

ARTICLE

Identification of a potent indigoid persister antimicrobial by screening dormant cells

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Email: tuw14@psu.edu**Abstract**

The subpopulation of bacterial cells that survive myriad stress conditions (e.g., nutrient deprivation and antimicrobials) by ceasing metabolism, revive by activating ribosomes. These resuscitated cells can reconstitute infections; hence, it is imperative to discover compounds which eradicate persister cells. By screening 10,000 compounds directly for persister cell killing, we identified 5-nitro-3-phenyl-1*H*-indol-2-yl-methylamine hydrochloride (NPIMA) kills *Escherichia coli* persister cells more effectively than the best indigoid found to date, 5-iodoindole, and better than the DNA-crosslinker cisplatin. In addition, NPIMA eradicated *Pseudomonas aeruginosa* persister cells in a manner comparable to cisplatin. NPIMA also eradicated *Staphylococcus aureus* persister cells but was less effective than cisplatin. Critically, NPIMA kills Gram-positive and Gram-negative bacteria by damaging membranes and causing lysis as demonstrated by microscopy and release of extracellular DNA and protein. Furthermore, NPIMA was effective in reducing *P. aeruginosa* and *S. aureus* cell numbers in a wound model, and no resistance was found after 1 week. Hence, we identified a potent indigoid that kills persister cells by damaging their membranes.

KEYWORDS

indole, persisters

1 | INTRODUCTION

Nearly all bacterial cells are stressed (e.g., lack of nutrients and antimicrobials; Kim, Chowdhury, Yamasaki, & Wood, 2018; Song & Wood, 2018), so they reduce their metabolism and a subpopulation becomes dormant (Bigger, 1944; Hobby, Meyer, & Chaffee, 1942); this dormant state is known as persistence. Beyond being prevalent in the environment, persistence is relevant in medicine since these cells likely reconstitute recurring infections (Van den Bergh, Fauvart, & Michiels, 2017). Because traditional antibiotics target growing cells and are largely ineffective against persister cells that lack the metabolic activity (Defrain, Fauvart, & Michiels, 2018), it is critical to identify new compounds for killing persister cells to control infections.

To target effectively persister cells with new compounds, it is germane to understand how they form and how they resuscitate. Cells have myriad ways to combat stress; for example, they utilize

sigma factors like RpoS in *Escherichia coli* that redirect gene expression upon nutrient depletion (Wang, Kim, et al., 2011), and most cells in a population employ such an active response. However, a subpopulation of cells, as a result of noisy gene expression or through elegant regulation, becomes dormant (Wood, Song, & Yamasaki, 2019). To reduce metabolism and become persistent, cells utilize toxin/antitoxin (TA) systems (Wang & Wood, 2011); direct evidence of the importance of specific TA systems in persistence is that deletion of toxins MqsR (Kim & Wood, 2010; Luidalepp, Jöers, Kaldalu, & Tenson, 2011), TisB (Dörr, Vulić, & Lewis, 2010), and YafQ (Harrison et al., 2009) reduces persistence, and production of toxins generally increases persistence (Chowdhury, Kwan, & Wood, 2016).

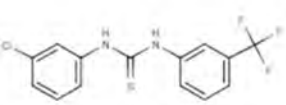
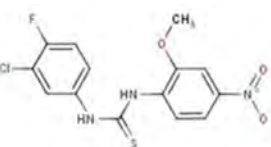
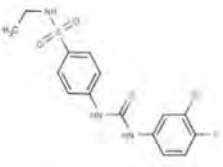
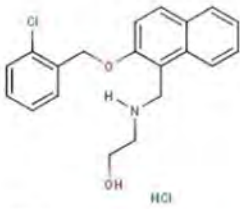
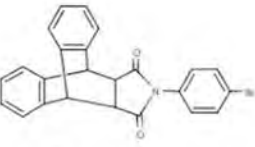
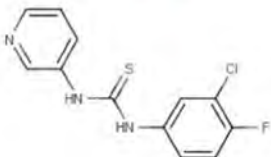
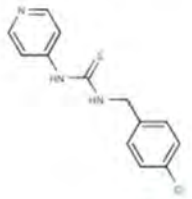
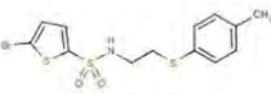
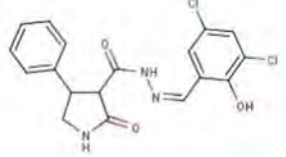
After surviving stress through dormancy, single-cell experiments demonstrate *E. coli* persister cells resuscitate by activating ribosomes (Kim, Yamasaki, Song, Zhang, & Wood, 2018). Resuscitation is heterogeneous as some cells wake immediately and others do not

TABLE 1 Persister cell inhibitors and their structures that were identified in the initial screen

Name	Structure	Name	Structure
[5-nitro-3-phenyl-1 <i>H</i> -indol-2-yl)methyl]amine hydrochloride (NPIMA)		2-({2-[(4-chlorophenyl)amino]-4-quinazoliny]amino)ethanol hydrochloride	
1-[2-(4-chlorophenoxy)-2-methylpropanoyl]-4-methylpiperazine		<i>N</i> -benzyl- <i>N</i> -{[3-(4-methoxyphenyl)-1-phenyl-1 <i>H</i> -pyrazol-4-yl]methyl}acetamide	
<i>N</i> -(3,4-dichlorophenyl)- <i>N'</i> -(3-fluorophenyl)thiourea		2-({2-[(4-bromophenyl)amino]-4-quinazoliny]amino)ethanol hydrochloride	
<i>N</i> -2-(4-ethoxyphenyl)-2,4-quinazolinediamine hydrochloride		4-{{(5-nitro-2-thienyl)methylene}amino}benzamide	
2-[(6-phenyl-2,3,4,9-tetrahydro-1 <i>H</i> -carbazol-1-yl)amino]ethanol		2-bromo-4-chlorophenyl phenylcarbamate	
1-(3,6-dichloro-9 <i>H</i> -carbazol-9-yl)-3-(2-methyl-1 <i>H</i> -imidazol-1-yl)-2-propanol		<i>N</i> -(3-chloro-4-fluorophenyl)- <i>N'</i> -[2-(difluoromethoxy)phenyl]thiourea	
2-(butyryloxy)-1 <i>H</i> -benzo[de]isoquinoline-1,3(2 <i>H</i>)-dione		<i>N</i> -phenyl- <i>N'</i> -[(1-phenylcyclopentyl)methyl]thiourea	
<i>N</i> -[2-(4-fluorophenyl)ethyl]- <i>N'</i> -(4-nitrophenyl)thiourea		2,4-dichloro-5-(5-nitro-2-furyl)benzoic acid	

