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DNA-Crosslinker Cisplatin Eradicates Bacterial Persister Cells

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ABSTRACT: For all bacteria, nearly every antimicrobial fails since a subpopulation of the bacteria enter a dormant state known as persistence, in which the antimicrobials are rendered ineffective due to the lack of metabolism. This tolerance to antibiotics makes microbial infections the leading cause of death worldwide and makes treating chronic infections, including those of wounds problematic. Here, we show that the FDA-approved anti-cancer drug cisplatin [*cis*-diamminodichloroplatinum(II)], which mainly forms intra-strand DNA crosslinks, eradicates *Escherichia coli* K-12 persister cells through a growth-independent mechanism. Additionally, cisplatin is more effective at killing *Pseudomonas aeruginosa* persister cells than mitomycin C, which forms inter-strand DNA crosslinks, and cisplatin eradicates the persister cells of several pathogens including enterohemorrhagic *E. coli, Staphylococcus aureus*, and *P. aeruginosa*. Cisplatin was also highly effective against clinical isolates of *S. aureus* and *P. aeruginosa*. Therefore, cisplatin has broad spectrum activity against persister cells.

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KEYWORDS: cisplatin; persistence; biofilm; DNA-crosslinking

Introduction

Bacterial infections are the leading cause of death (Rasko and Sperandio, 2010); for example, there are 17 million new biofilm infections every year in the U.S.A., which lead to 550,000 fatalities (Wolcott and Dowd, 2011). Current antibiotics are becoming increasingly ineffective due to extensive bacterial resistance and also since a subpopulation of bacterial cells in these bacterial infections become dormant (Kwan et al., 2013; Lewis, 2007; Wood et al., 2013) and escape the antibiotic treatment; these cells are known as persisters and grow after the course of antibiotics and re-establish the infection (Fauvart et al., 2011). Hence, there is an urgent need to develop new antimicrobials that target persister cells in order to eliminate bacterial infections.

We recently discovered that mitomycin C (MMC) is effective in killing a broad-range of bacterial persister cells including those formed in biofilms and wounds (Kwan et al., 2015). MMC is produced by *Streptomyces caespitosus* (Tomasz, 1995) and is an FDA-approved drug that has previously been used to treat breast, lung, and prostate cancer (Bradner, 2001). MMC crosslinks the neighboring guanine bases on different DNA strands as a result of the sequence 5′-CG (Tomasz, 1995); critically, MMC crosslinks the DNA of persister cells (Kwan et al., 2015). Corroborating this initial success, MMC has also been shown to be effective against persister cells of the spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease (Sharma et al., 2015).

Cisplatin (*cis*-diamminodichloroplatinum(II), Fig. 1 inset) is also an FDA-approved drug that has been used to treat testicular, ovarian, bladder and head and neck cancers (Eastman, 1987). The biological effects of complexes of platinum were found serendipitously in 1965 in an electrophoresis study where platinum from an electrode was found to inhibit cell division and increase *Escherichia coli* cell length by as much as 300 fold (Rosenberg et al., 1965). Like MMC, cisplatin crosslinks DNA but the crosslinks occur primarily on the same strand of DNA with intra-strand crosslinks between purines at adjacent guanines (54–65%), at AG sequences (17–19%), and at GNG sequences where N can be any nucleotide (<8%) (Eastman, 1987). Therefore, because of its potent crosslinking

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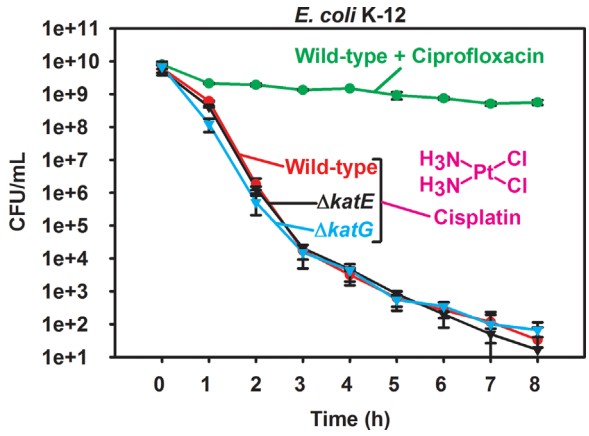

Figure 1. Cisplatin eradicates late stationary-phase *E. coli* K-12 cells. Time course dependent killing of late-stationary phase (16 h of growth) cells of wild-type *E. coli* K-12 in buffered LB by ciprofloxacin (5 μg/mL, 100× MIC, green line) and cisplatin (500 μg/mL, 5× MIC, red line). Killing with cisplatin (500 μg/mL) for two isogenic catalase mutants Δ*katE* (black line) and Δ*katG* (blue line) is also indicated. The structure of cisplatin is shown as an inset.

activity, we reasoned that cisplatin may have activity toward bacterial persister cells.

We found that cisplatin is effective against persister cells in a broad range of bacteria including commensal *E. coli* K-12 as well as pathogenic strains of *E. coli*, *Staphylococcus aureus* (frequently found in wounds), and *Pseudomonas aeruginosa* (including clinical, multi-drug resistant strains). We also demonstrate that cisplatin eradicates persister cells more effectively than MMC. Therefore, cisplatin has broad-spectrum activity against growing, non-growing, and persister cells, and has potential for use in the treatment of recalcitrant infections.

Materials and Methods

Bacterial Strains and Growth Media

The bacterial strains and plasmids used are listed in Table I. Bacterial strains were grown at 37°C with shaking at 250 rpm. *E. coli* and *P. aeruginosa* were grown in lysogeny broth (LB) (Sambrook et al., 1989) at pH 6.9 or M9-glucose (0.4%) at pH 7.0 (Rodriguez and Tait, 1983), and *S. aureus* was grown in tryptic soy broth (TSB) at pH 6.8. Kanamycin was added at 50 μg/mL for the *E. coli* isogenic mutants and 15 μg/mL gentamicin was used for the *P. aeruginosa* isogenic mutants.

Stocks of cisplatin (2 mg/mL, Sigma–Aldrich, St. Louis, MO) were prepared in 0.1 M of sodium perchlorate ($NaClO_4$) (Chvalova et al., 2007) and MMC (0.5 mg/mL, Fisher Scientific, Pittsburgh, PA) was prepared in water. Stocks of busulfan (2 mg/mL, Cayman Chemical, Ann Arbor, MI), melphalan (2 mg/mL, Cayman Chemical), dynemicin A (5 mg/mL, AdooQ, Irvine, CA), and tirapazamine (0.5 mg/mL, Sigma–Aldrich) were prepared in dimethylsulfoxide (DMSO), the stock of cyclophosphamide (0.5 mg/mL, Cayman Chemical) was prepared in water, and the stock of carmustine (214 mg/mL, Enzo Life Sciences, Farmingdale, NY) was prepared in ethanol.

