**INTRODUCTION**

The high frequency of multidrug-resistant (MDR) bacterial infections and the lack of new antibiotics threaten the future of our health care system as we approach a postantibiotic era (1–3). In 2013, antibiotic-resistant infections in the United States cost an estimated $20 billion in direct health care costs and an additional $35 billion in lost productivity (2). Enterobacteriaceae, including carbapenem-resistant (CRE) *Escherichia coli* and extended spectrum β-lactamase (ESBL)–producing *Klebsiella pneumoniae (KPN)* are severely antibiotic-resistant and were recently designated priority 1 critical class bacterial pathogens in urgent need of effective antibiotics by the World Health Organization (Fig. 1A and table S1) (3). The gravity of the situation is highlighted by the fact that clinical isolates of these strains have up to 1000-fold higher 50% growth inhibition concentrations (GIC50) of antibiotic relative to sensitive/resistant breakpoint. Further, superoxide-generating nanoparticles in combination with ciprofloxacin reduced bacterial load in epithelial cells infected with *S. enterica* serovar Typhimurium and increased *Caenorhabditis elegans* survival upon infection with *S. enterica* serovar Enteritidis, compared to antibiotic alone. This demonstration highlights the ability to engineer superoxide generation to potentiate antibiotic activity and combat highly drug-resistant bacterial pathogens.

**ANTIBIOTICS**

Potentiating antibiotics in drug-resistant clinical isolates via stimuli-activated superoxide generation

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The rise of multidrug-resistant (MDR) bacteria is a growing concern to global health and is exacerbated by the lack of new antibiotics. To treat already pervasive MDR infections, new classes of antibiotics or antibiotic adjuvants are needed. Reactive oxygen species (ROS) have been shown to play a role during antibacterial action; however, it is not yet understood whether ROS contribute directly to or are an outcome of bacterial lethality caused by antibiotics. We show that a light-activated nanoparticle, designed to produce tunable flux of specific ROS, superoxide, potentiates the activity of antibiotics in clinical MDR isolates of *Escherichia coli*, *Salmonella enterica*, and *Klebsiella pneumoniae*. Despite the high degree of antibiotic resistance in these isolates, we observed a synergistic interaction between both bactericidal and bacteriostatic antibiotics with varied mechanisms of action and our superoxide-producing nanoparticles in more than 75% of combinations. As a result of this potentiation, the effective antibiotic concentration of the clinical isolates was reduced up to 1000-fold below their respective sensitive/resistant breakpoint. Further, superoxide-generating nanoparticles in combination with ciprofloxacin reduced bacterial load in epithelial cells infected with *S. enterica* serovar Typhimurium and increased *Caenorhabditis elegans* survival upon infection with *S. enterica* serovar Enteritidis, compared to antibiotic alone. This demonstration highlights the ability to engineer superoxide generation to potentiate antibiotic activity and combat highly drug-resistant bacterial pathogens.

Recent studies indicate that the presence and level of reactive oxygen species (ROS) during antibiotic treatment can increase antibiotic lethality (4–8), affect survival of persisters (4, 9), and contribute to the development of drug resistance (10). ROS, including superoxide radical *O2*−, peroxide *O2*2−, and hydroxyl radicals *OH*, are present in bacteria at low levels during normal aerobic respiration and are mitigated by antioxidant defenses in bacteria including superoxide dismutase (SOD) and catalase. However, at elevated levels, ROS can overwhelm bacterial defenses and cause significant damage to DNA and iron sulfur clusters, which reduces metalloenzyme activity (11). Here, we exploit the toxic nature of ROS by using an engineered quantum dot (QD) nanoparticle to controllably produce intracellular superoxide and enhance our ability to inhibit clinical MDR bacteria. We show that the engineered production of intracellular superoxide leads to robust potentiation of both bactericidal and bacteriostatic antibiotics in a range of clinical MDR bacteria despite their high level of resistance. Our work sheds light on the current debate regarding whether ROS contribute directly to or are a byproduct of bacterial lethality in the presence of antibiotics (4–7, 9, 12), and provides support for the former hypothesis because of the pathway topology inferred from synergistic interactions (13).

QDs are nanoparticles made of semiconducting materials that, when illuminated with light, generate excited electrons and holes across their nominal energy band gap (14), which are then available, at energy levels specific to the engineered QD size and material, for reduction and oxidation (redox) reactions (Fig. 1B). Unlike photodynamic therapy that uses light activation of different dyes and small molecules to produce nonspecific ROS (15), engineered QDs with tailored redox potentials allow for the generation of desired ROS that can perturb the cellular redox environment (16). Because superoxide has been shown to be the principal initial ROS with relatively longer radical lifetime and diffusion lengths, which gives rise to a variety of physiologically relevant primary and secondary reactive oxygen/nitrogen species (17), we developed a specific ROS perturbation approach using superoxide radicals. In bacteria, superoxide disrupts enzyme iron sulfur clusters, resulting in...
an increased free ferrous iron pool (18). This free iron further localizes at the DNA, proteins, and lipids, allowing Fenton chemistry to occur within the diffusion lengths of hydroxyl radical for increased deleterious effects from ROS species (19). Furthermore, it was recently shown that enhancing the endogenous production of superoxide and peroxide in *E. coli* by single-gene deletions potentiated antibiotic activity (20); however, this study was constrained in the level of superoxide generation biologically possible in *E. coli*. Here, we create a platform for potentiating antibiotic activity without genetic manipulation, with concentration and stimuli-dependent control of superoxide generation by designing a stimuli-responsive nanoparticle to produce specific ROS superoxide.