(Continues)

TABLE 1 (Continued)

Name	Structure	Name	Structure
<i>N</i> -(3-chlorophenyl)- <i>N'</i> -[3-(trifluoromethyl)phenyl]thiourea		<i>N</i> -(3-chloro-4-fluorophenyl)- <i>N'</i> -(2-methoxy-4-nitrophenyl)thiourea	
4-(((3-chloro-4-fluorophenyl)amino)carbonothioyl)amino)- <i>N</i> -ethylbenzenesulfonamide		2-(((2-((2-chlorobenzyl)oxy)-1-naphthyl)methyl)amino)ethanol hydrochloride	
17-(4-bromophenyl)-17-azapentacyclonadeca-2,4,6,9,11,13-hexaene-16,18-dione		<i>N</i> -(3-chloro-4-fluorophenyl)- <i>N'</i> -3-pyridinylthiourea	
<i>N</i> -(4-chlorobenzyl)- <i>N'</i> -4-pyridinylthiourea		5-bromo- <i>N</i> -{2-[(4-methylphenyl)thio]ethyl}-2-thiophenesulfonamide	
<i>N'</i> -(3,5-dichloro-2-hydroxybenzylidene)-2-oxo-4-phenyl-3-pyrrolidinecarbohydrazide			

divide or elongate until their ribosome levels are increased (Kim, Yamasaki, et al., 2018).

In contrast to ineffective traditional antibiotics, some compounds have been identified that kill persister cells. For example, two compounds approved by the U.S. Food and Drug Administration for anticancer treatments, mitomycin C and cisplatin, kill *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and EHEC persister cells by cross-linking their DNA while they are dormant (Chowdhury, Wood, Martínez-Vázquez, García-Contreras, & Wood, 2016; Cruz-Muñiz et al., 2016; Kwan, Chowdhury, & Wood, 2015). As originally suggested due to its toxicity (Chowdhury, Wood, et al., 2016), cisplatin has been shown to be

TABLE 2 MICs (mM) for 5-iodoindole, NPIMA, and cisplatin

Strain	5-Iodoindole	NPIMA	Cisplatin
<i>Escherichia coli</i> BW25113	2	0.1	0.3
<i>Staphylococcus aureus</i>	2	0.1	1
<i>Pseudomonas aeruginosa</i> PA14	2	0.25	0.15

Note: Values for cisplatin are from (Chowdhury, Wood, et al., 2016). Abbreviations: MIC, minimum inhibitory concentration; NPIMA, 5-nitro-3-phenyl-1*H*-indol-2-yl-methylamine hydrochloride.

effective when applied topically for treating *P. aeruginosa* infections in a murine keratitis model (Yuan et al., 2018). In addition, Trp/Arg-containing antimicrobial peptides kill persister cells by disrupting the cell structure of the dormant cells (Kwan, Chowdhury, et al., 2015), and ADEP4, an acyldepsipeptide antibiotic, combined with rifampicin, can eradicate *S. aureus* persisters by causing ClpP protease to degrade proteins nonspecifically (Conlon et al., 2013). By conjugating the traditional antibiotic vancomycin to the cell-penetrating transporter D-octarginine, *S. aureus* persisters and biofilm cells were killed (Antonoplis et al., 2018). In addition, the vitamin A derivatives, retinoids CD437 and CD1530, have been identified that kill *S. aureus* persisters by disrupting lipid bilayers after screening 82,000 small molecules (Kim, Zhu, et al., 2018). Since indole reduces persistence (Hu, Kwan, Osbourne, Benedik, & Wood, 2015; Kwan, Osbourne, Hu, Benedik, & Wood, 2015), indole derivatives, such as 5-iodoindole and 4-fluoroindole, have been tested and found to kill *E. coli*, *S. aureus*, and EHEC persister cells (Lee, Kim, Gwon, Wood, & Lee, 2016), but they are not effective against *P. aeruginosa*.

In the present study, by screening 10,000 compounds, we identified that 5-nitro-3-phenyl-1*H*-indol-2-yl methylamine hydrochloride (NPIMA) kills *E. coli* persister cells. In addition, we found the

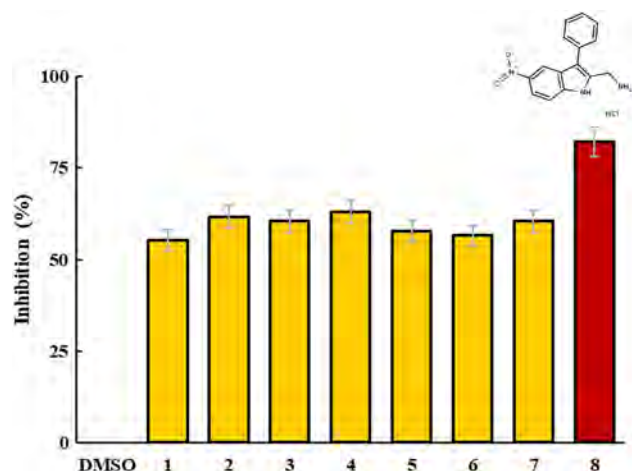


FIGURE 1 Most effective persister killing compounds identified from screening persister cells. *Escherichia coli* persister cells were treated for 24 hr at 100 μ M with 1: *N*-(3,4-dichlorophenyl)-*N'*-(3-fluorophenyl) thiourea, 2: 2-([2-[(4-bromophenyl)amino]-4-quinazolinyl]amino)ethanol hydrochloride, 3: 2-[(6-phenyl-2,3,4,9-tetrahydro-1*H*-carbazol-1-yl)amino] ethanol, 4: 1-(3,6-dichloro-9*H*-carbazol-9-yl)-3-(2-methyl-1*H*-imidazol-1-yl)-2-propanol, 5: *N*-[2-(4-fluorophenyl)ethyl]-*N'*-(4-nitrophenyl) thiourea, 6: *N*-(4-chlorobenzyl)-*N'*-4-pyridinylthiourea, 7: *N'*-(3,5-dichloro-2-hydroxybenzylidene)-2-oxo-4-phenyl-3-pyrrolidinecarbohydrazide, and 8: [(5-nitro-3-phenyl-1*H*-indol-2-yl)methyl]amine hydrochloride (NPIMA, structure shown in inset). See Table 1 for structures (those with a yellow background) [Color figure can be viewed at [wileyonlinelibrary.com](#)]

mechanism of killing is via cell lysis. Furthermore, we found that NPIMA is more effective than 5-iodoindole with the opportunistic pathogen *P. aeruginosa* and that NPIMA eradicates the persister cells of both *P. aeruginosa* and *S. aureus*.