Minimum Inhibitory Concentration (MIC) Assay

To determine the MICs of cisplatin for *E. coli* K-12, enterohemorrhagic *E. coli* (EHEC), *S. aureus*, and *P. aeruginosa* (PAO1 and PA14), freshly inoculated cultures (at least two independent cultures) in buffered LB (TSB for *S. aureus*) were incubated for 16 h with varying concentrations of cisplatin, and growth inhibition was assayed by the lack of turbidity.

Table I. Bacterial strains and plasmids used in this study.

Strain	Genotype	Source
E. coli K-12 BW25113	*rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568 rph-1*	Baba et al. (2006)
E. coli K-12 BW25113 Δ*katE*	BW25113 Δ*katE* Ω Km^R	Baba et al. (2006)
E. coli K-12 BW25113 Δ*katG*	BW25113 Δ*katG* Ω Km^R	Baba et al. (2006)
EHEC 86-24	EHEC O157:H7 $Stx2^+$	Sperandio et al. (2001)
S. aureus ATCC29213	Antibiotic-susceptible reference strain	ATCC
S. aureus 5B	Clinical isolate from diabetic food	Cervantes-Garcia et al. (2015)
S. aureus 50F	Clinical isolate from diabetic food	Cervantes-Garcia et al. (2015)
P. aeruginosa PAO1	Wild-type strain	Jacobs et al. (2003)
P. aeruginosa PA14	Clinical isolate	Liberati et al. (2006)
PA14 Δ*tpbA* (PA3885)	PA14_13660 Ω *Mar2xT7*, Gm^R	Liberati et al. (2006)
PA14 Δ*katA* (PA4236)	PA14_09150 Ω *Mar2xT7*, Gm^R	Liberati et al. (2006)
P. aeruginosa RME-101[a]	Clinical isolate	Adult pneumonia patient
P. aeruginosa INP-37	Clinical isolate	Pediatric cystic fibrosis patient
P. aeruginosa INP-57M	Clinical isolate	Pediatric cystic fibrosis patient
P. aeruginosa INP-64[b]	Clinical isolate	Pediatric cystic fibrosis patient

Km^R, Kanamycin resistance; Gm^R, Gentamicin resistance.

[a]Resistant to 18 antibiotics: carbenicillin, ticarcillin, piperacillin, ticarcillin/clavulanic acid, piperacillin/tazobactam, ceftazidime, ceftriaxone, cefotaxime, cefepime, imipenem, meropenem, aztreonam, amikacin, gentamicin, tobramycin, ciprofloxacin, norfloxacin, and levofloxacin.

[b]Resistant to 12 antibiotics: carbenicillin, ticarcillin, piperacillin, ticarcillin/clavulanic acid, ceftazidime, ceftriaxone, cefotaxime, cefepime, meropenem, ciprofloxacin, lomefloxacin, and levofloxacin.

Persister Cell Killing and Eradication Assays (Planktonic Cells)

For the time course experiment, late stationary phase cells of *E. coli* K-12 (wild-type and isogenic mutants) were prepared by growing cells overnight (16 h) in LB medium buffered with 100 mM KPO_4 at pH 7.0 (buffered LB). A 1 mL culture was used for a drop (10 μL × 3) assay (Donegan et al., 1991) to check cell viability at 0 h (i.e., before antibiotic treatment). An 18 mL culture was treated with ciprofloxacin (5 μg/mL, for the wild-type strain) and cisplatin (500 μg/mL) for 8 h (250 mL flask, 250 rpm). The concentrations of ciprofloxacin and cisplatin were at least 5× MIC to minimize survival of potential spontaneous resistant mutants. At 1 h intervals, 1 mL culture was centrifuged at 10,000 rpm for 3 min, the supernatant was discarded, the cells were resuspended in 1 mL of normal saline (0.85% NaCl), and the cell suspension was serially diluted for a drop assay to check cell viability. Experiments were performed with at least two independent cultures.

To test for eradication, the wild-type *E. coli* K-12 cells were treated with cisplatin for 12 h, a 1 mL sample was centrifuged, the cell pellet was resuspended in 150 μL of normal saline, and the cells were spread on LB agar to check cell viability. Another 1 mL culture was diluted 1:10 with fresh buffered LB and the cells were incubated to allow any remaining cells to replicate. After 12 h, 18 h, and 24 h, 1 mL samples were centrifuged, resuspended in 150 μL of normal saline and spread on LB agar plates to check cell viability. At least two independent cultures were used.

Rifampicin-pretreatment was used to convert nearly all the *E. coli* exponentially growing cells to persister cells (Kwan et al., 2013). In brief, overnight cultures (16 h) of *E. coli* BW25113 were diluted 1:1000 in buffered LB and grown to a turbidity of 0.8 at 600 nm (250 mL flask, 250 rpm). Cultures were treated with rifampicin (100 μg/mL) for 30 min (250 mL flask, 250 rpm), centrifuged, and resuspended in fresh medium to remove the rifampicin. A 1 mL culture was used for a drop assay to check cell viability at 0 h. A 5 mL culture was treated with ciprofloxacin (5 μg/mL), cisplatin (500 μg/mL) or equivalent amount of 0.1 M $NaClO_4$ (as a solvent control for cisplatin) for 3 h (125 mL flask, 250 rpm). After 3 h, 1 mL of culture was centrifuged at 10,000 rpm for 3 min, the supernatant was discarded, the cells were resuspended in 1 mL of normal saline, and the cell suspension was serially diluted for a drop assay to check cell viability. In addition, 1 mL samples were centrifuged, and the cell pellet resuspended in 150 μL of normal saline and spread on LB agar. Experiments were performed with at least two independent cultures.

To test whether lower concentrations than 500 μg/mL of cisplatin generate resistant mutants, 5 mL cultures of rifampicin-induced persister cells were treated with 200 μg/mL of cisplatin and cells were enumerated by the drop assay using LB agar, LB agar with 200 μg/mL of cisplatin (LBCP200), and LB agar with 500 μg/mL of cisplatin (LBCP500). Experiments were performed with at least two independent cultures.