**RESULTS AND DISCUSSION**

**Generation of superoxide via engineered nanoparticles**

We designed a cadmium telluride QD with a band gap energy of 2.4 eV (CdTe-2.4), whose oxidation potential is tuned for superoxide production from molecular oxygen (fig. S1). The ROS produced by CdTe-2.4 upon illumination were measured using electron paramagnetic resonance (EPR) spectroscopy. To identify short-lived radical species produced by CdTe-2.4, we used 5,5-dimethyl-1-pyrroline N-oxide (DMPO), a spin trapping reagent, whose resonance double bonds react with oxygen-centered radicals to form more stable radical adducts (Fig. 1C, left). These adducts were then exposed to a varied external magnetic field to measure characteristic energy differences of unpaired electron spins (21, 22). We measured CdTe-2.4 suspensions with and without illumination to identify adducts produced via their characteristic EPR spectra (see Materials and Methods) (14, 23). In the dark, CdTe-2.4 produced negligible unpaired spins or radical adducts, and values were subtracted from the illuminated CdTe-2.4 spectra before analysis (fig. S1). With illumination, CdTe-2.4 has characteristic superoxide and hydroxyl signals (Fig. 1C, middle). Immediately following photoexcitation, a dominant signal is observed from the superoxide radical (Fig. 1C, right). As time progresses, the superoxide radicals dismute to form hydroxyl radicals (Fig. 1C, right).

**Fig. 1. Light-activated QDs engineered to produce superoxide in MDR isolates.** (A) MDR clinical isolates for this study demonstrated high resistance to different classes of antibiotics. Sensitive (blue line)/resistant (red line) breakpoint minimum inhibitory concentrations; filled diamonds are biological replicates, and the open diamond represents replicate average. (B) Schematic showing MDR bacteria inhibited with previously ineffective concentrations of antibiotics, with superoxide-producing CdTe-2.4 potentiation of antibiotic activity. (C) Superoxide or hydroxyl adducts identified and measured by EPR in (C) and (D) (left). Confirmation of superoxide production from CdTe-2.4 by signal quenching upon the addition of SOD (middle). Hydroxyl signal is observed upon the addition of iron as Fenton chemistry occurs in solution. Production of superoxide by CdTe-2.4 and dismutation to hydroxyl as a function of time (right). (D) Concentration dependence of ROS production from CdTe-2.4. Dark CdTe-2.4 spectra are subtracted for (C) and (D). (E and F) Evidence of superoxide production by CdTe-2.4 in vitro. Overexpression (left) or deletion (right) of sodB in *E. coli* reduced or increased the inhibitory effect of CdTe-2.4, respectively, compared to control or wild-type (WT) strain, shown as normalized optical density at 24 hours (E). Corresponding growth curves are shown in (F), no trt, no treatment. (G) Micrographs of MDR bacterial clinical isolates with 100 nM CdTe-2.4 in light or dark and treated with DCFH-DA demonstrating light-activated ROS intracellularly. *P < 0.05.
radicals in solution, and the EPR adducts observed are predominantly hydroxyl (see Supplementary Discussion). We hypothesized that if the CdTe-2.4 was only producing superoxide, then the addition of SOD, an enzyme that dismutates superoxide to hydrogen peroxide (24), should eliminate the EPR signal of superoxide and, as a consequence, hydroxyl radical as well. With the addition of SOD, the EPR signal for both superoxide and hydroxyl radical is quenched, confirming the direct production of superoxide from CdTe-2.4 (Fig. 1C, middle). Further, we argued that the addition of Fe(II) should provide a pathway for hydrogen peroxide to dismute to hydroxyl radical through Fenton chemistry. As expected, with the addition of Fe(II) to SOD and CdTe-2.4, we observe a recovery of hydroxyl radical signal. We further demonstrated that with the same illumination, increased quantities of ROS were produced with higher CdTe-2.4 concentrations (Fig. 1D). These data indicate that illumination of CdTe-2.4 generates superoxide and stimuli-dependent superoxide radicals, which allows us to controllably increase the flux of superoxide in bacteria.