2 | MATERIALS AND METHODS

2.1 | Bacteria and growth conditions

The bacteria used in this study are *E. coli* K-12 BW25113 (Baba et al., 2006), *S. aureus* ATCC29213, and *P. aeruginosa* PA14 (Liberati et al., 2006). Lysogeny broth (LB; Bertani, 1951) was used at 37°C for culturing the bacteria. 2-(Aminomethyl)-indole was obtained from Sigma-Aldrich (catalog number 563838), and 2-methyl-5-nitro-3-phenyl-1*H*-indole was obtained from ChemBridge (San Diego, CA).

2.2 | Persister cells

E. coli persister cells were prepared following our previous method (Kim, Yamasaki, et al., 2018; Kwan, Valenta, Benedik, & Wood, 2013). Exponentially-growing cells (turbidity of 0.8 at 600 nm) were treated with rifampicin (100 μ g/ml for 30 min) to stop transcription, washed, and any remaining nonpersister cells were lysed by ampicillin in LB (100 μ g/ml for 3 hr). Cells were harvested by centrifugation (17,000g for 1 min) and washed with 1 \times phosphate-buffered saline buffer (PBS, 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 , and 0.2 g KH_2PO_4 per 1,000 ml) twice to remove all possible carbon sources, then resuspended with 1 \times PBS.

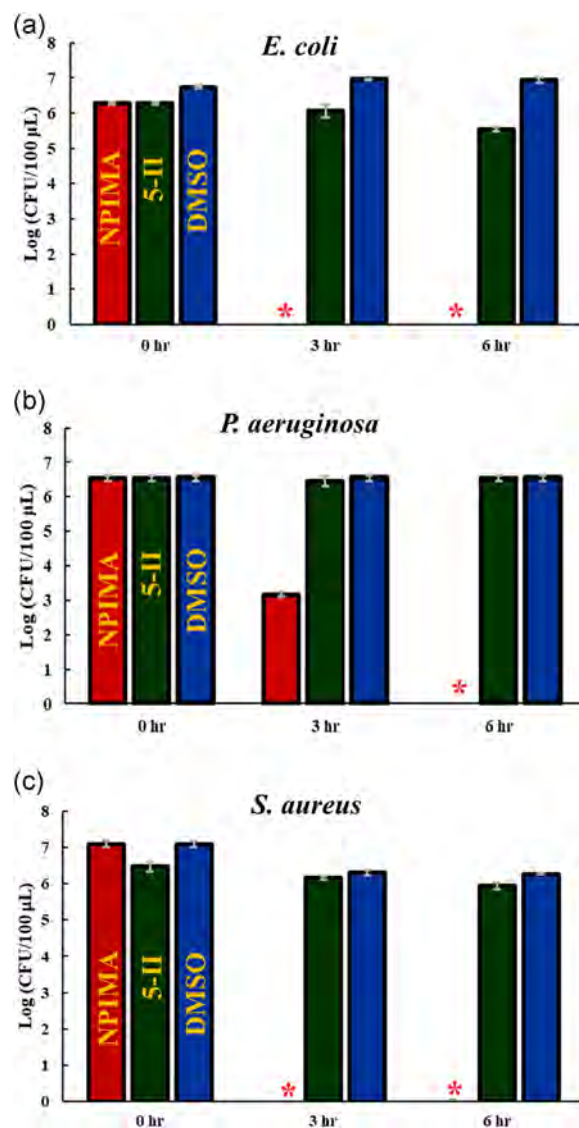


FIGURE 2 NPIMA eradicates *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* exponential cells. Survival after 6 hr for (a) *E. coli* BW25113 with NPIMA and 5-iodoindole at 100 μ M, (b) *P. aeruginosa* PA14 with NPIMA and 5-iodoindole at 100 μ M, and (c) *S. aureus* with NPIMA and 5-iodoindole at 200 μ M. Red asterisk indicates no viable cells detected. NPIMA, 5-nitro-3-phenyl-1*H*-indol-2-yl-methylamine hydrochloride [Color figure can be viewed at [wileyonlinelibrary.com](#)]

Natural *E. coli* persister cells were generated by treating stationary-phase cells (turbidity of 6 at 600 nm) with ampicillin (100 μ g/ml) for 3 hr.

P. aeruginosa PA14 persister cells were prepared by incubating to the stationary phase, diluting sixfold, and treating with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 50 mg/ml stock solution in dimethyl sulfoxide [DMSO]) to stop adenosine triphosphate (ATP) production (200 μ g/ml for 3 hr), washed twice with 0.85% NaCl (5,000g for 10 min), and any nonpersister cells were killed by ciprofloxacin (5 μ g/ml) in LB for 3 hr. Following the antibiotic treatment, bacteria were washed twice with 0.85% NaCl (5,000g for 10 min).

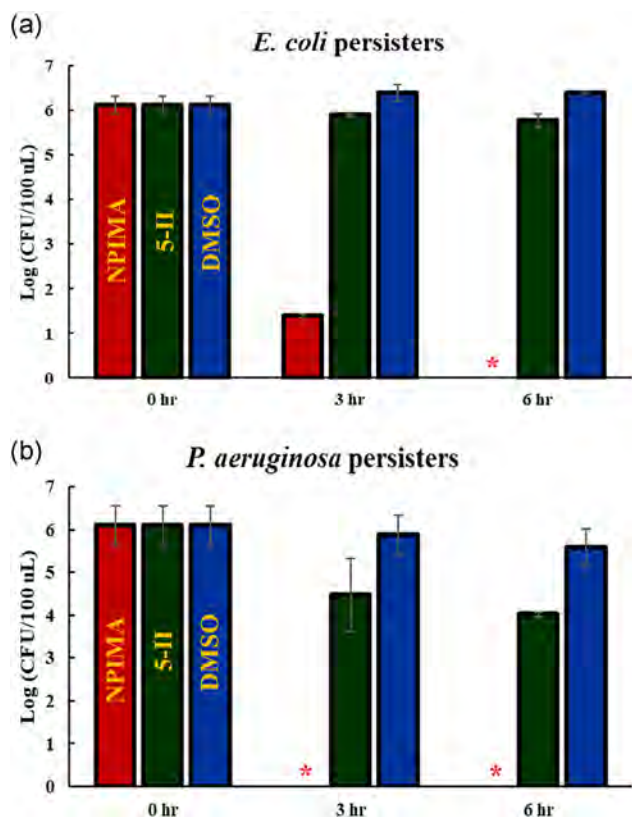


FIGURE 3 NPIMA kills *Escherichia coli*, *Pseudomonas aeruginosa*, persister cells. Survival of (a) *E. coli* BW25113 and (b) *P. aeruginosa* PA14 after treatment with NPIMA (100 μM), 5-iodoindole (5-II, 100 μM), and DMSO for 6 hr. Red asterisk indicates no viable cells detected. DMSO, dimethyl sulfoxide; NPIMA, 5-nitro-3-phenyl-1H-indol-2-yl-methylamine hydrochloride [Color figure can be viewed at wileyonlinelibrary.com]

