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"Science" 339, 1210 (2013);
DOI: 10.1126/science.1232751

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Cell Death from Antibiotics Without the Involvement of Reactive Oxygen Species

Yuanyuan Liu and James A. Imlay*

Recent observations have suggested that classic antibiotics kill bacteria by stimulating the formation of reactive oxygen species (ROS). If true, this notion might guide new strategies to improve antibiotic efficacy. In this study, the model was directly tested. Contrary to the hypothesis, antibiotic treatment did not accelerate the formation of hydrogen peroxide in *Escherichia coli* and did not elevate intracellular free iron, an essential reactant for the production of lethal damage. Lethality persisted in the absence of oxygen, and DNA repair mutants were not hypersensitive, undermining the idea that toxicity arose from oxidative DNA lesions. We conclude that these antibiotic exposures did not produce ROS and that lethality more likely resulted from the direct inhibition of cell-wall assembly, protein synthesis, and DNA replication.

In recent decades, the growing number of antibiotic-resistant pathogens has spurred efforts to further understand and improve the efficacy of the basic antibiotic classes. Most clinically used antibiotics target cell-wall assembly, protein synthesis, or DNA replication. However, recent reports have raised the possibility that although these antibiotics block growth by directly inhibiting the targets mentioned above, they may owe their lethal effects to the indirect creation of reactive oxygen species (ROS) that then damage bacterial DNA (1–10). The evidence supporting this proposal included the observation that cell-penetrating dyes were oxidized more quickly inside antibiotic-treated bacteria (3–8). Furthermore, iron chelators (3, 4, 7–9), which suppress hydroxyl-radical–generating Fenton chemistry, and thioreua (4, 6, 8–10), a potential scavenger of hydroxyl radicals, lessened toxicity. Mutations that diminish fluxes through the tricarboxylic acid cycle were protective (3–5), suggesting a key role for respiration, and DNA-repair mutants were somewhat sensitive (4, 8). Systems analysis of aminoglycoside-treated *Escherichia coli* suggested a model that fits these data (5). It was postulated that interference with ribosome progression would release incomplete polypeptides, some of which are translocated to the cell membranes where they might trigger envelope stress. The Arc regulatory system is perturbed, potentially accelerating respiration and thereby increasing the flux of superoxide and hydrogen peroxide into the cell interior. These two oxidants have known sequelae that ultimately lead to DNA damage. Specifically, superoxide and hydrogen peroxide damage the iron-sulfur clusters of dehydratases (II, 12), releasing iron atoms and elevating the pool of intracellular unincorporated iron (13, 14). This iron can then react with hydrogen peroxide in the Fenton reaction, generating hydroxyl radicals that either directly damage DNA (15) or indirectly oxidize the deoxyribonucleotide pool, which is subsequently incorporated into DNA (16). This scenario could explain the observed oxidation of intracellular dyes, protection by scavengers and chelators, a requirement for respiration, and the sensitivity of DNA-repair mutants.

This model is plausible, so we devised experiments to directly test the molecular events that underpin it. The bacterial strain (*E. coli* MG1655), growth medium (LB), and antibiotic doses were chosen to match those of previous studies (4). Kanamycin was used to target translation, ampicillin to block cell-wall synthesis, and norfloxacin to disrupt DNA replication. Superoxide and hydrogen peroxide are generated inside cells when flavoenzymes inadvertently transfer a fraction of their electron flux directly to molecular oxygen (15, 17). Thus, neither of these ROS can be formed under anoxic conditions.

**Fig. 1.** Antibiotic efficacy does not require oxygen or H₂O₂. (A to C) Wild-type cells were treated with ampicillin (A), norfloxacin (B), or kanamycin (C) in the presence (solid squares) or absence (open squares) of oxygen. In (C), anoxic killing was also tested in the presence of 40 mM KNO₃ (gray squares). (D to F) Wild-type cells (solid squares, MG1655) and congenic *Hpx*⁻ cells (open diamonds, AL427) were treated with antibiotics ampicillin (D), norfloxacin (E), or kanamycin (F). Results are representative of at least three biological replicates.

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that of the housekeeping gene cells, and the expression of OxyR-responsive genes was quantified by qRT-PCR. Signals were normalized to additionally treated with exogenous H2O2 at room temperature for 10 min before mRNA collection. (Hpx before and after antibiotic incubations (C). As a positive control, expression was measured in wild-type and ongoing rate of H2O2 production was measured. The redox-cycling compound paraquat (H) was included with the inhibitory effect of norfloxacin on DNA metabolism (22) (Fig. 1E).

We directly tested the prediction that antibiotic treatment would augment respiration and thereby increase superoxide and H2O2 formation. The respiratory chain is normally a minor source (<20%) of these oxidants (17), so a very large flux increase would be needed to substantially amplify total intracellular oxidant production. However, measurements of oxygen consumption with a Clark-type electrode showed that respiration slowly declined, rather than increased, when cells were treated with kanamycin (Fig. 2A). This trend persisted throughout the period during which the number of viable cells declined by >99%. Norfloxacin had little effect on respiration. Ampicillin transiently increased respiration, perhaps by causing envelope damage that dissipated the back-pressure of the proton motive force, before cell lysis ended metabolism. In no case did respiration accelerate enough to be a sufficient explanation for toxicity.