We investigated CdTe-2.4 superoxide generation intracellularly by studying the response of an E. coli SOD B (sodB) deletion strain, as well as an E. coli overexpressing sodB to treatment with CdTe-2.4. We found that with the addition of CdTe-2.4, there is significant growth inhibition in the sodB deletion strain compared to wild type (BW25113), whereas the overexpression of sodB in E. coli had reduced growth inhibition relative to control (E. coli MG1655 with pZE21MCS plasmid). These results indicate that SodB can modulate the phototoxic effects of CdTe-2.4 and further supports the idea that superoxide radical stress is the antibacterial mechanism (Fig. 1, E and F, and fig. S2). These data also suggest that CdTe-2.4 particles produce superoxide intracellularly, given that the small size (<3 nm) of CdTe-2.4 (fig. S1) is amenable to transport across bacterial cell membranes, that SodB is an established cytosolic enzyme specific to superoxide, and that under physiological conditions, superoxide radical does not cross Gram-negative cellular membranes (25). This is also supported by the lack of response from the SodC mutant, given that it is localized in the periplasmic space (fig. S2A). After confirming intracellular superoxide production by CdTe-2.4 upon illumination, we investigated whether light-activated CdTe-2.4 activates a ROS sensor, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), in three Gram-negative MDR clinical isolates: a CRE E. coli, an ESBL-producing strain of KPN, and an MDR strain of Salmonella enterica serovar Typhimurium (STm). Exposure to light-activated CdTe-2.4 stimulates conversion of DCFH-DA to a green fluorescent product (Fig. 1G and fig. S2B) consistent with oxidation by ROS (26). These results further indicate that light-activated CdTe-2.4 particles increase intracellular ROS.

### Potentiation of antibiotics in MDR clinical isolates

Given that CdTe-2.4 produces intracellular superoxide in Gram-negative pathogens, we hypothesized that it would increase bacterial inhibition when used in combination with antibiotics. We performed checkerboard-stain assays with the set of bactericidal (ceftriaxone, ciprofloxacin, and streptomycin) and bacteriostatic (clindamycin and chloramphenicol) antibiotics of varied mechanisms of action mentioned above and CdTe-2.4 (12.5, 25, or 50 nM). Five concentrations of each antibiotic were tested for every strain and were determined specific to the isolate’s antibiotic GIC_{50} to survey concentrations above and below the sensitive/resistant breakpoint and antibiotic GIC_{50} (Fig. 1A, figs. S3 to S7, and table S2), with a total of 480 unique treatment conditions measured in biological triplicates. All four clinical isolates had increased sensitivity to one or more antibiotics in the presence of light-activated CdTe-2.4 superoxide generation (Fig. 2A and figs. S8 to S13).

We evaluated the combinatorial nature of antibiotic and CdTe-2.4 using the Bliss Independence model (27). The S parameter dictates deviation from no interaction and is defined as $S = \frac{OD_{AB}}{OD_{AB} - OD_{A} - OD_{B}}$, where OD_A is the optical density (OD) at 8 hours in antibiotic only, OD_B is the OD at 8 hours in no treatment, OD_{AB} is the OD at 8 hours in only CdTe-2.4, and OD_{ABQD} is the OD at 8 hours in the combination of antibiotic and CdTe-2.4 (Fig. 2B) (27). Combinations were removed from the analysis if the OD of bacteria in either monotherapy did not reach 0.1 by 8 hours. We observed synergy ($S > 0$) between antibiotics and CdTe-2.4 in 76.4% of all combinations tested ($n = 271$), with greater than 95% confidence in 41% of the cases (Fig. 2, B and C, and fig. S14). With both bactericidal and bacteriostatic antibiotics, the degree of potentiation increased ($S > 0$) with increasing doses of CdTe-2.4, highlighting that antibiotic potentiation can improve with higher superoxide flux (Fig. 2C and fig. S14). Antagonism ($S < 0$) was observed in a few cases where the monotherapy concentration was ineffective, consistent with previous studies demonstrating that the type and strength of antibiotic interactions are dose-dependent (fig. S14) (28). The distribution of $S$ values across all conditions was significantly greater than 0, as indicated by a right-tailed t test ($P < 0.001$) (Fig. 2C). The synergistic effect results in the antibiotic GIC_{50} of many clinical isolates dropping below the sensitive/resistant breakpoint values of antibiotic with the addition of CdTe-2.4 (Fig. 2D). In cases of streptomycin-treated ESBL KPN and MDR Salmonella Typhimurium, the GIC_{50} goes to 100-fold below the breakpoint, demonstrating a strong potentiation of antibiotic activity. These data indicate that superoxide potentiates both bactericidal and bacteriostatic antibiotics with a broad range of mechanisms across diverse Gram-negative pathogens.