2.3 | ChemBridge screen

To identify compounds that kill *E. coli* persister cells, 10,000 compounds of the DIVERset Library from ChemBridge (San Diego, CA) were tested by adding 4 μl of each (in DMSO, final concentration 100 μM) to 186 μl of LB in 96-well plates along with 10 μl of persister cells; the persister cells were added after the ChemBridge chemical since LB wakes persister cells (Kim, Yamasaki, et al., 2018). For the negative control, pure DMSO (final concentration 2 vol%) was used. The degree of inhibition was determined by the change in turbidity at 600 nm after 24 hr. The best 26 compounds were re-tested with the same conditions.

2.4 | Minimum inhibitory concentration (MIC)

To determine the MICs of NPIMA and 5-iodoindole for *E. coli* K-12, *S. aureus*, and *P. aeruginosa* PA14, cells were inoculated into LB at varying concentrations and grown for 24 hr. The MIC was determined as the lowest concentration that prevented an increase in growth as evidenced by a lack of change of turbidity.

2.5 | LIVE/DEAD assay

Cell viability after treating with NPIMA was determined using the using the LIVE/DEAD BacLight Bacterial Viability Kit (catalog number, L7012; Molecular Probes, Inc., Eugene, OR). The fluorescence signal was analyzed via a Zeiss Axioscope.A1 using excitation at 485 nm and emission at 530 nm for green fluorescence and using excitation at 485 nm and emission at 630 nm for red fluorescence.

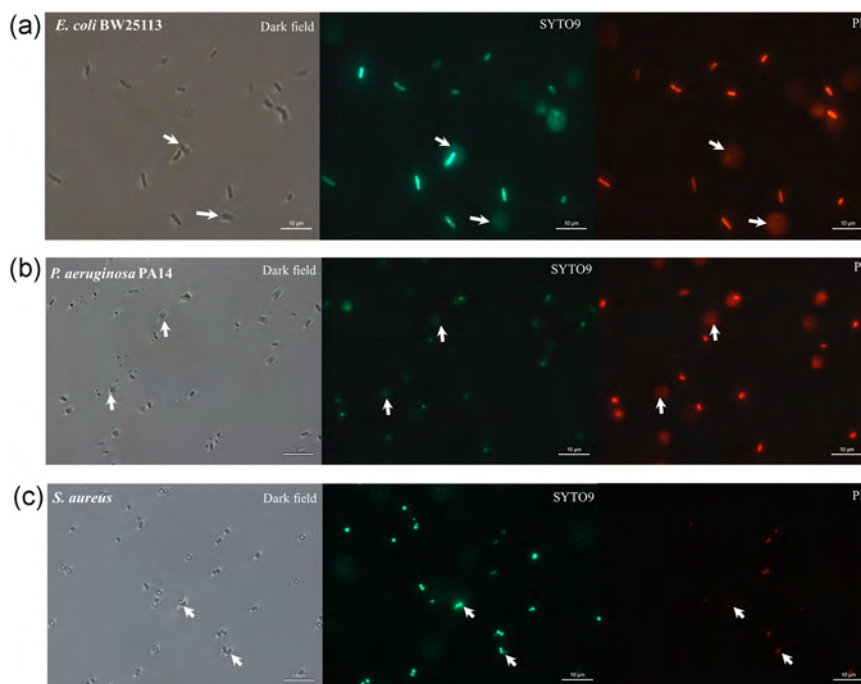


FIGURE 4 NPIMA lyses *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. LIVE/DEAD staining of (a) *E. coli* BW25113 after 0.75 hr, (b) *P. aeruginosa* PA14 after 1 hr, and (c) *S. aureus* after 1 hr treatment with NPIMA at 100 μM. Left shows dark field, middle shows all cells stained by Syto9, and right shows dead cells stained by propidium iodide. White arrows indicate lysed cells. Representative images are shown. Scale bar = 10 μm. NPIMA, 5-nitro-3-phenyl-1H-indol-2-yl-methylamine hydrochloride [Color figure can be viewed at wileyonlinelibrary.com]

2.6 | In vitro wound model

Overnight cultures of *S. aureus* and *P. aeruginosa* PA14 were diluted in wound-like media (45% Bolton broth, 50% bovine plasma, and 5% laked horse blood; Sun, Dowd, Smith, Rhoads, & Wolcott, 2008) to a turbidity of 0.5 at 600 nm in 1 ml. Each culture (1%) was used to inoculate fresh 5 ml of wound-like media. The combined culture (200 μ l/well) was placed into 96-well plates and incubated at 37°C with shaking for 24 hr. The nongel liquid was removed, then the gel was washed once with PBS. NPIMA (0.1 and 0.5 mM) and DMSO were then added (200 μ l/well) to the plates, which were incubated for another 6 hr with shaking. By triturating, the gel with cells was removed and added to 0.8 ml of PBS, and the cell viability was measured by spreading 100 μ l of diluted culture on LB plates.

2.7 | Transmission electron microscopy (TEM)

For transmission electron microscopy (TEM), *E. coli* BW25113 was grown to a turbidity of 0.8 at OD₆₀₀, contacted with NPIMA at 100 μ M for 0.75 hr in PBS, centrifuged at 8000g, and resuspended in PBS. The samples were fixed with buffer (2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) and negative stained with 2% uranyl acetate in the dark for 1 hr, then dehydrated. The sectioned specimens were stained again with uranyl acetate and lead citrate after dehydration and resin embedded. TEM images were obtained using a JEOL JEM 1200 EXII instrument.

2.8 | Lysis assays

Exponentially-growing *E. coli* BW25113 cells (turbidity 0.8 at 600 nm) were washed twice with 0.85% NaCl, resuspended in 1 ml of 1× PBS, and NPIMA (100 μ M) was added for 1 hr for *E. coli* and *P. aeruginosa* and 6 hr for *S. aureus* with shaking at 250 rpm (0.1% DMSO was used as the negative control). Cell supernatants were collected after centrifuging at 6,500g (4°C for 15 min), and total protein was measured by the Bicinchoninic Acid (BCA) protein assay Kit (Prod#23227; Pierce). DNA in the supernatant (199 μ l) was detected by adding 1 μ l of Picogreen (P7589; Invitrogen) and incubating for 5 min at room temperature in the dark room. The fluorescence signal was read by a Tecan microplate reader (Infinite M200PRO) with 480 nm excitation and 520 nm emission by utilizing a calibration curved made with plasmid pEX18Ap at 0, 0.004, 0.008, 0.016, 0.063, 0.125, 0.25, and 0.5 ng/ μ l.

