Scientific). Quantification was completed with a BD FACSAria II flow cytometer or a SpectraMax M5 microplate reader at an excitation/emission of 510/580 nm.

Oxidative DNA, protein, and lipid damage

8-OHdG levels were quantified with the OxiSelect Oxidative DNA Damage ELISA Kit (Cell Biolabs), protein carbonylation was measured with the Protein Carbonyl ELISA Kit (Enzo Life Sciences), and lipid peroxidation was measured with the Lipid Peroxidation MDA Assay Kit (Abcam). All assays were performed according to the manufacturer's protocol and are described in the Supplementary Materials and Methods.

Mitochondrial ETC complex activity

Direct inhibition of the activity for each of the five ETC complexes was measured with the MitoTox Complete OXPHOS Activity Assay Panel (Abcam), following the manufacturer's protocol. Each of the five complexes was captured from isolated bovine heart mitochondria in their functionally active state with highly specific monoclonal antibodies attached to 96-well microplates. IC₅₀ values for known inhibitors of each of the five complexes were used as positive controls: rotenone (complex I, 17.3 nM), thenoyltrifluoroacetone (complex II, 30 µM), antimycin A (complex III, 22 nM), KCN (complex IV, 3.2 µM), and oligomycin (complex V, 8 nM). The same positive controls were tested on each complex and used as negative controls for off-target complexes (for example, rotenone was a negative control for complexes II to V). For each of the complexes treated with antibiotics [ciprofloxacin (10 µg/ml), ampicillin (20 µg/ml), kanamycin (25 µg/ml), tetracycline (10 µg/ml), or spectinomycin (100 µg/ml)], activity was determined by measuring the decrease in absorbance in milli-optical density per minute at room temperature and at specified wavelengths [340 nm (I and V), 600 nm (II), and 550 nm (III and IV)] in kinetic mode [every minute for 2 hours (I) and 1 hour (II, IV, and V) and every 20 s for 5 min (III)] with a SpectraMax M5 microplate reader.

Metabolic activity, mitochondrial potential, and ATP

Metabolic activity was assayed with an XTT cell proliferation kit (ATCC), cellular ATP was measured with the ATPlite Luminescence Assay kit (Perkin Elmer), and mitochondrial potential was calculated with the ratio of TMRE to MitoTracker Green (Life Technologies). All assays were performed according to the manufacturer's protocol and are described in the Supplementary Materials and Methods.

Mitochondrial morphometry

Primary human mammary epithelial cells (HMEC) were plated in glass-bottom confocal dishes in treatment medium 24 hours before addition of antibiotics. Cells were washed once with PBS and treated with antibiotics for 24 or 96 hours. After treatment, cells were washed with 1× PBS and incubated in 7 nM TMRE and 10 μM MitoTracker Green for 30 min. After incubation, cells were returned to fresh treatment medium with only TMRE (7 nM) and immediately imaged on a Zeiss LSM710 confocal microscope.

Mitochondrial morphometry was carried out with the particle analysis function in the image processing software ImageJ [National Institutes of Health (NIH)] (39), as described in the Supplementary Materials and Methods.

Mitochondrial oxygen consumption

OCRs were measured at 37°C with an XF24 extracellular analyzer (Seahorse Bioscience). MCF10A cells were seeded at a density of 40,000 cells per well on XF24 tissue culture plates overnight and then treated with antibiotics for 6 and 96 hours. Measurements of OCR were done according to Seahorse Bioscience protocols as described in the Supplementary Materials and Methods.