<table>
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<tr>
<th>Clinical strain</th>
<th>Ceftriaxone</th>
<th>Chloramphenicol</th>
<th>Clindamycin</th>
<th>Streptomycin</th>
<th>Ciprofloxacin</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>GIC_{50}</td>
<td>MIC</td>
<td>GIC_{50}</td>
<td>MIC</td>
</tr>
<tr>
<td>CRE E. coli</td>
<td>&gt;512</td>
<td>512–1024</td>
<td>&gt;256</td>
<td>32–64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>ESBL KPN</td>
<td>&gt;16,384</td>
<td>2048–4096</td>
<td>&gt;256</td>
<td>8–16</td>
<td>&gt;16</td>
</tr>
<tr>
<td>MDR STm</td>
<td>&gt;16,384</td>
<td>1–4</td>
<td>&gt;8</td>
<td>2</td>
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</tr>
<tr>
<td>MDR E. coli</td>
<td>&gt;16,384</td>
<td>8192–16,384</td>
<td>8</td>
<td>4–8</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>

| Table 1. Antibiotic MIC and GIC_{50} (micrograms per milliliter) for respective strains and antibiotics.
Reduction of bacterial load in the infection models with the addition of stimuli-activated superoxide

To establish whether CdTe-2.4 potentiates antibiotic activity not only in broth but also during infection, we investigated a tissue culture and an animal model system. Intestinal epithelial cells are infected by *S. enterica* in gut-associated and systemic infections, are permissive for uncontrolled *S. enterica* growth in culture, and have little endogenous or inducible ROS activity (29). To test whether CdTe-2.4 potentiates ciprofloxacin, we infected HeLa cells (epithelial cells) with STm [strain SL1344 expressing green fluorescent protein (GFP) from the chromosome (30)] (Fig. 3A), treated with monotherapy or combinatorial therapy, and then lysed the HeLa cells after 18 hours of infection to enumerate intraepithelial colony-forming units (CFU). All CdTe-2.4 concentrations used were minimally lethal to HeLa cells, as determined with a lactate dehydrogenase release assay and MitoTracker staining (fig. S15). CdTe-2.4 significantly reduced CFU at concentrations of 80 nM and higher, suggesting that CdTe-2.4 could be a useful monotherapy for intracellular infection ($P < 0.05$; Fig. 3B). Ciprofloxacin has high efficacy against SL1344 and, for combinatorial experiments, was set at 0.06125 μg/ml, which alone reduced bacterial load 50-fold (Fig. 3B). Incubation of infected HeLa cells with a dosage range of CdTe-2.4 in combination with ciprofloxacin (0.06125 μg/ml) significantly reduced recoverable bacteria compared to ciprofloxacin treatment alone ($P < 0.05$; Fig. 3, C and D). To test the effectiveness against extracellular pathogens, such as those involved in wound and burn infections, we treated a clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA). We demonstrate that chloramphenicol activity was increased against MRSA upon the addition of 160 nM CdTe-2.4 in coculture with HeLa cells (Fig. 3, E and F). The observed synergistic interactions in this infection model (Fig. 3G) further highlight the ability of superoxide to increase the sensitivity of a range of bacteria to antibiotic therapy.

Testing the efficacy in a nematode animal model

To establish whether CdTe-2.4 may potentiate antibiotics in an animal model of gut colonization and killing, we used the nematode Caenorhabditis elegans. First, we screened 46 clinical isolates of KPN, *E. coli*, and *S. enterica* in gut-associated and systemic infections, are permissive for uncontrolled *S. enterica* growth in culture, and have little endogenous or inducible ROS activity (29). To test whether CdTe-2.4 potentiates ciprofloxacin, we infected HeLa cells (epithelial cells) with STm [strain SL1344 expressing green fluorescent protein (GFP) from the chromosome (30)] (Fig. 3A), treated with monotherapy or combinatorial therapy, and then lysed the HeLa cells after 18 hours of infection to enumerate intraepithelial colony-forming units (CFU). All CdTe-2.4 concentrations used were minimally lethal to HeLa cells, as determined with a lactate dehydrogenase release assay and MitoTracker staining (fig. S15). CdTe-2.4 significantly reduced CFU at concentrations of 80 nM and higher, suggesting that CdTe-2.4 could be a useful monotherapy for intracellular infection ($P < 0.05$; Fig. 3B). Ciprofloxacin has high efficacy against SL1344 and, for combinatorial experiments, was set at 0.06125 μg/ml, which alone reduced bacterial load 50-fold (Fig. 3B). Incubation of infected HeLa cells with a dosage range of CdTe-2.4 in combination with ciprofloxacin (0.06125 μg/ml) significantly reduced recoverable bacteria compared to ciprofloxacin treatment alone ($P < 0.05$; Fig. 3, C and D). To test the effectiveness against extracellular pathogens, such as those involved in wound and burn infections, we treated a clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA). We demonstrate that chloramphenicol activity was increased against MRSA upon the addition of 160 nM CdTe-2.4 in coculture with HeLa cells (Fig. 3, E and F). The observed synergistic interactions in this infection model (Fig. 3G) further highlight the ability of superoxide to increase the sensitivity of a range of bacteria to antibiotic therapy.