2.9 | Viability and cytotoxicity assays

For bacterial viability, cells were washed twice with 0.85% NaCl, resuspended in 1×PBS, and cell counts were determined via the drop assay (Donegan, Matyac, Seidler, & Porteous, 1991). For human cell viability, pre-cultured human cancer HT-29 cells were dispensed in 98 μ l in 96-well plates with approximately 5,000 cells/well. NPIMA was added (2 μ l) to produce concentrations of 5, 10, 100, and 200 μ M. For controls, Triton X-100 (positive control) and DMSO (negative solvent control), were used, and the medium was used for background. Plates

were incubated in a humidified incubator (37°C, 5% CO₂) for 24 hr. Cell viability was determined via a cell counting kit (CCK-8 Kit, ab228554; Abcam), and cytotoxicity was determined via the lactate dehydrogenase (LDH) assay (LDH Assay Kit, MK401; Takara).

3 | RESULTS

3.1 | NPIMA kills *E. coli* persister cells

To identify compounds capable of killing *E. coli* persister cells, we created a population that consists solely of persister cells and has their population increased by 10⁵-fold by pretreating exponentially-growing cells with rifampicin (100 μ g/ml) for 30 min to stop transcription followed by ampicillin treatment (100 μ g/ml) for 3 hr to kill any nonpersister cells (Kwan et al., 2013). This method for generating persister cells has been evaluated eight ways (Kim, Yamasaki, et al., 2018) and used by us to determine that persister cells wake via ribosome resuscitation (Kim, Yamasaki, et al., 2018) and to show that the cells capable of resuscitation in a viable but not

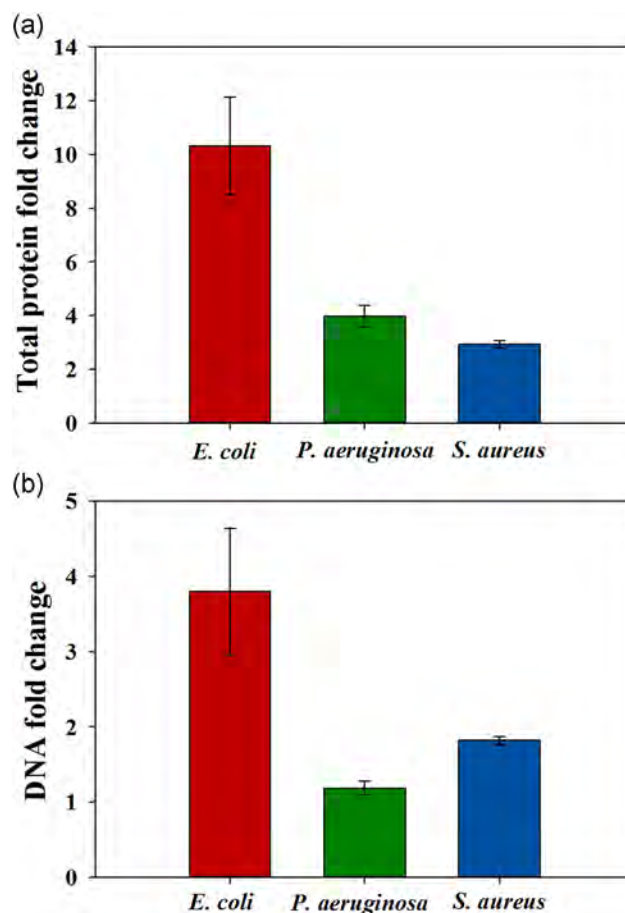


FIGURE 5 NPIMA lyses *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Total protein (a) and DNA (b) found in supernatants as evidence of cell lysis after treatment of cells at a turbidity of 0.8 at 600 nm with 100 μ M NPIMA after 1 hr for *E. coli* and *P. aeruginosa* and 6 hr for *S. aureus*. NPIMA, 5-nitro-3-phenyl-1H-indol-2-yl-methylamine hydrochloride [Color figure can be viewed at wileyonlinelibrary.com]

culturable population are equivalent to persister cells (Kim, Chowdhury, et al., 2018). In addition, this method has been adopted by at least six independent groups (Cui et al., 2018; Grassi et al., 2017; Narayanaswamy et al., 2018; Pu et al., 2019; Sulaiman, Hao, & Lam, 2018; Tkhalishvili, Lombardi, Klatt, Trampuz, & Di Luca, 2018).

To screen directly for killing persister cells, a high-throughput approach using 96-well microtiter plates was devised that consisted of (a) washing persister cells formed from exponentially-growing cells using rifampicin pretreatment followed by ampicillin treatment, (b) adding 10 μ l of the persister cells to 190 μ l of LB containing one each of the 10,000 compounds of the DiverSet library dissolved in dimethyl sulfoxide (100 μ M final concentration), and (c) monitoring for growth via change in turbidity for 24 hr. With this approach, we allowed for up to a 140-fold change in turbidity (0.005 could be increased to 0.69).

Table 1 shows the 25 persister cell inhibitors and their structures that were identified in the initial screen. Of these 25 persister inhibitors, a second screen was performed under the same conditions as the original screen; 8 of these 25 compounds were selected as the most potent (Figure 1) with NPIMA substantially more effective than the other compounds. Critically, NPIMA was the only compound which reduced the turbidity during the first and second screen, suggesting NPIMA lyses persister cells. Hence, we focused on this compound.

3.2 | NPIMA kills *E. coli* exponential cells

Since NPIMA was identified as killing persister cells, we tested whether it is effective on both persister cells and exponential cells. The MIC for NPIMA with *E. coli* was determined to be 100 μ M (15 μ g/ml) (Table 2); hence, we tested it at 100 μ M (1 MIC) and found NPIMA eradicates exponentially-growing *E. coli* within 3 hr (Figure 2a). Furthermore, NPIMA (100 μ M) also eradicated *E. coli* persisters in 6 hr (Figure 3a). Critically, persister cells generated with only ampicillin treatment ("natural persisters") were killed in an identical manner (Figure S1), which confirms our rifampicin-treatment persister model. Therefore, NPIMA kills both persister and exponentially-growing *E. coli*.

