Designer broad-spectrum polyimidazolium antibiotics

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For a myriad of different reasons most antimicrobial peptides (AMPs) have failed to reach clinical application. Different AMPs have different shortcomings including but not limited to toxicity issues, potency, limited spectrum of activity, or reduced activity in situ. We synthesized several cationic peptide mimics, main-chain cationic polyimidazoliums (PIMs), and discovered that, although select PIMs show little acute mammalian cell toxicity, they are potent broad-spectrum antibiotics with activity against even pan-antibiotic-resistant gram-positive and gram-negative bacteria, and mycobacteria. We selected PIM1, a particularly potent PIM, for mechanistic studies. Our experiments indicate PIM1 binds bacterial cell membranes by hydrophobic and electrostatic interactions, enters cells, and ultimately kills bacteria. Unlike cationic AMPs, such as colistin (CST), PIM1 does not permeabilize cell membranes. We show that a membrane electric potential is required for PIM1 activity. In laboratory evolution experiments with the gram-positive *Staphylococcus aureus* we obtained PIM1-resistant isolates most of which had menaquinone mutations, and we found that a site-directed menaquinone mutation also conferred PIM1 resistance. In similar experiments with the gram-negative pathogen *Pseudomonas aeruginosa*, PIM1-resistant mutants did not emerge. Although PIM1 was efficacious as a topical agent, intraperitoneal administration of PIM1 in mice showed some toxicity. We synthesized a PIM1 derivative, PIM1D, which is less hydrophobic than PIM1. PIM1D did not show evidence of toxicity but retained antibacterial activity and showed efficacy in murine sepsis infections. Our evidence indicates the PIMs have potential as candidates for development of new drugs for treatment of pan-resistant bacterial infections.

cationic antimicrobial polymers | bactericidal | colistin-resistant

AMPs and AMP mimics have attracted considerable attention as candidates for therapeutic development (1). The basic design elements include a region of charged residues, generally cationic residues, enabling interaction with bacterial cell surfaces, combined with a hydrophobic nature in AMPs (2). Unfortunately, AMPs and related polymers, in general, have one or more issues that limit their use as broad-spectrum antibiotics. Some are quite toxic to human cells, the potency of some is not adequate for human administration, others are sensitive to salt at levels present in human fluids, and some are too difficult and expensive to synthesize (3, 4). One broad-spectrum antimicrobial peptide, CST has seen increased recent use as a last resort antibiotic. CST is believed to kill bacteria by virtue of its ability to disrupt membrane integrity (5). This antibiotic requires intravenous administration and is nephrotoxic (6). The emergence of CST-resistant pathogens has also become a significant problem

(7). We are unaware of any new broad-spectrum AMPs that have advanced to clinical trials.

Imidazolium (IM) salts are antimicrobials (8), and there is an emerging literature on antimicrobial activity of side-chain and main-chain polyimidazolium (PIM) salts with chemical structures that are in some ways similar to those we describe. Although PIMs are potent antimicrobials, there are biocompatibility problems hindering their development, and some have somewhat limited activity spectra. As with other AMPs, there have been toxicity issues, potency issues, and delivery issues as many have large molecular masses, and there is little known about mammalian cell toxicity or mechanism of action (9–12).

Here we show that members of a series of PIMs we designed and synthesized are potent broad-spectrum antibacterial compounds. We selected two for further analysis and showed they retain activity even against pan-antibiotic-resistant bacteria. Unlike CST and many other AMPs, which disrupt bacterial membranes, our model PIM is bactericidal without disrupting

Significance

We designed and synthesized antimicrobial compounds effective in killing pan-drug-resistant bacterial pathogens. These compounds have a large therapeutic window due to their low mammalian cell toxicity. A lead compound showed efficacy in treating murine model *S. aureus* and *P. aeruginosa* infections. In the case of the obligate respiratory bacterium *P. aeruginosa* we were unable to evolve strains resistant to these compounds. This paper points toward the potential of this type of compound to deal with the current threat of pan-resistant pathogens.

bacterial membranes. Our experiments provide insights about mechanism of action, the potential for the emergence of PIM resistance, and indicate PIMs are effective against a model gram-negative and a model gram-positive pathogen in murine infection models.

Results

Relationship between Alkyl-Chain Hydrophobicity, Antibacterial Activity, and Cytotoxicity. We first synthesized eight water-soluble main-chain alkylated PIM chloride salts with different degrees of hydrophobicity and relatively low molecular weights (Fig. 1). Except for PIM6 and PIM7, all showed significant antimicrobial activity (Table 1). The ethoxylated/carboxylated alkyl chains of PIM6 and PIM7 make them the least hydrophobic of the series, and PIM7 is zwitterionic rather than cationic. PIM0, which due to its short alkyl chain is less hydrophobic than PIM1 showed reduced activity. The difference between PIM1, which did not exhibit toxicity, and PIMs 2 and 3, which did, is that PIMs 2 and 3 have alkyl chains two or four carbons longer than PIM1, respectively. Small differences in the alkyl chain can affect mammalian cell toxicity dramatically.

We chose PIM1 for further study due to its potent antibacterial activity across a spectrum of pathogenic bacteria and the fact that it showed no measurable acute mammalian cell toxicity in our screen of the PIMs (Table 1). In a wider screen of bacterial
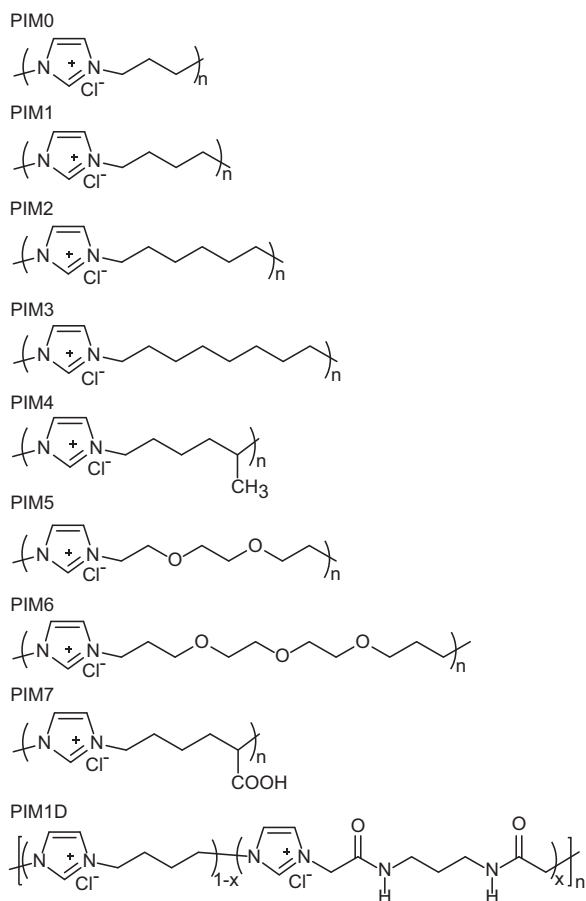
Fig. 1. Chemical structures of PIMs we synthesized and used in our experiments. The number of repeating subunits for each PIM was estimated by gel-permeation chromatography (*SI Appendix*, Fig. S4). Dispersity values for each PIM are also shown. We also provide MIC values for different synthetic batches of PIM1 and PIM1D in *SI Appendix*, Tables S1 and S2. There was very little batch-to-batch variation in either potency or dispersity values.
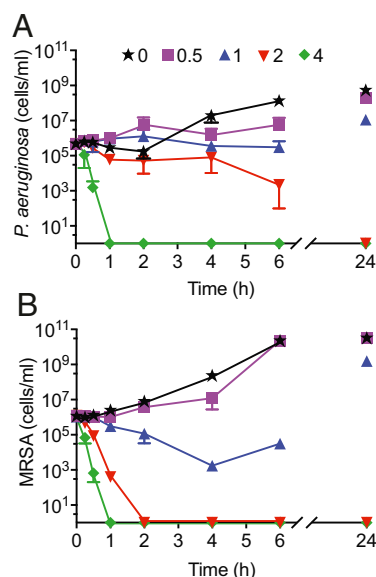
Fig. 2. Viability of (*A*) *P. aeruginosa* PAO1 and (*B*) MRSA LAC* treated with PIM1 (0.5–4 times the MIC for each bacterial species) in comparison to a control with no added PIM1. Cells were incubated at 37 °C in growth media and sampled at times indicated. Cell numbers were determined as cfu per milliliter by plate counting.