**Fig. 2.** QDs potentiate bactericidal and bacteriostatic antibiotic activity and lower antibiotic GIC$_{50}$ values. (A) Growth curves of strains under respective treatments demonstrating increased growth inhibition upon combination of antibiotic and CdTe-2.4. (B) Evaluation of CdTe-2.4 and antibiotic synergistic interaction using the Bliss Independence model. $S > 0$ (red scale) indicates a synergistic interaction, where $S >> 0$ is the higher deviation from no interaction between treatments. $S < 0$ indicates antagonistic interaction (gray scale). CLI, clindamycin; CIP, ciprofloxacin; CHL, chloramphenicol; STR, streptomycin. (C) Histogram of $S$ values for all combinations of antibiotic and CdTe-2.4 across all clinical isolates tried in this investigation ($n = 271$; left). The $S$ value distribution average is significantly higher than 0 ($P < 0.05$; right-tailed $t$ test). Demonstration of increased potentiation of antibiotic activity with increasing CdTe-2.4 concentration (right). At constant antibiotic concentration, the addition of greater CdTe-2.4 increases the $S$ value, indicating a more synergistic relationship. $S$ values shown in (B) and (C) are the average of three biological replicates. (D) GIC$_{50}$ of respective antibiotic with the addition of CdTe-2.4 at various concentrations. The addition of CdTe-2.4 potentiates the activity of antibiotics to allow for successful inhibition of 50% or greater at or below sensitive (blue line)/resistant (red line) breakpoint values. The effect is seen as a sharp decrease in GIC$_{50}$ corresponding with the increased addition of CdTe-2.4.
species for high mortality of *C. elegans* (fig. S17) and selected a *Salmonella Enteritidis* isolate. We then transferred mature *C. elegans* to sparse lawns of control *E. coli* OP50 (standard *C. elegans* food) or the MDR clinical isolate of *Salmonella Enteritidis* and incubated for 3 days. Infected worms were then transferred to S medium under illumination and were dosed with respective treatments every 24 hours. After 4 days of treatment in the presence of light, *C. elegans* were stained with SYTOX orange viability dye, and live nematodes were enumerated (Fig. 3H). Only 35 ± 10% of untreated,
Salmonella Enteritidis–infected C. elegans survived infection. Monotherapy treatment with ciprofloxacin (0.5 μg/ml) or CdTe-2.4 (75 nM) increased survival to 51.5 ± 1.0 and 49.2 ± 1.6%, respectively. However, combinatorial therapy led to a 69 ± 5.0% survival rate of infected worms (Fig. 3I). This additive interaction leads to a slight increase in survival rate over monotherapy (P = 0.076 compared to antibiotic only and P = 0.063 compared to CdTe-2.4 only), which suggests that superoxide in combination with an antibiotic could protect nematodes from bacterial killing.

**Light penetration for antibiotic potentiation**

After investigating antibiotic potentiation in these two infection systems, we modeled light penetration through human skin and calculated the skin depth at which antibiotic potentiation could occur. To determine the bacterial inhibition at various skin depths with combination therapy, we used the light attenuation through human epidermal and dermal layers of the skin, as measured in in vivo experiments (31), and determined the superoxide flux from CdTe-2.4 at that light intensity. On the basis of our measurements of CdTe-2.4 toxicity in human epithelial cells (fig. S15), we chose a nontoxic concentration of 160 nM of CdTe-2.4 for our calculations and assumed uniformly dispersed antibiotic and CdTe-2.4 photoexcited by light-emitting diode (LED) light at 520 nm (1.6 mW/cm2). This wavelength of green LED light has also been shown useful for wound healing at low intensity (0.25 to 8 mW/cm2)(32).

We used our experimental data and Beer’s law for light attenuation to obtain skin depths using

\[
I_{\text{LED,520nm}} \times 10^{-\varepsilon l(520nm)z} \times \left(1 - 10^{-\varepsilon l(520nm)d}\right) \times QE
\]

where \(I_{\text{LED,520nm}}\) is the intensity of therapeutic light, \(z\) is the skin depth, \(a\) is the molar absorptivity of the epidermis at \(\lambda < 0.007\) cm and the dermis at \(\lambda > 0.007\) cm (31), \(\varepsilon\) is the extinction coefficient of CdTe-2.4 (33), \(C\) is the concentration of CdTe-2.4, \(I_{\text{light source}}\) is the intensity of light used in our measurements, \(c\) is the concentration of CdTe-2.4 in experimental cultures, \(l\) is the path length of experimental cultures, and QE is the quantum efficiency for the generation of superoxide by CdTe-2.4 via absorption of a photon above its nominal band gap (Fig. 4A). Our experiments at higher light intensities and varied CdTe-2.4 concentrations also validate the estimated increase in superoxide flux and the resulting increase in bacterial growth inhibition (fig. S19). The estimated skin depths required for 50 and 75% growth inhibition within the skin (GIC50 and GIC75) demonstrate the potential of combination therapy with an external LED sheet using CdTe-2.4 and antibiotic (Fig. 4B and fig. S18). In addition, we estimated skin depths for 50 and 90% inhibition (GIC50 or GIC90; Fig. 4C) at the respective CLSI resistance breakpoint or lower antibiotic concentration for all antibiotics and MDR strains used and found that sufficient light penetrates ~1 to 2 cm for CdTe-2.4 antibiotic potentiation. This model suggests that CdTe-2.4 and antibiotic combination therapy could be useful for treating skin and burn infections.