3.3 | NPIMA damages the cell membrane and causes cell lysis

To initially explore how NPIMA kills cells, we treated exponentially-growing *E. coli* BW25113 cells with 100 μ M NPIMA and stained with the LIVE/DEAD kit. Remarkably, we found that cells treated with 100 μ M NPIMA lysed as evidenced by the extracellular DNA seen surrounding cells that was stained by both Syto9 and propidium iodide of the LIVE/DEAD kit (Figure 4); note there were no dead cells

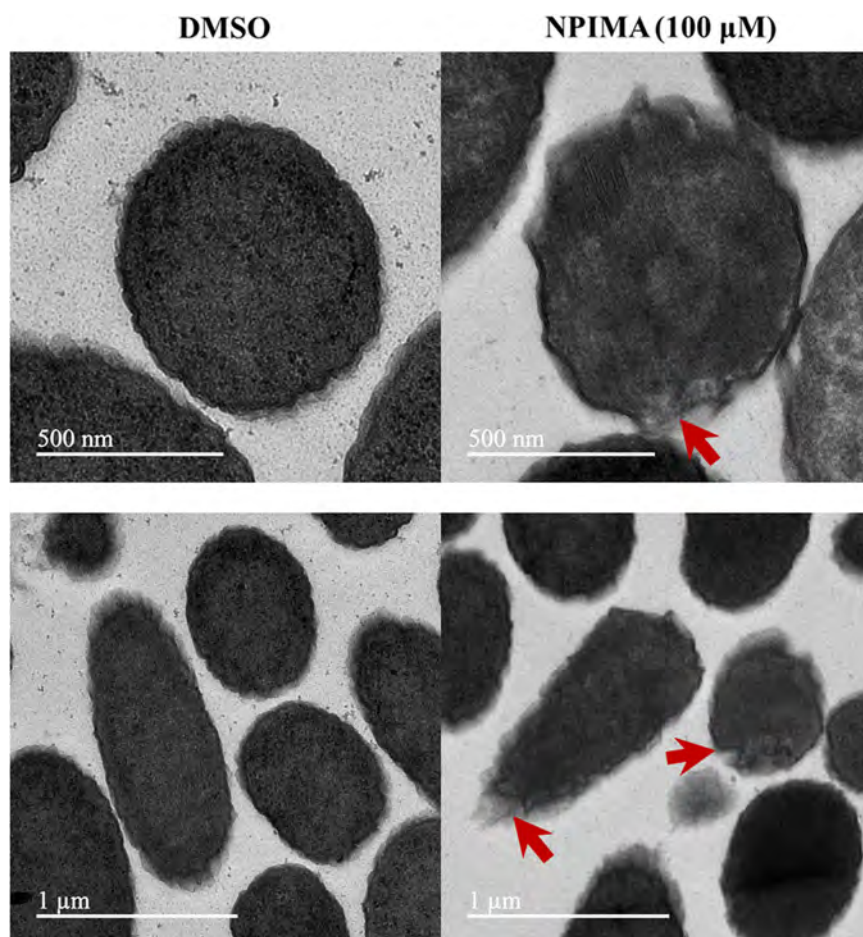


FIGURE 6 NPIMA damages the *Escherichia coli* cell membrane. Transmission electron microscopical (TEM) images of *E. coli* BW25113 after NPIMA (100 μ M) treatment for 40 min. Two representative images are shown. The red arrow indicates membrane damage. NPIMA, 5-nitro-3-phenyl-1*H*-indol-2-yl-methylamine hydrochloride [Color figure can be viewed at wileyonlinelibrary.com]

seen with the solvent control (DMSO). Corroborating the cells lysis seen with the DNA dyes, treatment of exponentially-growing *E. coli* with 100 μ M NPIMA for 1 hr led to both a 10 ± 2 increase in total cell protein in supernatants as well as a 3.8 ± 0.8 increase in DNA in the supernatants compared to the addition of DMSO alone (Figure 5). To investigate further the cell lysis caused by NPIMA, we used transmission electron microscopy and found clear cell envelope damage (Figure 6). Together, these five lines of evidence show NPIMA lyses *E. coli* cells.

3.4 | NPIMA is more effective with *E. coli* persisters than 5-iodoindole

Since 5-iodoindole is the most effective indigo derivative for killing *E. coli* persister cells (kills 99.993% of persisters at 1 mM; Lee et al., 2016), we compared the effectiveness of this compound to NPIMA. Using both compounds at 100 μ M, as indicated above, NPIMA eradicated both exponential (Figure 2a), persister cells (Figure 3a) of *E. coli* whereas 5-iodoindole was much less effective. Corroborating these results, we found the MIC for 5-iodoindole to be 2 mM (Table 2).

3.5 | NPIMA has broad activity

We also tested whether NPIMA was effective at killing *P. aeruginosa* and *S. aureus* persister cells. At 100 μ M, NPIMA eradicated exponentially-growing *P. aeruginosa* PA14 cells in 6 hr (Figure 2b) as well as eradicated *P. aeruginosa* persister cells in 3 hr (Figure 3b). Similarly, NPIMA eradicated *S. aureus* cells at 200 μ M in 3 hr (Figure 2c). In contrast, 5-iodoindole was ineffective with both *P. aeruginosa* at 100 μ M and *S. aureus* at 200 μ M. Corroborating these results, the MIC for 5-iodoindole for *P. aeruginosa* was 2 mM versus 0.25 mM for NPIMA, and the MIC for 5-iodoindole for *S. aureus* was 2 mM versus 0.1 mM for NPIMA (Table 2). Therefore, NPIMA is highly effective with *P. aeruginosa* and *S. aureus*.

3.6 | NPIMA lyses *S. aureus* and *P. aeruginosa*

We also investigated the mechanism by which NPIMA kills *S. aureus* and *P. aeruginosa* persister cells. Using the LIVE/DEAD staining Kit, we found after 1 hr, 100 μ M NPIMA killed 27% for the *S. aureus* cells (Figure 4c). Critically, we also saw evidence of *S. aureus* cell lysis in the form of hazy staining with Syto9 only around cells with NPIMA. Hence, we checked for the presence of extracellular DNA and protein as evidence of lysis and found 2.9 ± 0.1 -fold total protein and 1.82 ± 0.05 -fold DNA released due to 100 μ M NPIMA treatment after 6 hr compared with the addition of DMSO alone (Figure 5).