pathogens (Table 2) we found that PIM1 showed potent antibacterial activity against a variety of pan-antibiotic-resistant gram-positive and gram-negative bacteria including even CST-resistant *Burkholderia thailandensis* and a CST-resistant *P. aeruginosa* mutant. We note that PIM1 is also a potent anti-*Mycobacterium* compound. By comparison, PIM1 had a broader activity spectrum than CST and polymyxin B, which are not particularly effective as antibiotics for gram-positive bacteria (Table 2). These findings suggest PIM1 has a mode of action distinct from that of CST. Finally, in tests with four different mammalian cell lines, toxicity was not evident or evident at only the highest concentrations tested (Table 3).

PIM1 Is Bactericidal. The experiments described above do not discriminate between PIM1 serving to block bacterial growth or kill bacteria. To make this determination, we inoculated growth media with a model gram-negative pathogen *P. aeruginosa* or a gram-positive pathogen MRSA and determined viable cell number over time in the presence of different concentrations of PIM1 (Fig. 2). Bacterial growth was evident in the absence of PIM1 or in the presence of PIM1 at a level of one-half of the MIC. At twice the MIC, both *P. aeruginosa* and *S. aureus* were killed by PIM1. From these experiments, we conclude that PIM1 is bactericidal.

Antibacterial Activity of PIM1 Involves a Novel Mode of Action. Because we designed the PIMs to have moderately hydrophobic alkyl chains with cationic IM moieties, it seemed possible that, like antimicrobial peptides (5), their activity involves permeabilizing cell membranes. To test this hypothesis, we compared uptake of the fluorescent dye propidium iodide (PI) into PIM1-treated and CST-treated *P. aeruginosa*. Viable cells with intact cell membranes exclude PI. If the membrane is permeabilized, PI can enter cells. As expected, almost all cells treated with CST were stained, but most cells treated even with high concentrations of PIM1 excluded PI (Fig. 3). These results support the view that PIM1 activity does not involve membrane disruption as does CST. In further support of this view, we used the lipophilic fluorescent dye 3,3′-dipropylthiodicarbocyanine iodide [DiS-C3-(5)] to monitor the membrane electrical

Table 1. Antibacterial and cytotoxic effects of PIM0-7

	Minimum inhibitory concentration (μg/mL)*							
	PIM0	PIM1	PIM2	PIM3	PIM4	PIM5	PIM6	PIM7
Bacteria[†]								
Staphylococcus aureus	4	1–2	1–2	2	4–8	8–16	256	>256
Enterococcus faecium	32	1–2	2–4	4	8	8	256	>256
Klebsiella pneumoniae	16	2–4	1–2	2	4	8	64	>256
Acinetobacter baumannii	8–16	2	2	4	4	8–16	>256	>256
Pseudomonas aeruginosa	16	2	2–8	4–8	8–16	16–32	>256	>256
Escherichia coli	32	4–8	4	4	8–16	16–32	>256	>256
Enterobacter cloacae	16	2	2	2–4	8	8	>256	>256
Mouse cells[‡]	155	>1,024	206	20	503	>1,024	>1,024	525

*The minimum PIM concentration required to inhibit bacterial growth by, at least, 90% (MIC_{90}) or the half-maximal inhibition (IC_{50}) of 3T3 cell viability. Values are the ranges of three independent experiments.
[†]The gram-positive bacterial strains were *S. aureus* ATCC 29213, *E. faecium* ATCC 19434, and the gram-negative bacterial strains were *K. pneumoniae* ATCC 13883, *A. baumannii* ATCC 19606, *P. aeruginosa* PAO1, *E. coli* ATCC 8739, and *E. cloacae* ATCC 13047.
[‡]Mouse fibroblast 3T3 cells.

potential ($\Delta\Psi$) in *P. aeruginosa*. Whereas treatment with the proton ionophore gramicidin resulted in a dramatic increase in DiS-C3-(5) fluorescence, indicative of $\Delta\Psi$ dissipation, PIM1 did not show such an effect (Fig. 4).

That PIM1 does not disrupt membranes and does not dissipate $\Delta\Psi$ led us to speculate that it might be taken up by cells. To address this question, we synthesized a fluorescent derivative of PIM1, a PIM1-fluorescein isothiocyanate (FITC) conjugate and treated *P. aeruginosa* with this fluorescent PIM1 derivative. As shown in Fig. 5, PIM1-FITC entered cells. We hypothesized that, as is true of cationic antibiotics (for example, gentamicin [GEN]), association with cells and antimicrobial activity of PIM1 might depend on $\Delta\Psi$. If so, activity should be high when *P. aeruginosa* is in alkaline environments and reduced in acidic environments. In bacteria, such as *P. aeruginosa*, the proton motive force (PMF) remains relatively constant over a range of external pH values as does the cytoplasmic pH (mildly basic).