**CONCLUSIONS**

In conclusion, we established that superoxide generation potentiates bactericidal and bacteriostatic antibiotics against a range of MDR Gram-negative clinical isolates despite their high drug resistance. We highlight the utility of engineered stimuli-responsive nanoparticles to produce the specific ROS of interest, namely, superoxide. We show that combinatorial treatment inhibits MDR clinical isolates to levels where the antibiotic GIC50 is below the clinical sensitive/resistant breakpoint and, in some cases, 1000-fold lower. The synergy observed in our study provides new insight into superoxide’s impact on
bactericidal and bacteriostatic antibiotic lethality. The demonstration of combinatorial therapy functioning additively, or in some cases synergistically, to outperform antibiotic monotherapy in two different infection models, epithelial cell and nematode gut infection, further highlights the potential to improve antibiotic efficacy with the addition of superoxide generation. Considering the low absorption of light through human skin at 520 nm and the significant antibiotic potentiation obtainable at skin depths up to 1 to 2 cm, this demonstration holds promise for the application of this technology for treating skin infections and wound healing. Furthermore, a wide variety of tunable nanomaterial parameters, including size, reduction, and oxidation potentials, and surface moieties provide an opportunity to engineer tools to control redox perturbation for mitigating multidrug resistance and improving antibiotic efficacy.

MATERIALS AND METHODS

QD synthesis and characterization
CdTe-2.4 QDs were synthesized and filtered for experiments, as described in the study by Courtney et al. (16). Size distribution analysis of the used particles was obtained from transmission electron micrographs (fig. S1) using ImageJ (d = 2.8 ± 0.3 nm). Images were acquired using a Phillips CM 100 transmission electron microscope at an acceleration of 80 kV. Samples were prepared using Cu-Formvar grids, which were made hydrophilic using a glow-discharge plasma treatment.

Culture conditions
For nonclinical isolates bacterial growth, liquid LB broth (2% LB; L3022, Sigma-Aldrich) or solid LB (2% LB and 1.5% agar; 214010) was used for all experiments. For clinical isolate growth liquid, cation-adjusted Mueller Hinton broth (CAMHB) (212322, Becton, Dickinson and Company) or solid CAMHB (1.5% agar) was used. Dulbecco modified Eagle’s medium (Sigma-Aldrich) supplemented with fetal bovine serum (10%), L-glutamine (2 mM), sodium pyruvate (1 mM), β-mercaptoethanol (50 μM), and Heps (10 mM) was used for HeLa infection assays and maintenance. All cells were incubated at 37°C during growth. HeLa infection studies were carried out with 5% CO2, and liquid bacterial cultures were grown with shaking at 225 rpm. Worms were grown on solid nematode growth medium (NGM) [2.3% N1000 powder (United States Biological), 25 mM phosphate buffer, 1 mM CaCl₂, and 1 mM MgSO₄] for propagation and during infection. Worms were grown in liquid S medium [1 liter of S Basal [0.585% NaCl, 0.1% K₂HPO₄, 0.6% KH₂PO₄, and cholesterol (5 mg/liter)], 10 ml of 1 M potassium citrate, 10 ml of trace metals solution, 3 ml of 1 M CaCl₂, and 3 ml of 1 M MgSO₄] during treatment.

Bacterial strains
Clinical strains were obtained from N. Madinger at the University of Colorado Anschutz Medical Campus and were stored in 10% glycerol at −80°C for long-term storage. Biological replicates were started in liquid medium from individual, single colonies off of solid plates and grown for 16 hours before starting the experiments. OD was measured with a Tecan S1 and S3. All bacterial strains used are in tables S1 and S3.

Cloning of sodB overexpression plasmid
The SodB gene, including the RBS, was cloned from the E. coli MG1655 genome and inserted to the Bam HI and Mlu I sites of the pZEr21MCS plasmid (EXPRESSYS) for the expression in E. coli by the PtetO-1 promoter. The control plasmid was pZEr1MCS plasmid in E. coli MG1655 without insert. Plasmids were cloned into chemically competent E. coli MG1655 and maintained with kanamycin (25 μg/ml).

GIC₅₀ measurement
Overnight cultures of clinical isolates were diluted to a 0.5 McFarland standard in the media with respective test concentrations of antibiotic. Cultures were grown for 24 hours in 384-well microplates. After 24 hours of growth, Resazurin sodium salt (Sigma-Aldrich) solution was added, and the reaction was monitored to measure fluorescence every 5 min for 4 hours at 37°C with shaking at 225 rpm using 485/610-nm filters. The slope of Resazurin fluorescence was used as a quantitative measure of cell metabolism. The GIC₅₀ was determined as the lowest concentration of antibiotic that caused a 50% or greater reduction in slope compared to the same biological replicate in no treatment. GIC₅₀’s were compared to sensitive/resistant breakpoints from 2016/2017 CLSI guidelines or literature (34, 35) shown in table S4.