Similar to *S. aureus*, 100 μ M NPIMA also lysed *P. aeruginosa* as indicated by LIVE/DEAD staining that shows distinct extracellular DNA after treating for 1 hr for both Syto9 and propidium iodide (Figure 4b); however, unlike with *S. aureus*, all (100%) of the *P. aeruginosa* cells were killed in 1 hr. Therefore, we checked for the presence of extracellular DNA and protein as evidence of lysis and found a 1.19 ± 0.09 -fold increase in DNA and a

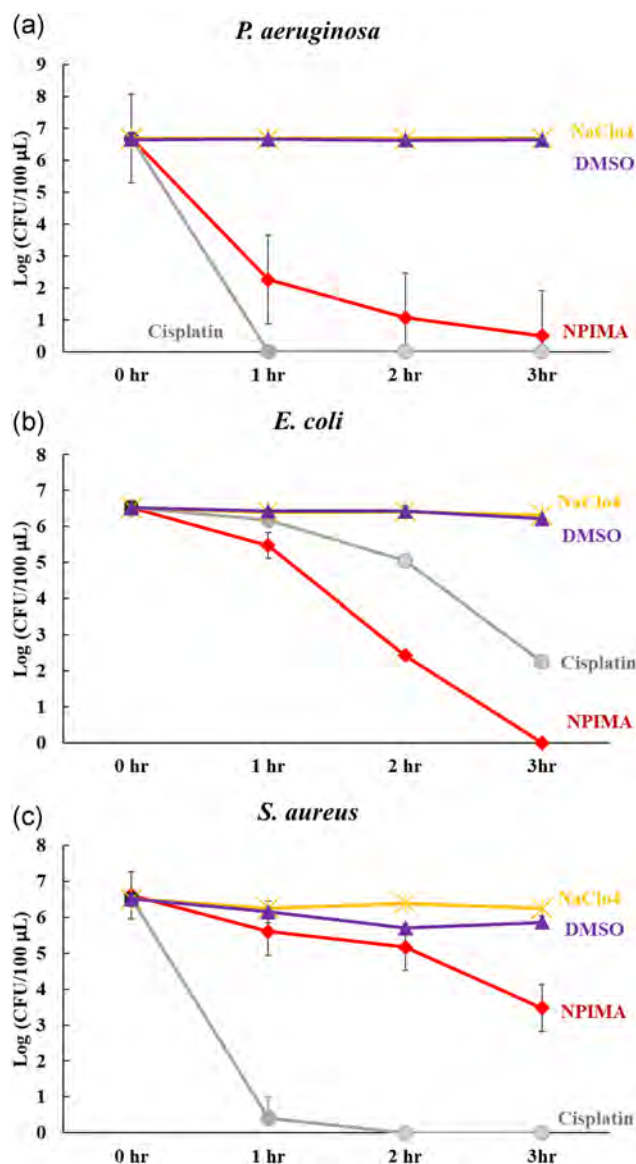


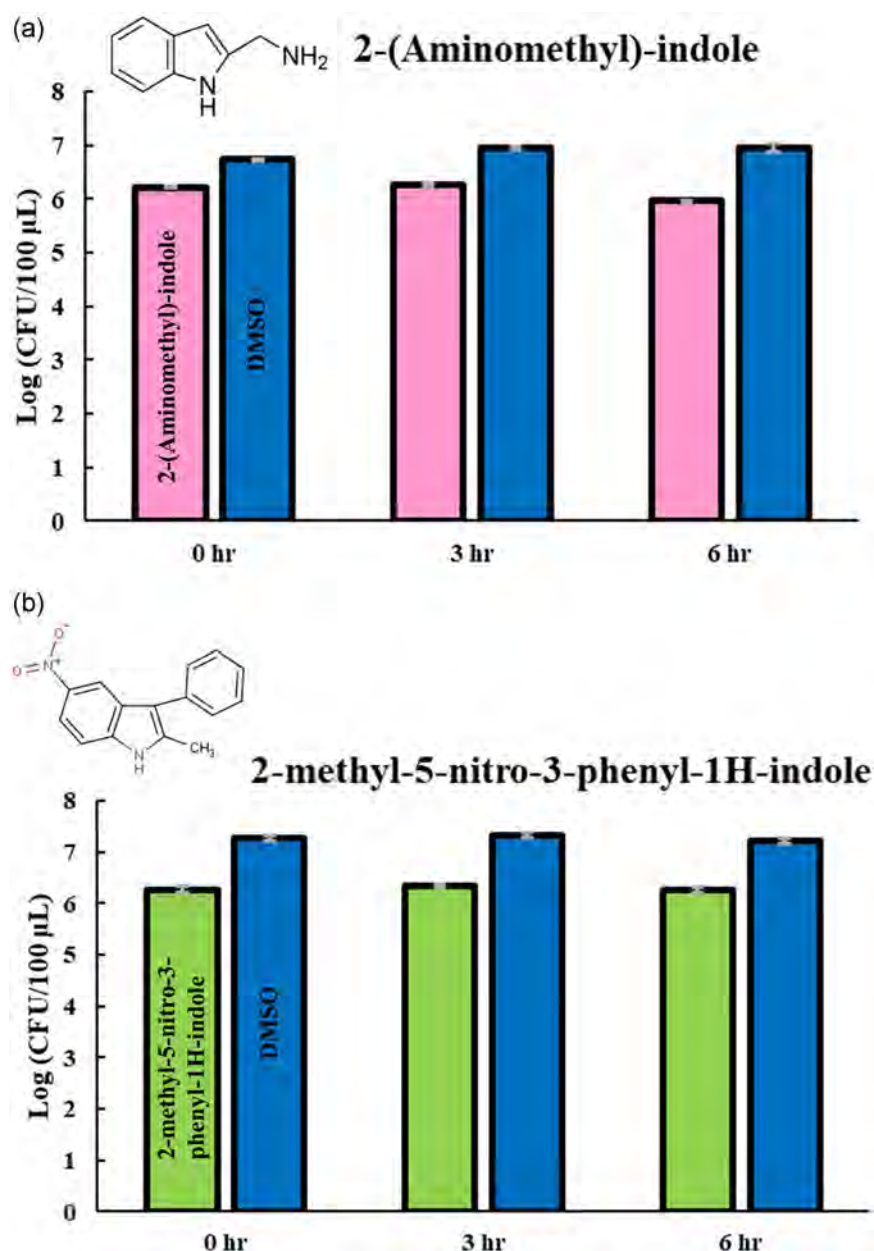
FIGURE 7 NPIMA compared with cisplatin for killing *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*. Survival of exponentially-growing cells of *P. aeruginosa* PA14 (a), *E. coli* (b), and *S. aureus* (c) treated with one MIC of NPIMA (♦) and cisplatin (●) for 3 hr in PBS. DMSO (▲) and NaClO4 (*) were used as negative controls. MIC, minimum inhibitory concentration; NPIMA, 5-nitro-3-phenyl-1*H*-indol-2-yl-methylamine hydrochloride [Color figure can be viewed at wileyonlinelibrary.com]

4.0 ± 0.4 -fold total protein released due to 100 μ M NPIMA treatment after 1 hr compared to the addition of DMSO alone (Figure 5). Hence, as with *E. coli*, NPIMA lyses both Gram-positive *S. aureus* and Gram-negative *P. aeruginosa*.