The total PMF consists of the $\Delta\Psi$ and the pH gradient across the cell membrane (ΔpH). Therefore, in mildly alkaline environments the cytoplasmic and external pH values are similar, and PMF is primarily in the form of a $\Delta\Psi$. In acidic environments the outside pH is lower than the cytoplasmic pH and PMF is primarily in the form of a ΔpH. In fact, the PIM1 MIC was dependent on external pH, and PIM1 showed poor antimicrobial activity at pH 5 (Fig. 5C). These findings suggest that PIM1 uptake is $\Delta\Psi$ dependent. To gain further insights, we tested the influence of the potassium ionophore valinomycin and the sodium potassium exchanger nigericin on PIM1 activity. At neutral pH, valinomycin reduces $\Delta\Psi$, and nigericin collapses ΔpH (16). Results were consistent with our hypothesis: the PIM1 MIC for *P. aeruginosa* was increased by valinomycin treatment and not affected greatly by nigericin (Fig. 5D). From our results we conclude that PIM1 is taken up by cells in a $\Delta\Psi$-dependent

Table 2. Antibacterial effect of select PIMs compared to the activity of CST and polymyxin B on a panel of pan-resistant bacteria and naturally antibiotic-resistant bacteria

	Minimum inhibitory concentration (μg/mL)*			
Bacteria	PIM1	PIM1D	CST	Polymyxin B
S. aureus USA300 (MRSA[†])	2	4	>128	64–128
S. aureus BAA40 (MRSA)	2–4	2	>128	64–128
E. faecalis 583 (VRE[†])	4–8	8	>128	>128
E. coli 958 (MDR)	4	16	2	2
P. aeruginosa PAER	1	4	1	2
P. aeruginosa PAK *pmrB*12[†]	2	4	16	32
A. baumanii AB-1 (MDR)	2–4	4	2	2
A. baumanii X26 (MDR)	2–4	8–16	2	4
A. baumanii X39 (XDR[†])	8	8	4–8	8
B. thailandensis 700388[†]	4	16	>128	>128
K. pneumoniae KPNR (MDR)	2	8	2	4
E. cloacae CRE (MDR)	4	8	2	4
Salmonella enterica 13076	1	1	1	1
Mycobacterium bovis bacillus Calmette–Guérin	1	0.5	128	64
Mycobacterium smegmatis	2	1	128	32

*The concentration of antimicrobial inhibiting bacterial growth by, at least, 90%. Values are the ranges of three independent experiments.
[†]MRSA, methicillin-resistant *S. aureus*; VRE, vancomycin resistant *Enterococcus*; MDR, multidrug resistant; *P. aeruginosa* PAK *pmrB*-12 is a CST-resistant mutant derived from *P. aeruginosa* PAK (13); XDR, extensive drug resistant (14); *B. thailandensis* 700388 is a naturally CST-resistant close relative of the emerging pathogen *Burkholderia pseudomallei* (*B. pseudomallei* is also CST resistant) (15).

Table 3. Comparison of PIM1, PIM1D, CST, and polymyxin B cytotoxicity

	IC$_{50}$ (μg/mL)*			
	PIM1	PIM1D	CST	Polymyxin B
Human kidney (HEK293)	>1,024	716	64	240
Human liver (HepG2)	>1,024	>1,024	>1,024	765
Mouse fibroblast (3T3)	>1,024	>1,024	>1,024	920
Human epithelial (A549)	870	870	>1,024	879

*The concentration of antimicrobial that induced the half-maximal inhibition of mammalian cell viability. Values are the averages of triplicates with less than 10% SDs.

manner, but we cannot discern whether it is exerting its antimicrobial effects at the cellular membrane or in the cytoplasm.

In general, antibiotics have limited activity against nongrowing bacteria. For *P. aeruginosa* this is evident when comparing bactericidal activity of antibiotics, such as GEN on stationary phase cells incubated in the presence vs. the absence of an energy source (17, 18). Based on our finding that PIM1 does not appear to disrupt membrane integrity and that, as does GEN, it requires ΔΨ for activity, we hypothesized that its bactericidal activity on nutrient deprived bacteria might be limited. In fact, stationary phase cells were much less susceptible to PIM1 killing (or GEN killing as a control) than they were to killing by CST (Fig. 6A). Bactericidal activity of both PIM1 and GEN was restored when fumarate was supplied to the stationary-phase cells as an energy source (Fig. 6B). We conclude that, as is true of GEN and many other antibiotics, PIM1 will have limited utility as a bactericide against nongrowing bacteria. We also note that these experiments are consistent with our conclusion that PIM1 does not act by disrupting cell membranes and that it requires ΔΨ for activity.

Laboratory Evolution of PIM1 Resistance. To evaluate the potential of designer PIMs as therapeutics and perhaps gain further insights into the PIM mechanism of action we performed repetitive passaging experiments with escalating concentrations of PIM1 or ciprofloxacin (as a control) on *P. aeruginosa* and MRSA. With *P. aeruginosa*, ciprofloxacin-resistant mutants emerged but PIM1-resistant mutants did not (Fig. 7). PIM1-resistant MRSA emerged at a rate similar to the emergence of ciprofloxacin-resistant mutants.

To gain insights about the nature of the PIM resistance phenotype in our evolved MRSA populations, we isolated bacteria from the final passage. Of 21 resistant isolates characterized, all showed a small-colony variant (SCV) phenotype, 15 had PIM1 MICs more than 128 times that of the initial strain, and the other six had PIM1 MICs 64–128 times that of the parent strain. We sequenced the genomes of the 15 resistant isolates showing MICs >128 times than the unevolved strain (19). All but one had a mutation in a gene required for menaquinone biosynthesis (either a gene in the *menA-F* operon or *ispD*). Several isolates also had mutations in genes known to confer resistance to cationic peptides, specifically, *vraG* or *vraF*, *graR* or *graS*, or *fmtC* (20–22) (*SI Appendix*, Table S3). The genes coding for menaquinone synthesis were of particular interest because a relationship between menaquinones and PIM1 activity might provide some clue about the mode of PIM1 action. Therefore, we compared PIM1 susceptibility of a *menD* deletion mutant to its parent. This *menD* mutant cannot make menaquinone (23) and is growth restricted to fermentation. Like our evolved PIM1-resistant isolates, this mutant has a SCV phenotype. This is a characteristic phenotype of menaquinone synthesis mutants (24). The *menD* mutant showed an eightfold increase in PIM1 resistance over its parent (MIC of 16 μg/mL vs. 2 μg/mL for the