Bacterial killing

The growth and survival of untreated exponential-phase wild-type *E. coli* (MG1655) were compared to cultures treated with bactericidal antibiotics alone [ciprofloxacin (5 μg/ml), ampicillin (5 μg/ml), or kanamycin (5 μg/ml)], 10 mM NAC alone, or a combination of bactericidal antibiotics supplemented with NAC for 3 hours. Cells were grown overnight at 37°C and 300 rpm in a light-insulated shaker and then diluted 1:500 in 25-ml LB in a 250-ml flask. Cultures were grown to an optical density (OD₆₀₀) of about 0.3, as measured with the SPECTRAFluor Plus spectrophotometer (Tecan). Antibiotics were added at this point. For bacterial count measurements [colony-forming units (CFU)/ml], 100 μl of culture was collected 30 min and 1, 2, and 3 hours after addition of antibiotics, washed twice with filtered 1× PBS (pH 7.2) (Fisher Scientific), and then serially diluted in 1× PBS. Ten microliters of each dilution was plated onto 120-mm square dishes (BD Biosciences) containing LB Agar (Fisher Scientific), and plates were incubated at 37°C overnight. Colonies were counted, and CFU/ml values were calculated with the following formula:

$$\frac{(\# \text{colonies}) \times 10^{\text{dilution factor}}}{(\text{volume plated}) \times 1000}$$

Animal studies and tissue collection

Six- to 8-week-old female FVB/NJ mice (Jackson Laboratory) were administered one of the following treatments: ciprofloxacin (12.5 mg/kg per day), ampicillin (28.5 mg/kg per day), kanamycin (15 mg/kg per day), with and without NAC (1.5 g/kg per day), tetracycline (13.5 mg/kg per day), and vehicle only (basic water, pH 8.0, or deionized water). Solu-

tions were made fresh every 3 days and administered in the drinking water for mice to feed ad libitum. Doses were achieved on the basis of an average mouse weight of 20 g and an approximate intake of drinking water of 4 ml per day. After 2 or 16 weeks, the animals were bled and euthanized. Mammary tissue was collected from each mouse, preserved in RNAlater (Ambion, Life Technologies) for real-time qPCR and 10% buffered formalin for immunohistochemistry, and flash-frozen for protein extraction. The Institutional Animal Care and Use Committee approved all mouse experiments. Quantitative reverse transcription PCR of oxidative stress genes is described in the Supplementary Materials and Methods.

Mouse blood oxidative stress

To measure general oxidative stress, 1×10^7 peripheral blood cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Life Technologies), dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich), at a final concentration of 0.4 mM at 37°C for 15 min at 5% CO₂. Reduced glutathione levels were measured by labeling 2×10^7 blood cells with mercury orange (Sigma-Aldrich), dissolved in DMSO, at a final concentration of 40 μM for 3 min at room temperature. To measure lipid peroxidation, we washed 2×10^7 blood cells with PBS and labeled with *N*-(fluorescein-5-thiocarbonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethyl-ammonium salt (fluor-DHPE) (Life Technologies), dissolved in ethanol, at a final concentration of 50 μM for 1 hour at 37°C in a 5% CO₂ incubator with continuous agitation. After the incubation period with each dye, blood cells were washed twice with PBS to remove unbound label, resuspended in PBS, and analyzed by flow cytometry (FACS Fortessa, Becton Dickinson). A 488-nm argon laser beam was used for excitation. Blood cells labeled with DCF and fluor-DHPE were detected by FL-1 PMT with linear amplification, whereas mercury orange-labeled red blood cells were detected by FL-2 PMT with log amplification. For each assay, treated and untreated unstained cells were used as controls. Instrument calibration and settings were performed with CaliBRITE-3 beads (Becton Dickinson). The mean fluorescence channel of the entire population was calculated for DCF, glutathione, and lipid peroxidation by the fluorescence-activated cell sorting (FACS)-equipped BD FACSDiva software.

UTI mouse model

Eight-week-old C57BL/6 female mice were inoculated with 50 μl of 8% (w/v) mucin solution in sterile saline containing 2×10^9 *E. coli* (MG1655) cells, via transurethral catheterization into their bladders, as described previously (40). Briefly, mice were anesthetized with 2 to 4% isoflurane. Urinary catheters (30-gauge × 1/2-inch hypodermic needle aseptically covered with polyethylene tubing) were coated in medical-grade, sterile lubricating jelly. The bladder of the mouse was gently massaged to expel urine. The lubricated catheter was inserted into the urethral opening. It was then pushed into the urethra until the base of the needle reached the urethral opening. Once fully inserted, 50 μl of the inoculum (containing 2×10^9 *E. coli* cells) was injected directly into the bladder.