3.7 | Wound model

To test NPIMA against the pathogen *P. aeruginosa* and *S. aureus* in a realistic infection model, we chose the in vitro Lubbock chronic wound pathogenic biofilm model (Sun et al., 2008), since both pathogens are frequently found together in wounds (DeLeon et al.,

FIGURE 8 NPIMA structure function relationships. Exponential cells of *Escherichia coli* BW25113 were treated with (a) 2-(aminomethyl) indole, and (b) 2-methyl-5-nitro-3-phenyl-1*H*-indole for 6 hr. NPIMA, 5-nitro-3-phenyl-1*H*-indol-2-yl-methylamine hydrochloride [Color figure can be viewed at wileyonlinelibrary.com]



2014) and this model mimics the conditions of polyclonal infections. We found NPIMA (0.5 mM) reduced the total viable number of cells of *S. aureus* and *P. aeruginosa* in the wound model 10-fold in 6 hr compared to the DMSO solvent control (Figure S2).

3.8 | NPIMA is more effective for *E. coli* but less effective for *P. aeruginosa* and *S. aureus* than the DNA crosslinker cisplatin

To gauge its effectiveness, we compared NPIMA to cisplatin, which has been shown to be effective for killing *P. aeruginosa* (Chowdhury, Wood, et al., 2016; Yuan et al., 2018), with each

compound used at one MIC (Table 2). As shown in Figure 7a, cisplatin eradicated *P. aeruginosa* cells in 1 hr whereas NPIMA was less effective. For *E. coli* (Figure 7b), NPIMA was more effective than cisplatin, but for *S. aureus* (Figure 7c), NPIMA was less effective than cisplatin.

3.9 | Resistance

To test if *E. coli* could obtain resistance easily to NPIMA, cells were propagated daily in LB with 0.25 MIC of NPIMA (25 µM) for 7 days. After 7 days, the sequentially-propagated *E. coli* cells were contacted with LB with 1 MIC of NPIMA (100 µM) and incubated overnight to

allow any putative resistant cells to grow and increase the turbidity. Critically, all of the *E. coli* cells were killed. Hence, resistance to NPIMA does not occur readily.

3.10 | Structure activity relationships

To discern insights about the importance of the three substituents on the indole ring of NPIMA, we tested the importance of both the nitro and phenyl groups by assaying the killing of 2-(aminomethyl)-indole with *E. coli* and found 2-(aminomethyl)-indole (100 μ M) is unable to kill *E. coli* (Figure 8). In addition, 2-methyl-5-nitro-3-phenyl-1*H*-indole was used to ascertain the importance of the amine group, and we found 2-methyl-5-nitro-3-phenyl-1*H*-indole (100 μ M) is unable to kill *E. coli* (Figure 8). Hence, all three substituents are important for NPIMA activity.

3.11 | NPIMA cytotoxicity

To determine the cytotoxicity of NPIMA, we performed both an LDH assay and CCK-8 assay with human cells. NPIMA was not toxic at 5 and 10 μ M but showed toxicity in both tests at concentrations of 50 μ M and higher (Figure S3).

4 | DISCUSSION

Previously, we demonstrated through two independent approaches that cell signaling through indole decreases persistence (Hu et al., 2015; Kwan, Osbourne, et al., 2015). Specifically, by producing the RNase toxin YafQ of the *E. coli* YafQ/DinJ TA system, we found YafQ cleaves the mRNA of tryptophanase, which produces indole from tryptophan; hence, there is less indole production and a dramatic increase in persistence (Hu et al., 2015). Additionally, we showed that producing the phosphodiesterase DosP reduces cAMP concentrations which in turn reduces tryptophanase and indole production which leads to a dramatic increase in persistence (Kwan, Osbourne, et al., 2015). Also, direct addition of both indole and halogenated indoles reduces persistence (Lee et al., 2016); hence, indole signaling reduces persister cell formation.

For compounds to kill persister cells by corrupting cytosolic functions, they must be able to enter the cytosol of the dormant cell through passive diffusion, like the DNA-crosslinkers mitomycin C (Kwan, Chowdhury, et al., 2015; Wood, 2016) and cisplatin (Chowdhury, Wood, et al., 2016) or they can attack the outside of the cell by damaging the membrane, like retinoids (Kim, Zhu, et al., 2018). Critically, we found here that NPIMA, a substituted indole, reduces persistence, not by changing indole signaling and altering tryptophanase activity (Hu et al., 2015; Kwan, Osbourne, et al., 2015), but by killing cells through lysis from membrane damage (Figures 4–6). This mode of killing was found to be general for both Gram-negative and Gram-positive bacteria since we found NPIMA was effective with *E. coli*, *P. aeruginosa*, and *S. aureus*.

NPIMA is probably less effective in the complete lysis of Gram-positive strains (Figure 4), which results in the release of cellular protein and DNA, due to the protective cell wall of Gram-positive strains that Gram-negative strains lack. Although there is less lysis of Gram-positive *S. aureus*, NPIMA kills *S. aureus* as well as *E. coli* (Figure 2); hence, complete lysis of Gram-positive strains must not be necessary for NPIMA to cause cell death. Furthermore, comparing Gram-negative strains, actively growing *P. aeruginosa* was lysed (Figures 4 and 5) and killed (Figure 2) less effectively than actively-growing *E. coli*, most likely due to the innate resistance of *P. aeruginosa* due to its active efflux (Chen et al., 2010). However, NPIMA was able to kill the most recalcitrant of cells, persister cells, of all three bacteria since it eradicated the generated persister cells of *E. coli* and *P. aeruginosa* equally well (Figure 3) as well as eradicated the complete population of *S. aureus* cells, which includes persisters (Figure 2c). Note that due to its cytotoxicity at 50 μ M to human cells, NPIMA would have to be used in combination with other antimicrobials or less toxic derivatives need to be found.

Previously, indole (Chimerel, Field, Piñero-Fernandez, Keyser, & Summers, 2012) and the indole derivative 1-geranylindole (Yang et al., 2017) [5-fluoro-[(E)-1-(3,7-dimethylocta-2,6-dien-1-yl)]-3-(piperidin-1-ylmethyl)-1*H*-indole] have been shown to disrupt the cell membrane. In addition, 1-geranylindole killed nongrowing *Mycobacterium bovis*; however, unlike NPIMA, 1-geranylindole has no effect on *E. coli* (Yang et al., 2017). Therefore, we have discovered a potent substituted indole that is effective in killing a wide-range of bacteria.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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