parent). Thus, we believe that menaquinones or a functional electron transport system are involved in the susceptibility of MRSA to PIM1, but that other factors must be involved in the very high PIM1 resistance of our evolved isolates. We reason that either PIM1 directly interferes with the electron transport
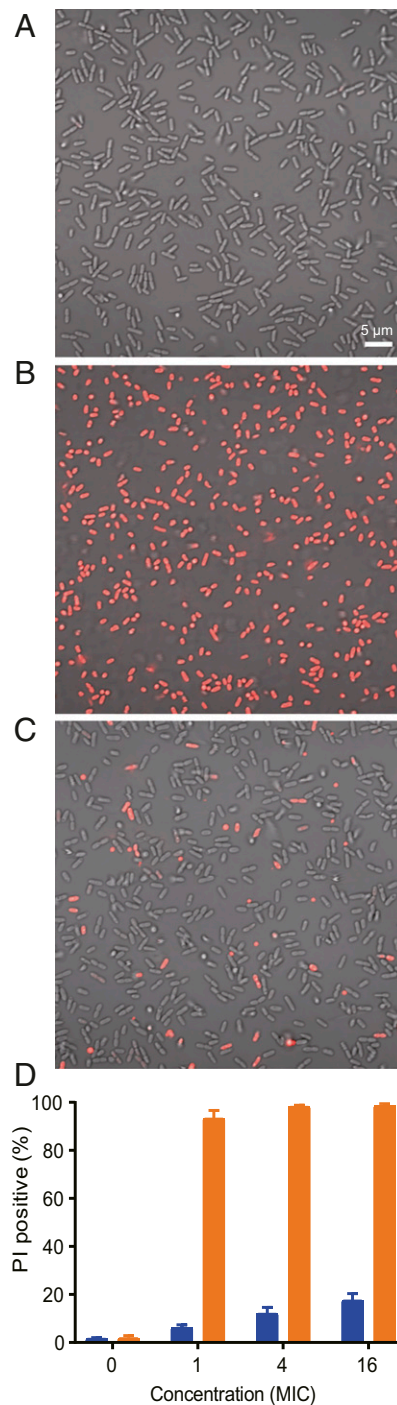
Fig. 3. PI staining of *P. aeruginosa* PAO1 cells. Fluorescence microscope images of (*A*) control cells (no antibiotic), (*B*) cells treated with CST (1 times the MIC), and (*C*) cells treated with PIM1 (1 times the MIC). (*D*) Percent of PI positive cells exposed to PIM1 (blue) or CST (orange) at the concentrations indicated as determined by flow cytometry. Cells were incubated for 1 h in the presence of the antibiotic indicated prior to either microscope examination or flow cytometry.
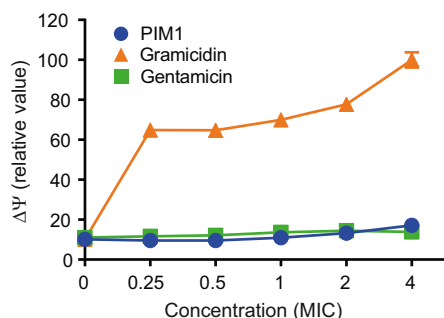
Fig. 4. Relative level of cell membrane electric potential ($\Delta\Psi$) of *P. aeruginosa* PAO1 cells exposed to increasing concentrations of PIM1, the ionophore gramicidin, or the antibiotic GEN. Relative membrane potential was assessed by using the $\Delta\Psi$-sensitive fluorescent membrane probe DiS-C3-(5). An increase in DiS-C3-(5) fluorescence corresponds to a dissipation of $\Delta\Psi$. The ionophore gramicidin is a control agent known to collapse $\Delta\Psi$, and the antibiotic GEN requires $\Delta\Psi$ for uptake but does not dissipate $\Delta\Psi$. Data are relative dye fluorescence values 30 min after addition of test compound. Each data point represents the average of two independent experiments each done in duplicate.

chain, and this leads to generation of toxic reactive oxygen species, or that during fermentative growth PIM1 uptake and, thus, its antimicrobial activity is diminished.

Efficacy of PIM1 Treatment in an Animal Infection. We tested the ability of PIM1 to control a carbapenem-resistant *P. aeruginosa* murine wound infection. We applied bacteria (10^6 colony-forming units [cfu]) to wound sites created on the shaved backs of mice. Four hours after infection, PIM1 solutions were applied to infected wounds. Imipenem (Imp) was used as a control. As expected, the Imp-resistant strain of *P. aeruginosa* increased in numbers over the next 24 h in untreated or Imp-treated wounds. In comparison to untreated or Imp-treated wounds, *P. aeruginosa* numbers were slightly reduced when treated once with PIM1 at 0.1 mg/kg and substantially reduced by about four logarithms when treated once with PIM1 at a dose of 1 mg/kg or above (Fig. 8).

Development of a PIM Lead (PIM1D) for Systemic Use. We first tested the safety of PIM1 when delivered to mice by intraperitoneal (IP) injection and found evidence of acute toxicity; that is, we observed a decrease in body weight over a period of 7 d after administration of a single 6 mg/kg dose (Fig. 9*A*). Therefore, we synthesized PIM1D, which has an amide linkage and is less hydrophobic than PIM1 (Fig. 1). In vitro antimicrobial activity of PIM1D was excellent, and it did not show toxicity in our mammalian cell experiments (Tables 2 and 3). Furthermore, mice treated with PIM1D (15 mg/kg) daily for 7 d did not show a significant weight loss (Fig. 9*A*). To gain further information on the potential for PIM1D toxicity when delivered IP, we analyzed blood chemistry and found a number of markers sensitive to drug toxicity were unchanged by the initial dosing or even after the last dose of PIM1D was delivered (Fig. 9).

We examined the ability of PIM1D to treat infections with carbapenem-sensitive and carbapenem-resistant *P. aeruginosa* as well as infection with MRSA USA300 (Fig. 10). When IP infections with the carbapenem-sensitive *P. aeruginosa* PAO1 were initiated, all untreated mice were dead within 24 h, and the infection had spread to kidneys, livers, and spleens. All mice treated with a single dose of either Imp or PIM1D 2-h postinfection survived the 7-d duration of the experiment and showed no overt signs of illness. Bacterial loads in affected organs were greatly reduced compared to levels in animals that did not receive Imp or PIM1D. Without treatment or with Imp treatment,

all mice infected with the Imp-resistant *P. aeruginosa* PAER had died within 1 or 2 d, whereas all mice treated with a single injection of PIM1D (15 mg/kg) survived for the 7-d duration of the experiment and showed no signs of illness. Likewise, PIM1D spared immunosuppressed mice infected with MRSA USA300 from illness and lowered bacterial burden in affected organs.
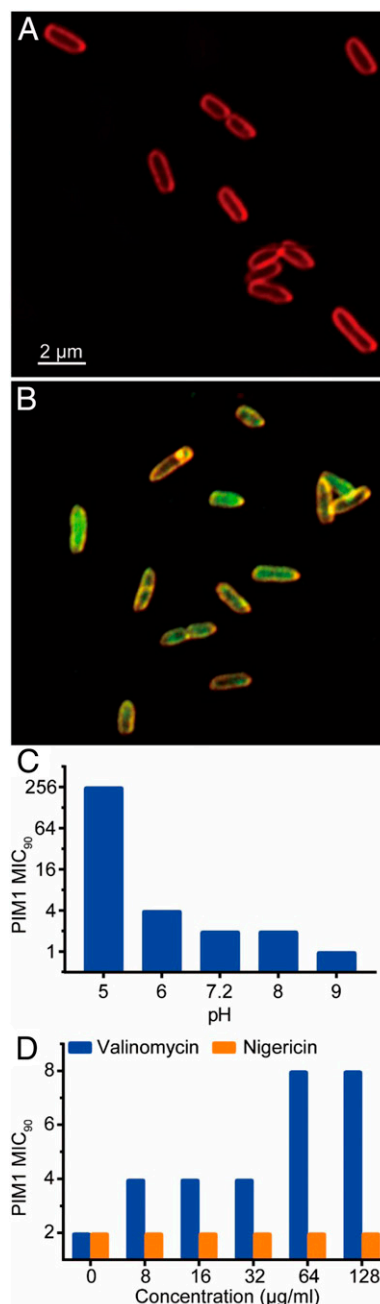