E. coli features a transcription factor, OxyR, that is activated by H2O2 whenever its levels approach the concentrations (0.5 to 1 µM) sufficient to damage enzymes or DNA (23). The OxyR regulon includes genes whose products assist in H2O2 scavenging (katG and ahpCF), freeiron control (dps, fer, and yaaA), and enzyme protection (mntH and sufABCDDE) (15, 24). When cells were treated with ampicillin and norfloxacin, they accumulated lethal damage within 1 hour; however, real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis indicated that OxyR-controlled genes were not induced (Fig. 2B). These antibiotic-treated cells robustly activated katG, ahpC, and yaaA when exogenous H2O2 was added as a control. (After 1 hour, kanamycin-treated cells failed to respond even to authentic H2O2, perhaps because OxyR protein was depleted in these translationally blocked cells.) This result was confirmed by measurements of katG::lacZ expression (Fig. 2, C and D). Thus, neither ampicillin nor norfloxacin create enough H2O2 stress to trigger the natural H2O2 sensor within the cell.

The rate of intracellular H2O2 formation can be directly measured using catalase/peroxide mutants. Endogenous H2O2 rapidly diffuses from these cells out into the growth medium, and the rate of its accumulation can be quantified by direct assay (Fig. S1) (17). When cells were treated with lethal concentrations of kanamycin and ampicillin, H2O2 production did not increase (Fig. 2, E to H). In norfloxacin-treated cells H2O2 formation increased slightly (~2-fold) after 1 hour but not at all within the first 30 min when >99% of cells accumulated lethal damage. In contrast, when cells were treated with a nonlethal dose of the redox-cycling drug paraquat, H2O2 production was stimulated ~7-fold. We conclude that these classic bactericidal antibiotics did not promote H2O2 formation.

There is one other way that hydroxyl-radical formation might be accelerated: by increases in the amount of unincorporated iron. Iron can accumulate inside cells under conditions of superoxide stress, due to the destruction of labile enzymic iron-sulfur clusters (13, 14), and this mechanism was postulated to occur during antibiotic exposure (4). We monitored the status of 6-phosphogluconate dehydrogenase, an iron-sulfur
enzyme that quickly loses activity in superoxide-stressed cells. We observed that the overall enzyme activity diminished during hours of antibiotic treatment, but the fraction of enzymes with incomplete clusters did not significantly rise (Fig. 3, A to C). In contrast, in control mutants that lack superoxide dismutase, the entire enzyme population was inactive due to cluster damage.

Levels of intracellular unincorporated iron were directly imaged by whole-cell electron paramagnetic resonance (EPR) spectroscopy (14). These levels did not rise in kanamycin- or norfloxacin-treated cells (Fig. 3, D and E). (Levels could not be measured after ampicillin treatment, as lysing cells could not be concentrated for the measurement.) In contrast, free-iron levels were increased 4-fold in fur regulatory mutants that oversynthesize iron import systems (Fig. 3F). Notably, although these fur mutants were slightly more sensitive to ampicillin, they were actually more resistant to kanamycin and norfloxacin (fig. S2). This result, also observed by others (3), indicates that intracellular free-iron levels do not correlate with antibiotic sensitivity.

DNA is substantially damaged when hydroxyl radicals are rapidly formed inside cells, and mutants that are deficient in the excision or recombinational repair of oxidative DNA lesions are particularly vulnerable (15). The extreme sensitivity of recA and polA mutants to exogenous H2O2 is illustrated in fig. S3A. However, recA strains exhibited little or no hypersensitivity to killing doses of kanamycin or ampicillin, suggesting that these antibiotics have little effect upon the rate of DNA damage (Fig. 3, G and H). The recA mutants were hypersensitive to norfloxacin (Fig. 3I), as expected, because this antibiotic directly introduces DNA strand breaks by interrupting the catalytic cycle of topoisomerases. The polA mutants were actually more resistant to kanamycin and ampicillin than were wild-type strains (fig. S3B), a phenomenon that likely reflects the slower growth rate of this mutant. In sum, these data indicate that, unlike H2O2 stress, these antibiotics do not introduce lethal damage into the DNA of cells.

Other workers noted that lower doses (1 to 2 µg/ml) of ampicillin slowly kill recA mutants while being merely bacteriostatic to wild-type strains (4, 8). We reproduced that result (fig. S4A). However, this behavior also occurred under anoxic conditions, when ROS are nonexistent (fig. S4B). The slight sensitivity of recA mutants may reflect defects in seption from their innate replication problems. At higher doses of ampicillin, i.e., at doses sufficient to kill wild-type bacteria, recombination proficiency had little or no impact on death rate.

Collectively, these data indicate that the known mechanisms of oxidative stress by ROS were not involved in causing the death of antibiotic-treated cells. Oxygen was dispensable for toxicity, and neither OxyR activity nor direct measurements of H2O2 formation indicated that ROS were formed at accelerated rates. Further, no customary markers of oxidative damage—enzyme damage, increases in free-iron levels, or hypersensitivity of DNA-repair mutants—were detected.