Infected animals received ciprofloxacin (50 μg/ml), NAC (10 mM), ciprofloxacin and NAC, or vehicle (PBS) only, via intraperitoneal delivery 24 hours after inoculation. After treatment, animals were observed for an additional 24 or 48 hours. At the end of the experiment, animals were euthanized by CO₂ asphyxiation followed by cervical dislocation. Bladders were collected in 1 ml of PBS and homogenized for 30 s for subsequent quantification of bacterial load. For the CFU/

bladder measurements, the homogenized bladder was serially diluted in PBS (pH 7.2). A 200- μ l portion of each dilution was plated in LB-agar plates and incubated overnight at 37°C. The colonies were counted, and CFU/bladder was calculated with the following formula:

$$\frac{5(\#\text{colonies})(\text{dilution factor})}{\text{volume plated}}$$

Histology and immunohistochemistry

Six- to 8-week-old female FVB/NJ mice (Jackson Laboratory) were administered ciprofloxacin (12.5 mg/kg per day), ampicillin (28.5 mg/kg per day), kanamycin (15 mg/kg per day), with and without NAC (1.5 g/kg per day), tetracycline (13.5 mg/kg per day), or vehicle only (basic or deionized H₂O). Solutions were made fresh every 3 days and administered in the drinking water for 16 weeks. Mice were euthanized, and mammary glands were collected and stored in 10% buffered formalin overnight at 4°C, transferred to 70% ethanol, and stored at room temperature until sectioning. Tissues were embedded in paraffin and sectioned for subsequent staining. Mammary gland sections were stained with anti-nitrotyrosine polyclonal antibody (Millipore), followed by anti-rabbit immunoglobulin G conjugated to HRP (Vector Laboratories). Slides were counterstained with hematoxylin. Image capture and analysis are described in the Supplementary Materials and Methods.

Statistical analysis

A one-tailed Student's *t* test was performed on measurements of oxidative stress in the blood, UTI model, and for comparisons between antibiotic-treated groups and groups treated with antibiotic and NAC. For all other statistical analyses, a two-tailed Student's *t* test was performed to compare treatment groups to untreated (control) groups. We assumed equal variance for all groups.

SUPPLEMENTARY MATERIALS

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Materials and Methods

- Fig. S1. Dose-response and time-course measurements of ROS levels in antibiotic-treated cells.
- Fig. S2. Effect of antibiotic treatment on cell viability in vitro.
- Fig. S3. ROS production measured across different mammalian cell types treated with antibiotics.
- Fig. S4. Dose-response and time-course measurements of mitochondrial superoxide production in antibiotic-treated cells.
- Fig. S5. Dose-response and time-course measurement of extracellular hydrogen peroxide in antibiotic-treated cells.
- Fig. S6. DNA damage in human mammary epithelial cells treated with antibiotics.
- Fig. S7. Effect of antibiotics on the function of isolated ETC complexes.
- Fig. S8. ROS production in mammalian cells lacking mtDNA treated with antibiotics.
- Fig. S9. NAC reduces bactericidal antibiotic-induced ROS levels.
- Fig. S10. OCR in antibiotic- and NAC-treated cells.
- Fig. S11. OCR in mammalian cells.
- Fig. S12. NAC rescues bactericidal antibiotic-induced DNA damage.
- Fig. S13. Bactericidal antibiotic efficacy is unaffected by NAC in vitro.
- Fig. S14. Bactericidal antibiotic efficacy is unaffected by NAC when tested in vivo in a murine UTI model.
- Fig. S15. Bactericidal antibiotics induce expression of antioxidant defense genes in mouse mammary gland tissue.

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