Fig. 5. Uptake of a PIM1-FTIC conjugate by *P. aeruginosa* PAO1 and relationship between PIM1 activity and membrane potential. (*A*) Fluorescence microscope image of control cells (without PIM1) stained with membrane dye FM 4–64FX. (*B*) Fluorescence microscope image of cells treated with PIM1-FITC (1 × the MIC) and stained with FM 4–64FX. (*C*) MIC₉₀ (microgram/milliliter) of PIM1 against *P. aeruginosa* pH-adjusted MHB. (*D*) MIC₉₀ (microgram/milliliter) of PIM1 against *P. aeruginosa* PAO1 in the presence of valinomycin or nigericin.
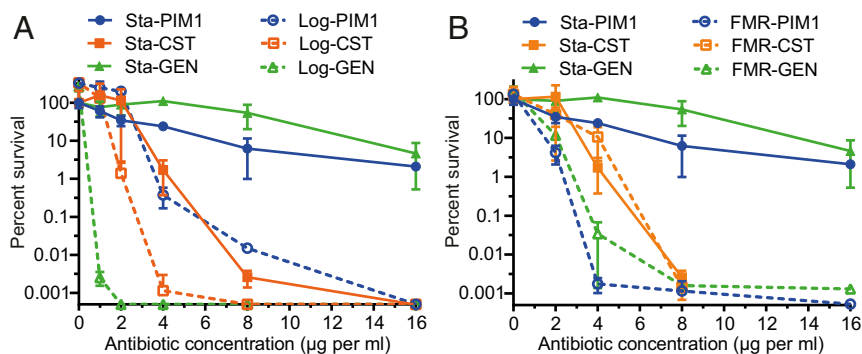
Fig. 6. Influence of metabolic status on *P. aeruginosa* PAO1 killing by PIM1. (*A*) Survival of stationary phase (Sta) bacteria and logarithmic phase (Log) bacteria after a 4-h exposure to PIM1, CST, or GEN. (*B*) Influence of fumarate (15 mM) on survival of stationary-phase bacteria. (Note) We used the same results for Sta-PIM1, Sta-CST, and Sta-GEN in *A* and *B*.

Discussion

Based on available literature on cationic antimicrobial peptides and antimicrobial activity of PIM compounds (8, 25, 26), we designed a series of soluble low-molecular weight compounds with modest hydrophobicity, having mostly delocalized cationic charges in the IM and chloride counterion (Fig. 1). Our rationale was that this design would result in reduced mammalian cell toxicity because a relatively high polymer positive charge might be required for electrostatic association with cell membranes, and mammalian cell membranes have a relatively low negative surface charge compared to bacterial cell membranes (27). This strategy led us to a compound we named PIM1, which has potent broad-spectrum bactericidal activity and little mammalian cell toxicity. Mouse infection experiments showed PIM1 was effective as a topical treatment for skin infections, but when delivered IP, it showed toxicity. We developed a PIM1 derivative PIM1D, which is less hydrophobic than PIM1 with the idea that it might be more readily cleared by mice than PIM1. Although we did not establish this to be the case, we showed IP delivery did not result in measurable toxicity. PIM1D was efficacious in treatment of infections with pan-resistant *P. aeruginosa* or multidrug-resistant *S. aureus*.

Although we have not uncovered a direct target for the antimicrobial activity of PIMs, our experiments primarily with PIM1 shed light on its excellent antibacterial activity with low mammalian cell toxicity. The antimicrobial activity of PIMs can tolerate some changes in the alkyl chain. It appears that the chain must impart sufficient hydrophobicity for maximum activity, and there was an apparent sweet spot for hydrophobicity in our series that corresponded to that of PIM1.

We expected that PIM1 would kill bacteria in a way similar to killing by the cationic peptide antibiotic CST, that it would form pores in the cell membrane. We were surprised to find that killing was not a result of membrane disruption. We present evidence that PIM1 activity requires a sufficient $\Delta\Psi$, and we suppose that its interaction with the cell membrane and subsequent uptake require an electrostatic interaction in much the same way as aminoglycoside antibiotics, such as GEN. In this regard, we tested the ability of PIM1 to kill stationary-phase cells of *P. aeruginosa*. Although many antibiotics including GEN have a limited ability to kill stationary-phase bacteria. CST by nature of its membrane disrupting activity not only kills actively growing bacteria, but also kills nongrowing stationary phase bacteria (5). With respect to activity against stationary phase *P. aeruginosa* PIM1 behaved more like GEN than CST. It has limited activity against stationary phase cells (Fig. 6). We show that a fluorescent PIM1 derivative is taken up by cells, but we have not established the site of PIM1 activity.

We gained some insights on mechanism of action from our experiments on PIM1 resistance. We failed in our attempt to evolve PIM1 resistance in the nonfermentative gram-negative *P. aeruginosa*. With the gram-positive *S. aureus*, we obtained highly resistant mutants almost all of which appeared to be defective in menaquinone synthesis. Such mutants are blocked in respiration, and growth is fermentative. One plausible explanation for our results is that PIM1 is a membrane-active antibiotic that serves as an electron shuttle diverting electrons from the electron transport chain with perhaps a resulting generation of toxic reactive oxygen species. Further studies are required to test this hypothesis and to develop a better general view of the mechanism of PIM action.