For the persister cell assay of the pathogens, stationary-phase cells were used. Cultures of EHEC, *P. aeruginosa*, and *S. aureus* were prepared by a 1:1000 dilution of overnight grown cultures (LB medium for EHEC and *P. aeruginosa*, TSB for *S. aureus*) into fresh buffered LB medium for EHEC, M9-glucose for *P. aeruginosa*, and TSB for *S. aureus* and re-growing the cells to a turbidity of 3–4 at 600 nm for EHEC and *S. aureus* and growing for 24 h for *P. aeruginosa*. Stationary-phase cultures of EHEC (buffered LB medium), *P. aeruginosa* (M9-glucose medium), and *S. aureus* (TSB medium) were then treated with cisplatin (500 μg/mL for EHEC and *S. aureus*, 250 μg/mL for *P. aeruginosa*) for 3 h (250 mL flask, 250 rpm).

To compare the killing efficiency of cisplatin with MMC, stationary-phase cells of *P. aeruginosa* PA14 in M9-glucose (24 h) were treated with 1× and 5× MIC of cisplatin (50 and 250 μg/mL, respectively) or 1× and 5× MIC of MMC (2 and 10 μg/mL, respectively) for 3 h. $NaClO_4$ (0.1 M) was used as a solvent control. The viability of the cells was determined before and after cisplatin and MMC treatment. At least two independent cultures of each strain were used in each experiment.

Persister Cell Killing Assay (Biofilm Cells)

Overnight (16 h) grown cultures in LB were diluted to a turbidity of 0.05 at 600 nm in M9-glucose and cultures were grown for 24 h at 37°C in 96-well plates (250 μL/well). Biofilm formation was assayed using the crystal violet staining method (Fletcher, 1977) and was normalized by planktonic cell growth (turbidity at 620 nm) to account for any differences in growth rates. Data were averaged from 6 replicate wells using at least two independent cultures. Biofilm cultures were treated for 3 h with 1× and 5× MIC of MMC (2 and 10 μg/mL, respectively) and 1× and 5× MIC cisplatin (50 and 250 μg/mL, respectively). $NaClO_4$ (0.1M) was used as a solvent control for cisplatin. Cell viability in biofilms was assayed before and after the MMC and cisplatin treatments by removing supernatants (planktonic cells) from each well carefully, resuspending the biofilm in normal saline, and sonicating twice at 3 W for 10 s (Ueda and Wood, 2009) to break apart the biofilm cells (due to the aggregative nature of *P. aeruginosa* Δ*tpbA* cells). Suspensions from multiple wells were combined to get an averaged sample. Then the cell suspensions were serially diluted in normal saline for a drop assay to check cell viability. Experiments were performed with at least two independent cultures.

Validation of Persisters

To validate that the persister cells are tolerant to antibiotics, not spontaneous resistant mutants, three rounds of the persister assay were performed. Overnight (16 h) cultures of *E. coli* BW25113 were diluted 1:1000 with fresh buffered LB and grown to a turbidity of ~0.8 at 600 nm. A 1 mL culture was used for the drop assay, and a 5 mL culture was treated with ciprofloxacin (5 μg/mL) and ampicillin (100 μg/mL) for 3 h (125 mL flask, 250 rpm). After the first round of antibiotic treatment, a 1 mL sample was centrifuged at 10,000 rpm for 3 min, and the cells were resuspended in 1 mL of normal saline. A 100 μL of cell suspension was used for the drop assay on both LB and LB with 5 μg/mL of ciprofloxacin (LBCipro5) agar (for ciprofloxacin treated samples) or LB with 100 μg/mL of ampicillin (LBAmp100) agar (for ampicillin treated samples). For the second and third rounds, cell suspensions were diluted 1:100 with fresh buffered LB and regrown to a turbidity of ~0.8 at 600 nm and used for antibiotic treatment followed by the drop assay. Experiments were performed with at least two independent cultures.

Results

Cisplatin Eradicates *E. coli* K-12 Persister Cells

Based on our success in killing persister cells with the DNA crosslinking agent MMC (Kwan et al., 2015), we tested seven other DNA crosslinking compounds: busulfan (100 μg/mL) (Frei and Holland, 2003), carmustine (100 μg/mL) (Frei and Holland, 2003), cisplatin (100 μg/mL) (Chvalova et al., 2007; Eastman, 1987), cyclophosphamide (100 μg/mL) (Frei and Holland, 2003), dynemicin A (200 μg/mL) (Konishi et al., 1989), melphalan (100 μg/mL) (Frei and Holland, 2003), and tirapazamine (10 μg/mL) (Shah et al., 2013). Of these, only cisplatin exhibited antimicrobial activity for *E. coli* K-12 cells, so we focused on this compound.

To evaluate the effectiveness of cisplatin against persister cells, cisplatin was compared with ciprofloxacin, a fluoroquinolone which inhibits DNA replication and kills both growing and non-growing cells but not persister cells (Sanders, 1988) and which is commonly used in persister studies (Conlon et al., 2013). Therefore, throughout this work, ciprofloxacin tolerance represents the baseline level of persistence with an effective antibiotic. Additionally, antibiotic treatments were at least 5× the minimum inhibitory concentration for *E. coli* (MIC, 100 μg/mL for cisplatin, Table II, and 0.05 μg/mL for ciprofloxacin (Kwan et al., 2015) to ensure eradication of non-persisters and to minimize the survival of potential spontaneous resistant mutants.

Previously, we demonstrated ciprofloxacin tolerance is due to persistence rather than spontaneous genetic resistance; hence, ciprofloxacin tolerance is an indicator of persistence (Kwan et al., 2015). To validate again that persister cells were generated, we performed three rounds of the persister assay with ciprofloxacin (100× MIC, 5 μg/mL) as well as with ampicillin at 100 μg/mL (10× MIC, MIC for ampicillin is 10 μg/mL, Kwan et al., 2015), since persister cells have different tolerances to different antibiotics (Chowdhury et al., 2016; Hofsteenge et al., 2013; Wu et al., 2015). We found there was no significant increase in *E. coli* K-12 cell viability after both ciprofloxacin or ampicillin treatments: the survival rates for ciprofloxacin were $0.045 \pm 0.008\%$, $0.03 \pm 0.01\%$, and $0.012 \pm 0.002\%$ after rounds 1, 2, and 3, respectively and the survival rates for ampicillin were $0.001 \pm 0.001\%$, $0.0001 \pm 0.0000\%$, and $0.0001 \pm 0.0000\%$ after rounds 1, 2, and 3, respectively. Moreover, no colonies were detected on either LBCipro5 or LBAmp100 agar, which confirmed that no resistant mutants were generated.