Fluorescein-based dyes such as hydroxy-phenyl fluorescein are more quickly oxidized within antibiotic-treated cells than in control cells, and this fact has been regarded as evidence that hydroxyl radicals are present (25). Indeed, thiourea, a presumptive scavenger of hydroxyl radicals, diminished the antibiotic-augmented fluorescence. However, we observed that thiourea efficiently blocked dye oxidation in a simple in vitro Fenton system, ethanol—another hydroxyl-radical scavenger of similar efficiency (26)—failed to do so, even at far higher doses (fig. S5, A and B). This result implies that the dye was actually oxidized by the high-valence iron (FeO2+) initially formed by the Fenton reaction, rather than by the hydroxyl radical to which it decomposes; thiourea, a sulfur compound, may have re-reduced the metal before it oxidized the dye. Indeed, the dye was very rapidly oxidized by horseradish peroxidase (fig. S5C), which has an analogous high-valence metal intermediate but does not release hydroxyl radicals (27). The dye was even oxidized by ferricyanide, a ferric-iron chelate of moderate oxidizing potential (+0.44 V) (fig. S5E).

Finally, the fluorescence of the oxidized dye
Killing by Bactericidal Antibiotics Does Not Depend on Reactive Oxygen Species

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Bactericidal antibiotics kill by modulating their respective targets. This traditional view has been challenged by studies that propose an alternative, unified mechanism of killing, whereby toxic reactive oxygen species (ROS) are produced in the presence of antibiotics. We found no correlation between an individual cell’s probability of survival in the presence of antibiotic and its level of ROS. An ROS quencher, thiourea, protected cells from antibiotics present at low concentrations, between an individual cell. Synthesis of these enzymes is also repressed when cells are fed glucose, and this treatment analogously diminished kanamycin sensitivity (fig. S7). Thus, it is advisable to follow up experiments that rely upon these agents with ones that employ more direct markers of oxidative stress.

We conclude that under our experimental conditions, these major classes of antibiotics did not exert their lethal actions through the known mechanisms of oxidative stress. Of course, this does not preclude the possibility that antibiotics trigger mechanisms of stress that do not involve the ROS that were tested here.

References and Notes


Acknowledgments: We thank D. Hassett (University of Cincinnati) for helpful conversations about this topic, and J. Collins, G. Walker, and members of their laboratories for generously sharing ideas, unpublished data, and strains. We also thank M. Nilges of the Illinois EPR Research Center for assistance. This work was supported by GM49060 from the National Institutes of Health. The authors declare no conflict of interest.

Supplementary Materials

www.sciencemag.org/cgi/content/full/339/6124/1210/DC1

Materials and Methods
Figs. S1 to S7
Table S1
References (30–36)
14 November 2012; accepted 24 January 2013
10.1126/science.1232751

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Bactericidal antibiotics are essential components of our antimicrobial arsenal. However, even the best bactericidal antibi-otics have limited efficacy against dormant persister cells, specialized survivors that are phenotypic variants of the wild type (1, 2). Bactericidal antibiotics from each of the three main classes have unique mechanisms of killing. Fluoroquinolones convert DNA gyrase/topoisomerase into an endonuclease (3); aminoglycosides cause mistranslation, leading to production of toxic peptides (4, 5); and β-lactams lead to autolysis (6, 7). In dormant persisters, targets of these antibiotics have limited activity, resulting in tolerance (2). Understanding the detailed mechanism of killing is essential for developing better therapeutics. Recently, an alterna-tive unified mechanism of antibiotic killing was proposed (8, 9), according to which bacteri-cidal compounds, irrespective of their mode of action, induce formation of reactive oxygen species (ROS) by activating the electron transport chain, which kills bacterial cells. The initial study reported that antibiotics induce ROS production and that their quenching by thiourea protects Escherichia coli cells from killing (8). Subse-quent studies report ROS-dependent killing in different bacterial species and detail the mechan-ism leading to cell death (10–15). The ROS hypothesis of antibiotic killing became widely accepted but does not account for many observations. For example, mutants lacking ROS production have not been reported among drug-resistant clinical isolates, and Streptococcus pneumoniae, which is highly susceptible to killing by bacte-ricidal antibiotics, lacks an electron transport chain (16, 17), the proposed source of ROS. Thus, we decided to reexamine the role of ROS in cell death and consequently found that killing by anti-biotics is unrelated to ROS production.

Thiourea was reported to protect E. coli from killing by norfloxacin, a fluoroquinolone anti-biotic (8). Norfloxacin was used at a fairly low concentration (0.25 μg/ml), only two to four times as high as the minimal inhibitory concentra-tion (MIC). The peak plasma concentration of norfloxacin has been reported to range from 1.3 to 1.6 μg/ml, with a half-life of 3 to 7 hours (18). We therefore examined the effect of thiourea on killing at a range of antibiotic concentrations that included clinically achievable levels.