Combinatorial experiments
Five antibiotic concentrations were chosen for each strain so that the levels tested would be below the GIC₅₀, near the CLSI or defined breakpoint, and near the GIC₅₀. Concentrations tested for each strain can be seen in table S2, with breakpoints shown in table S4. Concentrations of CdTe-2.4 were held constant for all strains at 12.5, 25, and 50 nM. Using these metrics, three biological replicates were tested from each strain with 15 combination test conditions as well as monotherapy controls and a no-treatment condition. Clinical strains were diluted 1:100 from the overnight cultures, into the test condition. Optical density was measured every 30 min for the first 3 hours and every hour subsequently until 8 hours. The optical density at 8 hours normalized to no treatment was used in the Bliss Independence model (27) to determine the combinatorial effect. We used optical density at 8 hours instead of growth rate because of the two-phase growth many of our conditions demonstrated, which yielded multiple growth rates. The optical density at 8 hours was normalized to account for the difference in starting cell viability and biological replicate colony variation.

C. elegans infection experiments
C. elegans CF512 ([fer-15(b26); fem-1(hc17)]) were used for infection experiments. CF512 do not replicate when grown at 25°C to allow for control of the worm count and determination of live and dead populations from starting t = 0. E. coli op50 was used both as the food source before infection and as the control strain during infection experiments. During the treatment phase of infection experiments, worms were suspended in standard liquid S medium, and all wash steps were carried out with standard M9 buffer (36). NGM plates were seeded with E. coli op50 or MDR strains by plating 100 μl of overnight culture onto NGM and incubating at 37°C for 8 hours. Worms were chunked onto NGM plates seeded with E. coli op50 and allowed to grow for 10 to 14 days at 15°C to allow for egg production. The eggs were then collected using NaOH and bleach and transferred to NGM and incubated at 25°C. After 24 hours, hatched worms were transferred to NGM seeded with op50 and grown at 25°C to allow for adult worms to grow without reproduction for 48 hours. Adult worms were collected and washed twice before being transferred to NGM seeded with infection strains. Infection on solid plates was carried out for 3 days. After infection, worms were collected and washed three times (30 s at 600g) before being transferred to S medium, with respective treatment conditions in 100 μl of cultures in 96-well plates at 25°C to limit reproduction. Every 24 hours,
hundred microliters of this solution was then mixed with 1 filter, as described above, and resuspended in water (pH 11). One for all EPR measurements, we used continuous-wave X-band EPR spectroscopy.  

For all EPR measurements, we used continuous-wave X-band EPR spectroscopy. QD samples were prepared for EPR measurements by filtering, as described above, and resuspended in water (pH 11). One hundred microliters of this solution was then mixed with 1 μl of DMPO (Dojindo) and sequestered from light exposure. Quartz capillaries were filled with the CdTe-DMPO mixture and measured in a Bruker Elexsys E 500 spectrometer equipped with an SHQE resonator. Exposure to ambient light was greatly minimized by preparing and recording every measurement in a dark room. A dark background was measured for each sample, which consisted of the average of 10 scans using a 200-G scan range (0.05-G resolution) centered on 3515 G with a microwave attenuation of 16 dB and power of 5 W and was subtracted from the light signal as negligible (fig. S1). Time-dependent scans were conducted over the same 200-G scan range (20.48 s) with a delay of 100 ms between scans. The SiO2 E− defect was present in all measurements but was subtracted during the analysis as part of the dark signal. The sample was then exposed to 9-mW/cm² white light and immediately remeasured to obtain the photoactivated spectrum. For confirmation of superoxide production, conditions were as follows: 10% (v/v) dimethyl sulfoxide in double distilled water (DDW), 1 to 2 mg of the SOD enzyme (Sigma-Aldrich) in 100 μl of DDW, or the degassed water was prepared by bubbling nitrogen through DDW for 1 hour. Each spectrum containing multiple species was simulated using the SpinFit module of the Bruker Xepr software (version 2.6b 149) to identify the radical adducts. The initial fit parameters were as follows: DMPO-OH, \(a_N = 14.90 \text{ G} \) and \(a_{Q1}^B = 14.93 \text{ G} \); DMPO-OOH, \(a_N = 14.2 \text{ G} \), \(a_{Q1}^B = 11.4 \text{ G} \), and \(a_{Q2}^B = 1.2 \text{ G} \). By providing a fixed and known active sample volume in each capillary, the concentration of DMPO adducts was calculated from the total number of spins detected using the Bruker SpinFit software. This software-reported concentration corresponded to the ROS generated multiplied by the number of capillaries in the cavity. Concentrations of ROS species are reported above, in the main text, for one capillary. Data in Fig. 1B (middle) are time-dependent EPR scan; successive scans were each saved as slices, and we averaged the results of the SpinFit from three replicate experiments. Data in Fig. 1B (right) show 10 successive scans that were averaged to minimize error.