Table II. MICs for cisplatin for the bacterial strains used in this study.

Strain	MIC, μg/mL
E. coli K-12 BW25113	100
EHEC 86-24	100
S. aureus ATCC29213	300
P. aeruginosa PAO1	50
P. aeruginosa PA14	50
S. aureus 5B	>400
S. aureus 50F	>400
P. aeruginosa RME-101	100
P. aeruginosa INP-37	400
P. aeruginosa INP-57M	400
P. aeruginosa INP-64	400

Initially, we tested whether cisplatin kills late stationary phase (16 h of growth) cells of *E. coli* K-12 in a time course experiment, since persister levels are high at this growth stage (Lewis, 2007). We found that cisplatin (500 μg/mL, 5× MIC) nearly eradicated these cells; however, ciprofloxacin, at a much higher MIC (5 μg/mL, 100× MIC) had little activity against the persister cells (Fig. 1).

We also tested two isogenic *E. coli* K-12 catalase mutants *katE* (Loewen, 1984) and *katG* (Loewen et al., 1985) to investigate whether cisplatin works via the production of reactive oxygen species (ROS). We did not find any significant differences in killing of the *katE* or *katG* mutants compared to the wild-type strain after prolong (8 h) treatment with 5× MIC of cisplatin (Fig. 1). Similar results were found with a catalase mutant *katA* (Heo et al., 2010) of *P. aeruginosa* strain PA14 compared to the wild-type strain (data not shown). Hence, reactive oxygen species do not seem to play a role in killing by cisplatin.

We also tested whether cisplatin completely eradicates *E. coli* K-12 persister cells and abolishes regrowth when cisplatin is withdrawn. We found that treatment with 5× MIC of cisplatin for 12 h completely eradicated *E. coli* persister cells since there were no colonies from 1 mL of culture, and there was no increase in turbidity or colonies (from 1 mL sample) when 1 mL of the cisplatin-treated culture was used to inoculate fresh medium and the medium was incubated for 12, 18, and 24 h.

Next, we tested cisplatin using persister cells of *E. coli* K-12 prepared by pretreating with rifampicin (Kwan et al., 2013). We found that after 3 h, ciprofloxacin (5 μg/mL, 100× MIC) was unable to kill $9 \pm 1\%$ of the persister cells; however, cisplatin (500 μg/mL, 5× MIC) reduced the number of persister cells below the limit of detection (100 cells/mL for drop assay) (Fig. 2A). To investigate further the number remaining cells after the 3 h cisplatin treatment, 1 mL samples were plated and found to produce 37 ± 5 colonies; hence, the surviving fraction was $0.00001 \pm 0.00000\%$. $NaClO_4$ was used as the solvent for cisplatin since DMSO inactivates cisplatin (Hall et al., 2014), and $NaClO_4$ did not show any killing effect (data not shown). Hence, cisplatin is highly effective against *E. coli* persister cells.

The use of lower concentrations than 5× MIC (500 μg/mL) of cisplatin was also tested to see if this generates any spontaneous resistant mutants. We found that treatment with 2× MIC (200 μg/mL) of cisplatin yields 0.08% survivors on LBCP200 agar plates; however, no colonies were found on LBCP500 agar plates. To confirm that the lack of colonies on the LBCP500 agar plates was not due to limits of detection of the drop assay (100 cells/mL), all the colonies from the LBCP200 agar plates were streaked on LBCP500 agar plates. However, no growth was observed. Similarly, none of the cells that survived from 3 h treatments with 5× MIC of cisplatin (1 mL spread counts) grew on LBCP500 agar plates. Therefore, treatment with 2× and 5× MIC of cisplatin does not generate spontaneous resistant mutants in these experiments.

Cisplatin Eradicates Pathogenic Persister Cells

Since MMC as an anticancer drug possesses broad spectrum activity against various bacterial pathogens (Kwan et al., 2015) and