The extraordinarily broad-spectrum antimicrobial activity and large selectivity index in comparison to mammalian cell toxicity of PIM1 led us to test its efficacy in animal infection models. As discussed above, PIM1 was effective as a topical treatment for skin wound infections, but it showed toxicity when delivered to
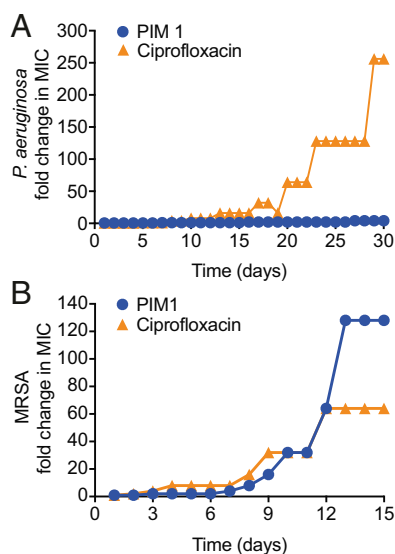

Fig. 7. Evolution of antibiotic resistance in (*A*) *P. aeruginosa* PAO1 and (*B*) MRSA LAC*. *P. aeruginosa* was grown in MHB and MRSA in TSB containing different concentrations of either PIM1 or ciprofloxacin. Bacteria showing visible growth at the highest concentration of antibiotic were transferred daily. Data are reported as the highest antibiotic concentration at which growth was observed and given as the fold increase in concentration relative to the MIC$_{90}$ on day 1.
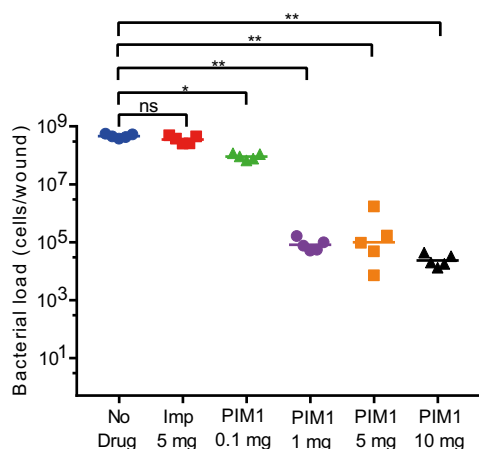
Fig. 8. PIM1 treatment of a skin wound infection. Wounds were infected with the pan-antibiotic-resistant *P. aeruginosa* PAER and treated with 5 mg/kg Imp (*P. aeruginosa* PAER is Imp resistant), or 0.1, 1, 5, or 10 mg/kg PIM1 4 h after infection. Bacterial numbers were determined by plate counting, and data for each individual mouse was reported. The horizontal lines indicate mean values and the bars ± SD. *$P < 0.05$, **$P < 0.01$, and ns indicates $P > 0.05$.

mice by IP injection. Our structure-function studies show that modifications to the main chain are allowable and it may be possible to develop PIMs with improved characteristics. It may also be possible to make some modifications to the IM ring. We also synthesized PIM1D. Design of this compound was aimed at making a PIM with reduced hydrophobicity compared to PIM1. This PIM did not show acute toxicity as did PIM1 when delivered IP. Future chemistry work will also be needed to improve PIM synthesis so that more precise control over the number of repeating subunits in a given PIM can be achieved and to further explore chemical space in PIM design.

We initiated our antibiotic development program with the idea that we could rationally design mimics of cationic peptide antimicrobials that would target bacterial cell membranes but show a reduced affinity to mammalian cell membranes. At the outset, we assumed such mimics would serve as antimicrobials by permeabilizing bacterial membranes by analogy to the action of many cationic peptide antibiotics (28). We succeeded to develop compounds with relatively low mammalian cell toxicity, but, at least, our model compound PIM1 does not appear to permeabilize membranes of our model gram-negative pathogen *P. aeruginosa*.

Materials and Methods

Chemicals. All chemicals used in the syntheses unless otherwise specified were purchased from Sigma-Aldrich Co. LLC. L-lysine and DiS-C3-(5) were purchased from Combi-Blocks, Inc. *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC.HCl) and 1-hydroxybenzotriazole (HOBt) were purchased from GL Biochem Ltd. Cyclophosphamide was purchased from MedChemExpress LLC. PI and FM 4–64FX were purchased from Thermo Fisher Scientific. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Alfa Aesar. FITC was purchased from Biotium, Inc. Technical grade solvents were purchased from SG Labware Pte Ltd. (Singapore) and directly used for column purification without any distillation. Pullulan standards were purchased from Polymer Standards Service.

Chemical Synthesis. The following general procedure was used to prepare PIM0-7. An aqueous acidic solution of diamine (100-mmol total) was maintained in an ice water bath for 30 min after which we added a mixture of formaldehyde (8.12 g, 100 mmol) and glyoxal (14.51 g, 100 mmol) dropwise. The reaction mixture was refluxed for 4.5 h at 80 °C with the exception of PIM5 which was refluxed for 12 h at 120 °C. During reflux the solutions changed from colorless to yellowish. We removed most of the solvent and the

unreacted monomers by rotary evaporation to give a yellow viscous oil, which was diluted with water and dialyzed against acidified water, pH 3 to 4 (1-kDa-cutoff Spectra/Por6 dialysis membrane, Repligen) for 1 d. The diamine for synthesis of PIM0 was 1,3-diaminopropane; for PIM1, 1,4-diaminobutane; PIM2, 1,6-diaminohexane; PM3, 1,8-diaminooctane; PIM4, 1,5-diamino-2-methylpentane; PIM5, 2,2′-(ethylenedioxy)bis(ethylamine); PIM6, 4,7,10-trioxa-1,13-tridecanediamine; and PIM7, L-lysine. NMR spectral data for final products were consistent with the structures shown in Fig. 1 (*SI Appendix*). Synthesis of PIM1D was more difficult than synthesis of PIM0–7, and it is described in detail in *SI Appendix*, Figs. S1–S4.

For preparation of the FITC-conjugated PIM1 we dissolved PIM1 in 0.1-M NaHCO₃ in water and stirred the mixture in this basic environment for 30 min after which we added FITC at a 1:1 molar ratio of FITC to PIM1 and continued stirring overnight in the dark. The PIM1-FITC conjugate was then dialyzed against acidified water (500–1,000-Da cutoff dialysis membrane) for 2 d to remove salts and unreacted dye. The resulting conjugate was lyophilized to achieve the final PIM1-FITC conjugate. Absorbance of FITC at 493 nm in phosphate-buffered saline (PBS) was used to establish the calibration curve, and from this, we estimated the molar ratio of FITC to PIM1 to be about 15%.