EPR was used to evaluate the effects of SOD and consequent hydroxyl radical formation through Fenton chemistry. CdTe-2.4 was filtered and washed as detailed above and dispersed in M9 medium to eliminate pH effects when combined with SOD. The stock of SOD contained 1 mg/ml of the enzyme and 0.1 M potassium phosphate. For the lysozyme control in fig. S1, 1 mg/ml of lysozyme was used. A 1 M solution of ferrous iron sulfate (FeSO₄) in M9 was used as a source of iron. Spectra were first measured in light and dark, as described above, for CdTe-2.4 alone. The resulting spectra were smoothed using a five-point moving average and are shown in Fig. 1C (middle).

DCFH-DA imaging

Respective strains were diluted 1:10 from overnight cultures into CAMHB and treated with respective concentrations of CdTe-2.4 for 1 hour in dark or light. Cells were pelleted and resuspended in phosphate-buffered saline (PBS) with DCFH-DA and incubated for 5 min before cells were imaged on glass slides with coverslips on an EVOS FL microscope. Images were processed identically for all conditions using ImageJ.

Gentamicin protection assays

HeLa cells were seeded at 1.0 × 10⁴ cells per well in 96-well tissue culture plates and grown for 24 hours. STm wild-type strain SL1344 with chromosomal rpsM::GFP (30) was grown overnight, diluted 1:10, and grown for 4 hours in LB before infections. Streptomycin antibiotic selection was used at 30 μg/ml. Salmonella Typhimurium in Gibco PBS was added at a multiplicity of infection of 30:1 (bacteria/HeLa) and centrifuged for 5 min at 500 g. After 45 min, cells were incubated for a further 1.25 hours at 37°C in fresh medium supplemented with gentamicin (40 μg/ml) to kill extracellular bacteria. Medium was then exchanged for fresh medium with treatment conditions supplemented with gentamicin (40 μg/ml) to inhibit extracellular bacterial growth. At 18 hours after infection, the wells were washed three times with 300 μl of PBS, and cells were lysed with 30 μl of 0.1% Triton X-100 for 15 min at room temperature. After 15 min, 270 μl of PBS was added to each well (serving as 100-fold dilution), serially diluted, plated onto 2% LB, 1.5% agar, and streptomycin (40 μg/ml), and then incubated at 37°C for 16 hours to enumerate CFU per milliliter. For staining and imaging, wells were stained with 100 nM MitoTracker, followed by fixation with 16% paraformaldehyde for 15 min. Wells were then washed twice with PBS, stained with DAPI for 20 min, washed twice with PBS, and stored in 100 μl of 90% glycerol and 1× PBS before imaging and/or storage at 4°C in dark. Images were acquired using an EVOS FL microscope and analyzed using ImageJ.

S. aureus and HeLa coculture

HeLa cells were seeded as described above. MRSA was grown overnight to saturation and added to HeLa at a multiplicity of infection of 5:1 (bacteria/HeLa) in medium supplemented with respective treatment conditions. At 24 hours after infection, the wells were agitated using pipetting, and 10 μl of the medium was transferred to PBS (serving at 10-fold dilution), serially diluted, plated on to 2% LB and 1.5% agar, and incubated at 37°C for 16 hours to enumerate CFU per milliliter.

Lactate dehydrogenase cytotoxicity assay

HeLa cells were seeded as described above. Cells were treated with respective conditions and incubated for 18 hours. Fifty microliters of the supernatant was used to determine lactate dehydrogenase (LDH) release as a measure of cytotoxicity using the Pierce LDH cytotoxicity assay kit. Cells lysed with the supplied lysis buffer served as a positive control.

Skin depth modeling

To estimate the penetration of light, excitation of CdTe-2.4, and potential of antibiotics in skin, we used in vivo absorption coefficients of the human skin, separated into epidermis and dermis, from List et al. (31). The light source for calculations of skin depth was an LED sheet with emission at 520 nm with an intensity of 1.6 mW/cm². We assumed that 160 nM CdTe-2.4 was dispersed in the skin, which had low toxicity in our LDH assay of human epithelial cells (HeLa). The skin depths where CdTe-2.4 superoxide flux and potentiation would be equal to 12.5, 25, and 50 nM in broth, 1.47, 1.32, and 1.16 cm, respectively, were calculated and then used to graph GIC₅₀ and GIC₇₅ skin depth isosurfaces (Fig. 4 and fig. S18). We then used an exponential decay fit for the
inhibition data at each antibiotic concentration to interpolate or extrapolate skin depths for GIC_{50} and GIC_{90} (Fig. 4 and Fig. S19).

**Error and significance analysis**

Error is represented as SD between technical replicates or SEM for biological replicates. Significance is defined as P < 0.05 and denoted by an asterisk (*) unless otherwise stated. Significance was determined using Student’s t tests, with a 95% confidence interval, unless otherwise stated.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/10/e1701776/DC1

**REFERENCES AND NOTES**

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Potentiating antibiotics in drug-resistant clinical isolates via stimuli-activated superoxide generation

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