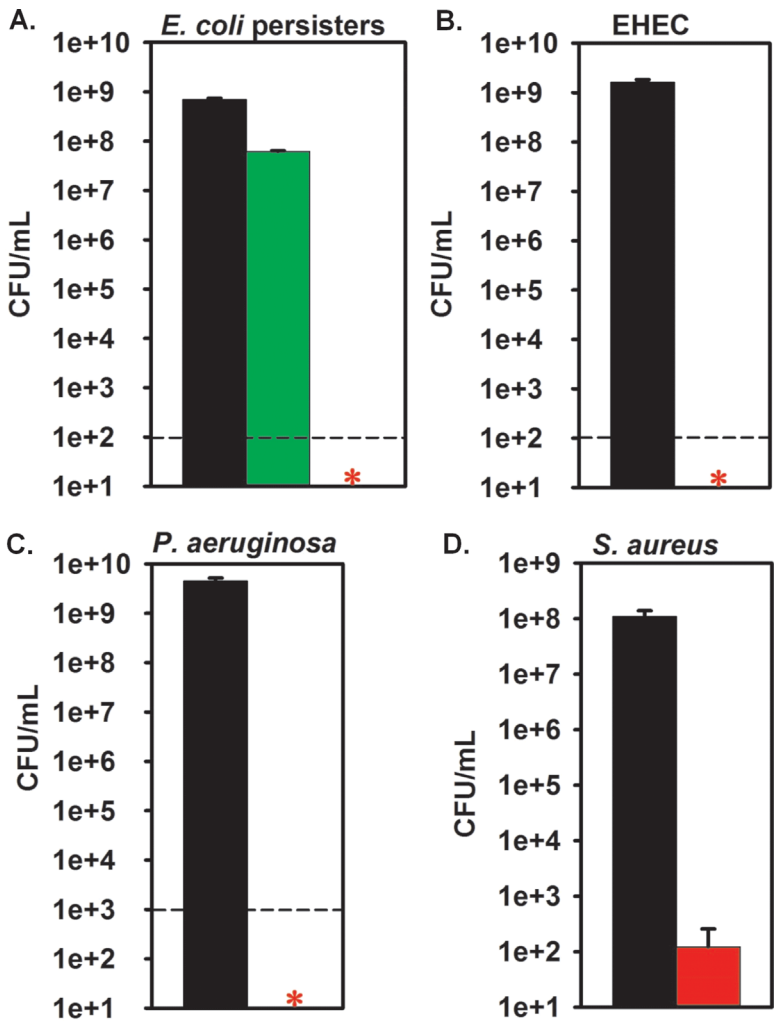

Figure 2. Cisplatin eradicates metabolically dormant persister cells and has broad spectrum activity against diverse bacterial pathogens. (**A**) Cell viability of rifampicin (30 min pretreatment with 100 μg/mL)-induced persister cells of *E. coli* K-12 in buffered LB after treatment with ciprofloxacin (5 μg/mL, 100× MIC) and cisplatin (500 μg/mL, 5× MIC). (**B**) Stationary-phase cells of EHEC in buffered LB after treatment with cisplatin (500 μg/mL, 5× MIC). (**C**) Stationary-phase cells of *P. aeruginosa* in M9-glucose (0.4%) after treatment with cisplatin (250 μg/mL, 5× MIC). (**D**) Stationary-phase cells of *S. aureus* in TSB medium after treatment with cisplatin (500 μg/mL, 1.7× MIC). Cell viability is shown before (black bar) and after 3 h treatment (green bar for ciprofloxacin and red bar or red asterisk for cisplatin). *Represents eradication of cells beyond the limit of detection (100 cells/mL).

since cisplatin is also an anticancer drug, we tested whether cisplatin can kill diverse bacterial pathogens. We used stationary-phase cells since persister levels are high at this growth stage (Lewis, 2007) and conditions have not been devised yet to convert these cells to persister cells using chemical pre-treatments as with *E. coli* K-12 (Kwan et al., 2013).

We found that cisplatin (500 μg/mL, 5× MIC) eradicated stationary-phase cells of enterohemorrhagic *E. coli* (EHEC), a common *E. coli* pathogen associated with food-borne illness in humans (Kaper, 1998) (Fig. 2B). Cisplatin was also tested against the Gram-negative opportunistic pathogen *P. aeruginosa* PA14 (Cross et al., 1983), and cisplatin (250 μg/mL, 5× MIC) eradicated stationary-phase cells of PA14 (Fig. 2C). Similar results were obtained using the related strain *P. aeruginosa* PAO1 (data not shown). Furthermore, cisplatin at 100 μg/mL (0.25× MIC or 1× MIC, Table II) nearly eradicated the persister cells of four clinical strains of *P. aeruginosa* (isolated from adult pneumonia patients and pediatric cystic fibrosis patients) which are resistant to multiple (12–18) antibiotics (Table I), and one of them, INP-57M, is resistant to the canonical quorum sensing quenching agent furanone C-30 (Garcia-Contreras et al., 2015); with cisplatin, there were 0.02 ± 0.03%, 0.05 ± 0.06%, 0.01 ± 0.00%, and 0.02 ± 0.00% survival for strains RME-101, INP-37, INP-57M, and INP-64M, respectively.

We also tested the effectiveness of cisplatin with a methicillin-sensitive strain of the Gram-positive human pathogenic bacterium *S. aureus* ATCC29213 (Archer et al., 2011) and two clinical isolates from diabetic food patients (Cervantes-Garcia et al., 2015). Cisplatin (500 μg/mL, 1.7× MIC) nearly eradicated (0.0001 ± 0.0001% survival) stationary phase cells of *S. aureus* ATCC29213 (Fig. 2D). Similarly, the stationary-phase cells of both clinical isolates were severely affected by 500 μg/mL of cisplatin (2.5 ± 3.5% survival and 10 ± 4% survival for the isolates 5B and 50F, respectively) and a higher concentration (800 μg/ mL) was

able to completely eradicate these cells. Together these results show that cisplatin is capable of eradicating the persister cells of three pathogens.

Cisplatin Is More Effective Than MMC With *P. aeruginosa*

Previously, we found that MMC was not effective in eradicating stationary-phase cells of *P. aeruginosa* PA14, and MMC was about as effective as ciprofloxacin with planktonic cells of this strain (Kwan et al., 2015). Hence, we compared the efficacy of cisplatin and MMC at 1× MIC (50 μg/mL for cisplatin [Table II] and 2 μg/mL for MMC) (Kwan et al., 2015) and at 5× MIC (250 μg/mL for cisplatin and 10 μg/mL for MMC). As observed before (Kwan et al., 2015), MMC failed to eradicate stationary phase cells of PA14 (5 ± 2% survival for 1× MIC and 0.0056 ± 0.0004% survival for 5× MIC); however, even 1× MIC of cisplatin completely eradicated PA14 persister cells (Fig. 3A). Similar results were obtained with *P. aeruginosa* strain PAO1 (data not shown). Therefore, cisplatin is a