Bacteria and Growth Conditions. *P. aeruginosa* PAO1, *E. faecalis* VRE583, and *E. coli* 958 were obtained from the Singapore Center for Environmental and Life Sciences Engineering (SCELSE). The pan-resistant *P. aeruginosa* PAER, the multidrug-resistant *A. baumannii* AB-1, the carbapenem-resistant *K. pneumoniae* KPNR, and the carbapenem-resistant *E. cloacae* CRE were obtained from Tan Tock Seng Hospital (TTSH) Singapore. MRSA USA300 LAC, the LAC derivative *S. aureus* LAC* and the LAC *menD* mutant have been described previously (29). The CST-resistant *P. aeruginosa* (PAK *pmrB*12) and *B. thailandensis* 700388 were provided by Samuel I. Miller, University of Washington. Multidrug-resistant *A. baumannii* X26, extensively drug-resistant *A. baumannii* X39, *M. bovis* bacillus Calmette–Guérin, and *M. smegmatis* mc²155 are from our collection. All other bacteria were purchased from the American Type Culture Collection. Unless otherwise specified, bacteria were grown in Mueller Hinton Broth (MHB) (Becton Dickinson) (30) at 37 °C with shaking. *S. aureus* was grown in Trypticase Soy Broth (TSB) (Becton Dickinson). Mycobacteria were grown in Middlebrook 7H9 broth medium (BD Difco) supplemented with 0.2% glycerol (Sigma-Aldrich), 0.05% Tween 80 (Sigma-Aldrich), and 10% ADS supplement (which is made via dissolving 25-g bovine serum albumin fraction V [Roche], 10-g D-dextrose [Sigma-Aldrich], and 4.05-g sodium chloride [Sigma-Aldrich] in 500-mL
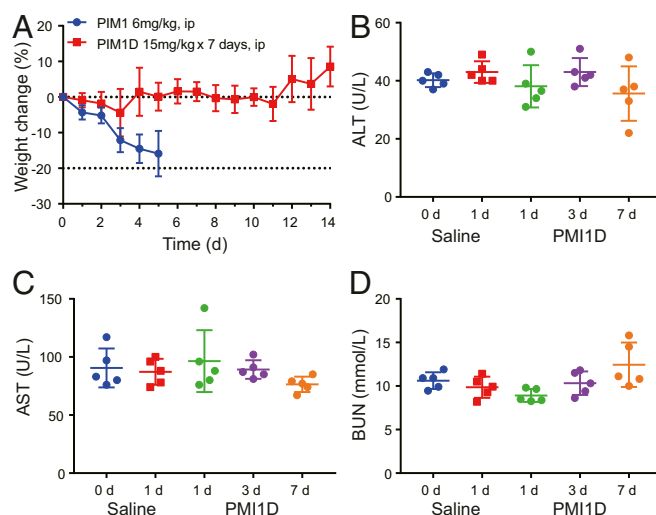

Fig. 9. PIM1 but not PIM1D has apparent toxicity. (*A*) Weight of mice treated with either a single 6 mg/kg dose of PIM1 (day 0) or daily doses of 15 mg/kg PIM1D for 1 wk (days 0–6) given via IP injections. There were five mice in each group. (*B*) alanine aminotransferase (ALT), (*C*) aspartate amino transferase (AST), and (*D*) urea nitrogen (BUN) levels in blood from mice treated with 15 mg/kg PIM1D daily for 7 d. Blood for mice given mock injections of saline solution was drawn just prior to initial injections and 1 d later. Blood for PIM1D-treated mice was drawn at 1, 3, and 7 d after administration of the first injection. There were five mice in each group, and data for individual mice were shown as well as means and SDs.
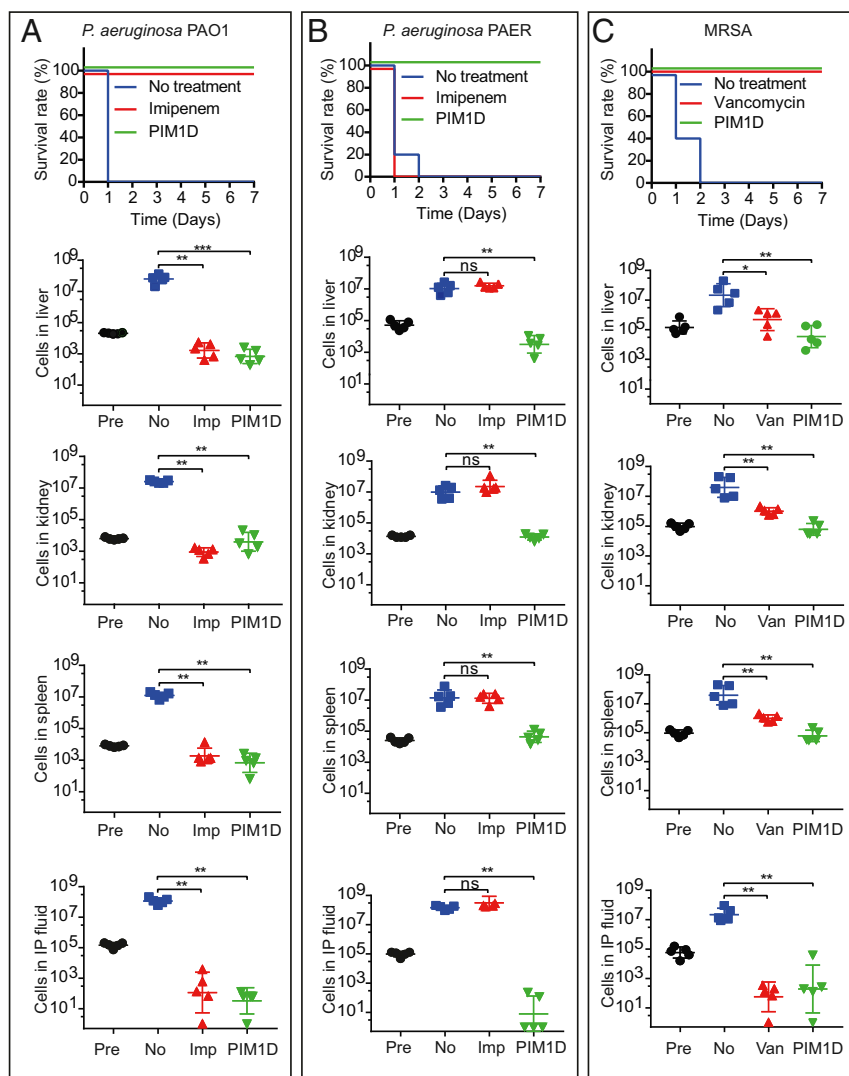
Fig. 10. Protection of mice against septicemia. Infection was via IP injection of (*A*) *P. aeruginosa* PAO1, (*B*) *P. aeruginosa* PAER or (*C*) MRSA USA300. In each panel, the top to bottom presents the Kaplan–Meier survival analysis and bacterial cells count in livers, kidneys, spleens, and peritoneum, respectively. For *P. aeruginosa* PAO1 and PAER, infection was with 10^5 cfu in 300 μL of mucin saline. Mice were then treated with a single IP injection of saline, no treatment (No), 15 mg/kg Imp, or 15 mg/kg PIM1D. Except for counts prior to treatment (Pre), bacterial cell numbers were determined 26 h after infection. Pretreatment bacterial cell counts were from animals 2-h postinfection. For MRSA infections were with 10^8 cells, and treatment consisted of two IP injections of 15 mg/kg the first at 2-h and the second at 26-h postinfection. Cell counts of PIM1 or antibiotic treated groups were from mice at 50-h postinfection, and vancomycin (Van) was used as the antibiotic control. The organs from the No group were harvested close to their death points (26-h/50-h postinfection). Bacterial counts for individual mice (with means and SD) are shown. ***$P \leq 0.001$, **$P \leq 0.01$, and ns is not significant (two-tailed Student *t* test).

water). Glycerol was not supplemented for *Mycobacterium* growth inhibition assay. For plating we solidified Lysogeny Broth (LB) (Becton Dickinson) with 1.5% agar (LB agar), and plates were incubated at 37 °C.