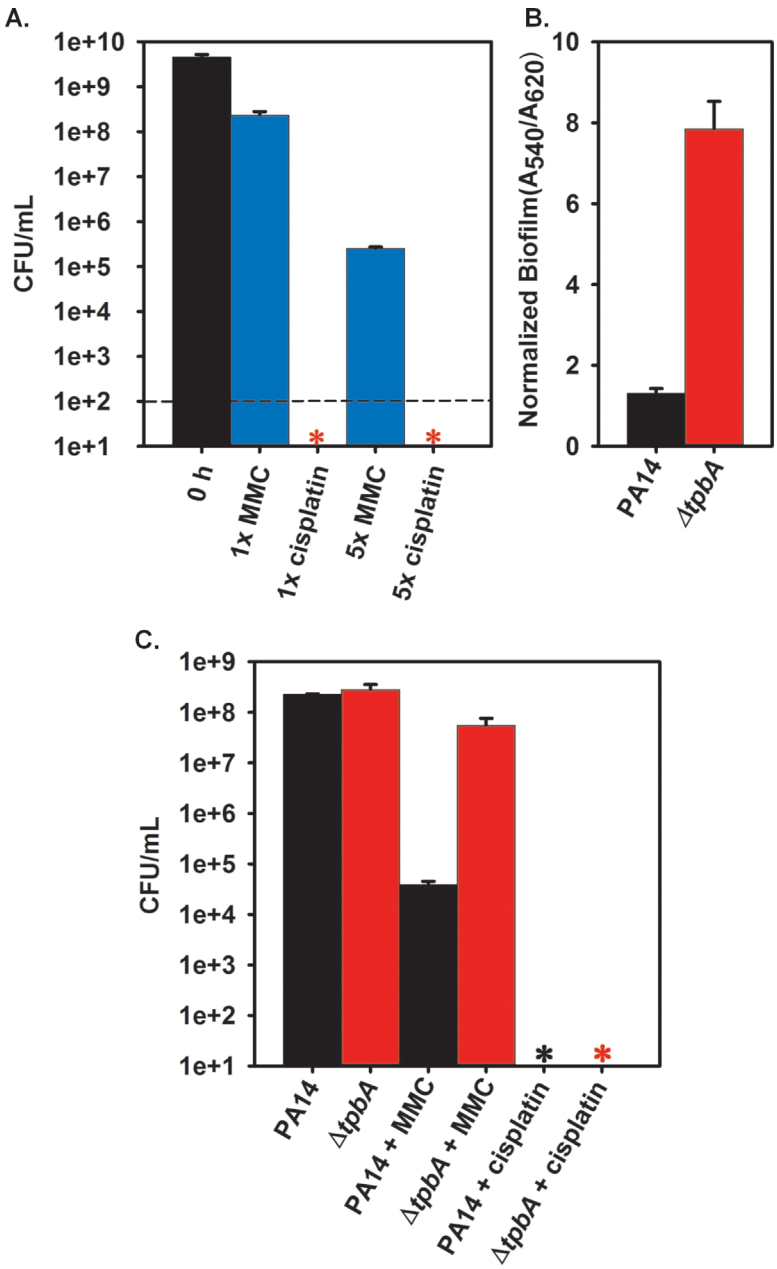

Figure 3. Cisplatin is more effective than mitomycin C (MMC) in killing *P. aeruginosa* planktonic and biofilm persister cells. (**A**) Cell viability of *P. aeruginosa* PA14 planktonic (stationary-phase) cells before (black bar) and after treatment for 3 h with 1× and 5× MIC of MMC (2 and 10 μg/mL, respectively; blue bar) and 1× and 5× MIC cisplatin (50 and 250 μg/mL, respectively; red asterisk). (**B**) Comparison of biofilm production between wild-type *P. aeruginosa* PA14 (black bar) and its isogenic mutant strain Δ*tpbA* (red bar) in M9-glucose (0.4%) medium. (**C**) Cell viability of biofilm cells of *P. aeruginosa* PA14 (black bar) and Δ*tpbA* (red bar) before and after treatment for 3 h with 10 μg/mL of MMC (5× MIC) or 250 μg/mL of cisplatin (5× MIC). *Represents eradication of cells beyond the limit of detection (100 cells/mL).

more effective than MMC in killing the persister cells of bacterial pathogens.

We then tested the efficacy of cisplatin versus MMC using biofilm cells of *P. aeruginosa*, since levels of persisters are also high in biofilms (Lewis, 2008), and they are a major cause of bacterial infections (Wolcott and Dowd, 2011). We utilized the Δ*tpbA* mutant of *P. aeruginosa* since it produces substantially more biofilm than PA14 due to elevated levels of cyclic diguanylate (Ueda and Wood, 2009); this elevated biofilm formation was confirmed since the Δ*tpbA* mutant produced sixfold more biofilm than PA14 in M9-glucose medium (Fig. 3B). We then treated biofilm cultures of PA14 and its Δ*tpbA* mutant with 5× MIC of cisplatin (250 μg/mL), and compared these results with those of treatment with 5× MIC of MMC (10 μg/mL). Cisplatin completely eradicated (beyond the limit of detection, 100 cells/mL) the biofilm cells of Δ*tpbA* and those of PA14. However, MMC could not eradicate biofilms of either strain (Fig. 3C). These results clearly demonstrate that cisplatin is more potent than MMC with *P. aeruginosa*.

Discussion

As we enter the post-antibiotic age, finding suitable alternatives to combat recalcitrant infections is mandatory. Hence, repurposing pharmaceuticals that are in current clinical use is an attractive strategy since it potentially saves time and resources and may benefit numerous patients (Rangel-Vega et al., 2015). For example, after screening 5,850 *P. aeruginosa* transposon mutants, we identified that uracil is a cell signal that increases biofilm formation and demonstrated that the anti-cancer drug 5-fluorouracil is effective in inhibiting biofilm formation (Ueda et al., 2009). Remarkably, clinical trials in humans demonstrated that coating with 5-fluorouracil was useful to prevent colonization and biofilm formation in central venous catheters, being even more effective than the positive control (chlorhexidine-silver-sulfadiazine) (Walz et al., 2010). Given that cisplatin is also an FDA-approved, anti-cancer agent, our results here indicate cisplatin may be repurposed for use in killing persister cells.

It is established now that chronic infections are facilitated by the survival of a small percentage of dormant persister cells (Fauvart et al., 2011), and since current antimicrobial agents target growth processes such as cell wall replication and protein and DNA synthesis, currently there are no drugs in clinical use that target persister cells. To address this need to combat persister cells, we demonstrated previously that MMC is effective for the eradication of persister cells (Kwan et al., 2015). In the current work, our results demonstrate that cisplatin is also highly effective in treating persister cells. We show that cisplatin can eradicate the persister cells formed from *E. coli*, EHEC, and *P. aeruginosa* (Figs. 1 and 2). We also found that cisplatin was highly effective against *S. aureus* and *P. aeruginosa* clinical strains. Hence it is effective against Gram-negative and Gram-positive bacteria that are among the most common causes of nosocomial infections. Furthermore, our results indicate that cisplatin is more effective than MMC with *P. aeruginosa* for both biofilm and planktonic cells (Fig. 3). Although the antimicrobial properties of cisplatin are well documented (Beck and Brubaker, 1973; Joyce et al., 2010; Rosenberg et al., 1965), prior to our study, this compound has never been tested on persister cells. Hence, this is the first study that shows cisplatin is highly effective in treating dormant persister cells.

Since *P. aeruginosa* biofilm persister cells were eradicated, cisplatin may be effective for treating wounds where biofilms are commonly formed from *P. aeruginosa* and *S. aureus* (DeLeon et al., 2014). This is important since wound care in the U.S. costs $18B/yr, and over 200,000 people die each year from wounds that do not heal properly (DeLeon et al., 2014).

Of the seven anticancer drugs tried in the report, only cisplatin showed potent anti-persister activity. The efficacy of cisplatin may be influenced by a number of factors, including that along with busulfan, carmustine, melphalan, and dynemicin, cisplatin is a direct alkylating agent. Of these direct alkylating agents, cisplatin is the only one that contains a metal; hence, its enhanced activity may also be related to the creation of ROS. However, we found here that catalase mutants of both *E. coli* and *P. aeruginosa* are not more susceptible to cisplatin. Therefore, the mechanism of killing persister cells by cisplatin includes its well-established direct crosslinking of DNA (Chvalova et al., 2007; Eastman, 1987; Zhen et al., 1992) that is independent of cell metabolism, which allows it to work on metabolically dormant persister cells. We also reconfirmed that cisplatin indeed crosslinks the same strand of DNA (Fig. S1). Moreover, cisplatin also crosslinks many types of RNA including siRNA, spliceosomal RNAs, tRNA, and rRNA (Dedduwa-Mudalige and Chow, 2015), thereby inhibiting vital cellular processes. Therefore, cisplatin killing probably depends on DNA repair systems, since an *E. coli* strain deficient in the DNA repair system UvrA (nucleotide excision repair pathway) and RecA (SOS repair pathway) are more sensitive to cisplatin (Beck and Brubaker, 1973). The cyclophosphamide that was also tested against persisters but found to be ineffective is a prodrug that produces phosphoramide mustard that is active only in cells that have low levels of aldehyde dehydrogenase (ALDH) (Emadi et al., 2009). Although persister cells are dormant, it is not known whether they have low levels of ALDH. Analogously, tirapazamine is activated to a toxic radical compound only at very low levels of oxygen (Denny and Wilson, 2000), and under our culture conditions, it is unlikely that the intracellular oxygen concentrations are hypoxic. Nevertheless, it may be informative to further test cyclophosphamide in combination with ALDH inhibitors as well as test tirapazamine at low oxygen conditions.

It should be noted that a possible drawback for the repurposing of anticancer drugs as antibacterial agents is the intrinsic toxicity of some of these compounds, nevertheless the beneficial effect of low cisplatin doses (0.1 and 0.5 mg/kg body weight) in combating bacterial sepsis has already been demonstrated in mice, increasing survival by the activation of peritoneal macrophages (Li et al., 2014). Hence, further animal tests should be performed in order to find the adequate treatment regimens and doses and to asses if the combination of cisplatin with conventional antibiotics improves bacterial clearance by attacking both metabolically active and dormant persister cells. Of course topical applications for wound treatments allow higher concentrations of compounds like cisplatin.

Critically, in light of our previous results demonstrating the effectiveness of MMC (Kwan et al., 2015), which crosslinks opposing strands of DNA, our results with cisplatin, which crosslinks the same strand of DNA, show that DNA crosslinking is

an effective means to combat persister cells. Furthermore, the enhanced anti-persister activity of cisplatin relative to MMC could be related to its more efficient crosslinking of DNA as well as RNA promoted by the platinum present in this compound.

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References

Archer NK, Mazaitis MJ, Costerton JW, Leid JG, Powers ME, Shirtliff ME. 2011. Staphylococcus aureus biofilms: Properties, regulation, and roles in human disease. Virulence 2:445–459.

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. Mol Syst Biol 2:2006.0008.

Beck DJ, Brubaker RR. 1973. Effect of cis-platinum(II) diamminodichloride on wild type and deoxyribonucleic acid repair deficient mutants of *Escherichia coli*. J Bacteriol 116:1247–1252.

Bradner WT. 2001. Mitomycin C: A clinical update. Cancer Treat Rev 27:35–50.

Cervantes-Garcia E, Garcia-Gonzalez R, Resendiz-Albor A, Salazar-Schettino PM. 2015. Infections of diabetic foot ulcers with methicillin-resistant *Staphylococcus aureus*. Int J Low Extrem Wounds 14:44–49.

Chowdhury N, Kwan BW, Wood TK. 2016. Persistence increases in the absence of the alarmone guanosine tetraphosphate by reducing cell growth. Sci Rep 6:20519.

Chvalova K, Brabec V, Kasparkova J. 2007. Mechanism of the formation of DNA-protein cross-links by antitumor cisplatin. Nucleic Acids Res 35:1812–1821.

Conlon BP, Nakayasu ES, Fleck LE, LaFleur MD, Isabella VM, Coleman K, Leonard SN, Smith RD, Adkins JN, Lewis K. 2013. Activated ClpP kills persisters and eradicates a chronic biofilm infection. Nature 503:365–370.

Cross A, Allen JR, Burke J, Ducel G, Harris A, John J, Johnson D, Lew M, MacMillan B, Meers P, Skalova R, Wenzel R, Tenney J. 1983. Nosocomial infections due to Pseudomonas aeruginosa: Review of recent trends. Rev Infect Dis 5(Suppl 5): S837–S845.

Dedduwa-Mudalige GN, Chow CS. 2015. Cisplatin targeting of bacterial ribosomal RNA hairpins. Int J Mol Sci 16:21392–21409.

DeLeon S, Clinton A, Fowler H, Everett J, Horswill AR, Rumbaugh KP. 2014. Synergistic interactions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an in vitro wound model. Infect Immun 82:4718–4728.

Denny WA, Wilson WR. 2000. Tirapazamine: A bioreductive anticancer drug that exploits tumour hypoxia. Expert Opin Investig Drugs 9:2889–2901.

Donegan K, Matyac C, Seidler R, Porteous A. 1991. Evaluation of methods for sampling, recovery, and enumeration of bacteria applied to the phylloplane. Appl Environ Microbiol 57:51–56.

Eastman A. 1987. The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. Pharmacol Ther 34:155–166.

Emadi A, Jones RJ, Brodsky RA. 2009. Cyclophosphamide and cancer: Golden anniversary. Nat Rev Clin Oncol 6:638–647.

Fauvart M, De Groote VN, Michiels J. 2011. Role of persister cells in chronic infections: Clinical relevance and perspectives on anti-persister therapies. J Med Microbiol 60:699–709.

Fletcher M. 1977. The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. Can J Microbiol 23:1–6.

Frei E, Holland JF. 2003. Cancer medicine-6 review: A companion to Holland-Frei Cancer medicine-6. Hamilton, ON: B.C. Decker.

García-Contreras R, Peréz-Eretza B, Jasso-Chávez R, Lira-Silva E, Roldán-Sánchez JA, González-Valdez A, Soberón-Chávez G, Coria-Jiménez R, Martínez-Vázquez M, Alcaraz LD, Maeda T, Wood TK. 2015. High variability in quorum quenching and growth inhibition by furanone C-30 in *Pseudomonas aeruginosa* clinical isolates from cystic fibrosis patients. Pathog Dis 73:ftv040.

Hall MD, Telma KA, Chang KE, Lee TD, Madigan JP, Lloyd JR, Goldlust IS, Hoeschele JD, Gottesman MM. 2014. Say no to DMSO: Dimethylsulfoxide inactivates cisplatin, carboplatin, and other platinum complexes. Cancer Res 74:3913–3922.

Heo YJ, Chung IY, Cho WJ, Lee BY, Kim JH, Choi KH, Lee JW, Hassett DJ, Cho YH. 2010. The major catalase gene (*katA*) of *Pseudomonas aeruginosa* PA14 is under both positive and negative control of the global transactivator OxyR in response to hydrogen peroxide. J Bacteriol 192:381–390.

Hofsteenge N, van Nimwegen E, Silander OK. 2013. Quantitative analysis of persister fractions suggests different mechanisms of formation among environmental isolates of *E. coli*. BMC Microbiol 13:25.

Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, Will O, Kaul R, Raymond C, Levy R, Chun-Rong L, Guenthner D, Bovee D, Olson MV, Manoil C. 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 100:14339–14344.

Joyce K, Saxena S, Williams A, Damurjian C, Auricchio N, Aluotto S, Tynan H, Demain AL. 2010. Antimicrobial spectrum of the antitumor agent, cisplatin. J Antibiot (Tokyo) 63:530–532.

Kaper JB. 1998. Enterohemorrhagic *Escherichia coli*. Curr Opin Microbiol 1:103–108.

Konishi M, Ohkuma H, Matsumoto K, Tsuno T, Kamei H, Miyaki T, Oki T, Kawaguchi H, VanDuyne GD, Clardy J. 1989. Dynemicin A, a novel antibiotic with the anthraquinone and 1,5-diyn-3-ene subunit. J Antibiot (Tokyo) 42:1449–1452.

Kwan BW, Chowdhury N, Wood TK. 2015. Combatting bacterial infections by killing persister cells with mitomycin C. Environ Microbiol 17:4406–4414.

Kwan BW, Valenta JA, Benedik MJ, Wood TK. 2013. Arrested protein synthesis increases persister-like cell formation. Antimicrob Agents Chemother 57:1468–1473.

Lewis K. 2007. Persister cells, dormancy and infectious disease. Nat Rev Microbiol 5:48–56.

Lewis K. 2008. Multidrug tolerance of biofilms and persister cells. Curr Top Microbiol Immunol 322:107–131.

Li Y, Wang Z, Ma X, Shao B, Gao X, Zhang B, Xu G, Wei Y. 2014. Low-dose cisplatin administration to septic mice improves bacterial clearance and programs peritoneal macrophage polarization to M1 phenotype. Pathog Dis 72:111–123.

Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, Villanueva J, Wei T, Ausubel FM. 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc Natl Acad Sci USA 103:2833–2838.

Loewen PC. 1984. Isolation of catalase-deficient *Escherichia coli* mutants and genetic mapping of *katE*, a locus that affects catalase activity. J Bacteriol 157:622–626.

Loewen PC, Triggs BL, George CS, Hrabarchuk BE. 1985. Genetic mapping of *katG*, a locus that affects synthesis of the bifunctional catalase-peroxidase hydroperoxidase I in *Escherichia coli*. J Bacteriol 162:661–667.

Rangel-Vega A, Bernstein LR, Mandujano-Tinoco EA, Garcia-Contreras SJ, Garcia-Contreras R. 2015. Drug repurposing as an alternative for the treatment of recalcitrant bacterial infections. Front Microbiol 6:282.

Rasko DA, Sperandio V. 2010. Anti-virulence strategies to combat bacteria-mediated disease. Nat Rev Drug Discov 9:117–128.

Rodriguez RL, Tait RC. 1983. Recombinant DNA techniques: An introduction. Menlo Park, CA: The Benjamin/Cummings Publiching Company, Inc.

Rosenberg B, Vancamp L, Krigas T. 1965. Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. Nature 205:698–699.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanders CC. 1988. Ciprofloxacin: In vitro activity, mechanism of action, and resistance. Rev Infect Dis 10:516–527.

Shah Z, Mahbuba R, Turcotte B. 2013. The anticancer drug tirapazamine has antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Clostridium difficile*. FEMS Microbiol Lett 347:61–69.

Sharma B, Brown AV, Matluck NE, Hu LT, Lewis K. 2015. *Borrelia burgdorferi*, the causative agent of lyme disease, forms drug-tolerant persister cells. Antimicrob Agents Chemother 59:4616–4624.

Sperandio V, Torres AG, Giron JA, Kaper JB. 2001. Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7. J Bacteriol 183:5187–5197.

Tomasz M. 1995. Mitomycin C: Small, fast and deadly (but very selective). Chem Biol 2:575–579.

Ueda A, Attila C, Whiteley M, Wood TK. 2009. Uracil influences quorum sensing and biofilm formation in *Pseudomonas aeruginosa* and fluorouracil is an antagonist. Microb Biotechnol 2:62–74.

Ueda A, Wood TK. 2009. Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885). PLoS Pathog 5:e1000483.
Walz JM, Avelar RL, Longtine KJ, Carter KL, Mermel LA, Heard SO. 2010. Anti-infective external coating of central venous catheters: A randomized, noninferiority trial comparing 5-fluorouracil with chlorhexidine/silver sulfadiazine in preventing catheter colonization. Crit Care Med 38:2095–2102.
Wolcott R, Dowd S. 2011. The role of biofilms: Are we hitting the right target?. Plast Reconstr Surg 127:28S–35S.
Wood TK, Knabel SJ, Kwan BW. 2013. Bacterial persister cell formation and dormancy. Appl Environ Microbiol 79:7116–7121.
Wu N, He L, Cui P, Wang W, Yuan Y, Liu S, Xu T, Zhang S, Wu J, Zhang W, Zhang Y. 2015. Ranking of persister genes in the same *Escherichia coli* genetic background demonstrates varying importance of individual persister genes in tolerance to different antibiotics. Front Microbiol 6:1003.
Zhen W, Link CJ, Jr., O'Connor PM, Reed E, Parker R, Howell SB, Bohr VA. 1992. Increased gene-specific repair of cisplatin interstrand cross-links in cisplatin-resistant human ovarian cancer cell lines. Mol Cell Biol 12:3689–3698.

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