Bacterial Growth Inhibition and Killing Assays. Minimum inhibitory concentrations (MICs) were determined by a slight modification of the broth microdilution method (30). Overnight cultures were subcultured, and cells in the Log growth phase were used to prepare suspensions of about 1×10^6 cfu/mL for use as inocula. A twofold dilution series of the compound to be tested in MHB in a 96-well plate was prepared (final volume 50 μL per well) and incubated at 37 °C with shaking for 10 min (orbital shaker at 225 revolutions per minute) prior to inoculation of each well with 50 μL of bacterial suspension. Plates were then incubated at 37 °C for 18 h statically after which the optical density at 600 nm was measured. For assays involving Mycobacteria, the compounds were serially diluted in twofold steps and 2 μL of this dilution series was spotted in 96-well plates, to which 200 μL of Log-phase bacteria at optical density at 600 nm (OD_{600}) of 0.005 (about 5×10^5 cfu/mL) were added. These plates were incubated for 48 h at 37 °C for *M. smegmatis* and incubated for 5 d at 37 °C for *M. bovis* bacillus Calmette–

Guérin. The MICs are reported as the concentration of the compound that inhibited bacterial growth by, at least, 90%, the MIC_{90}. Three independent experiments were conducted for each compound, and each bacterial strain tested, and the range of MIC values was reported for each compound.

To determine whether PIM1 was bactericidal or bacteriostatic, we inoculated MHB containing PIM1 with *P. aeruginosa* PAO1 or TSB containing PIM1 with *S. aureus* LAC* from Log phase cultures and determined total cfu in samples taken at different times after inoculation by plate counting on LB agar. Two independent experiments were performed and results are the means ± SD.

We used a method described elsewhere to determine the effect of PIM1 and other antibiotics on survival of stationary phase *P. aeruginosa* PAO1 (17) except that stationary-phase cells were obtained by overnight growth in MHB rather than LB, and we compared PIM1 to GEN rather than tobramycin. Results were compared to those with *P. aeruginosa* PAO1 harvested from a MHB culture at the mid-Log growth phase. We tested the ability of an energy source to potentiate PIM1 killing of stationary-phase *P. aeruginosa* by addition of fumarate (15 mM) to the stationary-phase cells.

Mammalian Cell Cytotoxicity Assays. We used four different mammalian cell lines: mouse embryonic fibroblast 3T3, HEK293, HepG2, and A549 cells. Briefly, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin) was used to culture 3T3. For culturing HepG2, HEK cells, and A549, we supplemented DMEM with 15% FBS. Cytotoxicity was measured by using a standard method (31). When 80% confluence was observed by microscopy, cells were treated with trypsin, concentrated, and counted by using a hemocytometer. We then used 1×10^4 cells to seed each well of a 96-well plate. After incubation of the 96-well plate for 24 h, we added PIMs or other test compounds. After a further 24-h incubation, cell viability was assessed by comparing the absorbance of formazan in wells with added antimicrobial agents to the absorbance of formazan in wells with untreated cells. IC_{50} values are reported as the test compound level that reduced viable cell number by 50%. Data are means of triplicate measurements. SDs were 10% or less.

PI Staining. We used *P. aeruginosa* PAO1 in PI experiments. Cells grown in MHB were harvested in the mid-Log phase and resuspended in fresh MHB. We then added PIM1 or CST (positive control) at the indicated concentrations and incubated the cells for an additional hour. After the 1-h incubation with the antimicrobials, we sampled the cell suspensions to determine numbers by plate counting. The remaining cells were washed with PBS and stained with 15 μg/mL PI following the manufacturer's protocol (Invitrogen, Thermo Fisher Scientific). We used flow cytometry to determine the percent of cells that had taken up PI (dead cells), and we used a Zeiss LSM800 confocal microscope to image cells on a polylysine-coated Petri dish (MatTek Corporation).

Monitoring Membrane Electric Potential. We used the membrane potential-sensitive dye DiS-C3-(5) to monitor ΔΨ in *P. aeruginosa* by using a procedure described elsewhere (32). *P. aeruginosa* PAO1 cells were harvested from mid-Log phase cultures by centrifugation and suspended in 5-mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer containing 100-mM KCl and 0.2-mM ethylenediaminetetraacetic acid to permeabilize the outer membrane for DiS-C3-(5) entry. The bacterial suspension was then adjusted to an OD_{600} of 0.02 and DiS-C3-(5) was added (final concentration 1 μM). The cell suspension (180 μL) was then added to each well of a 96-well plate, and test compounds were added to wells as indicated to bring the final mixture to 200 μL. Fluorescence was measured in each well every 2 min in a Spark 10-M microtiter plate reader (Tecan, Switzerland) with excitation at 622 nm and emission at 670 nm. Data shown are at 30 min after addition of the test compound. Two independent experiments were conducted, data are mean values ± SD.

Monitoring PIM1-FITC Uptake by *P. aeruginosa*. We monitored PIM1-FITC uptake as described elsewhere (33) with slight modifications. Briefly, cells grown in MHB were harvested in the mid-Log phase and suspended in fresh MHB containing PIM1-FITC at 1 MIC (the MIC of PIM1-FITC was the same as PIM1) for 30 min. Cells were then harvested by centrifugation, washed once with PBS, and then fixed with 4% paraformaldehyde in PBS for 15 min. Fixed cells were washed twice with PBS and then incubated with 5 μg/mL FM 4–64FX (Invitrogen, Thermo Fisher Scientific) for 10 min on ice. The cells were again washed twice with PBS, then sealed in slides by using Fluoromount aqueous mounting medium (Merck & Co.), and subsequently imaged using a Zeiss Super Resolution System ELYRA PS.1 with a LSM 800 system.

Influence of Valinomycin and Nigericin on the MIC of PIM1 against *P. aeruginosa*. Valinomycin, nigericin, and PIM1 were dissolved in MHB. Stock solutions were added to wells in a microtiter plate to give a volume of 50 μL to which 50 μL of a Log phase *P. aeruginosa* culture was added. Minimum inhibitory concentrations (MIC_{90}s) were determined as described above.

Evolution of PIM1 Resistance. Experiments on evolution of spontaneous PIM1 resistance and ciprofloxacin resistance involved sequential passage as described elsewhere (34). We used either *P. aeruginosa* PAO1 grown in MHB or MRSA LAC* grown in TSB. The inoculum for the initial transfer was 10^7 cells/mL with varying amounts of antibiotic in 1 mL or 100 μL using 2-mL test tubes and 96-well plates for *P. aeruginosa* and MRSA, respectively. The larger volumes for experiments with *P. aeruginosa* were to increase the cell number because resistance did not emerge with smaller culture volumes of this species. Growth was monitored at 24-h intervals. Transfers were daily, and the inocula for transfers (100-fold dilution) were from the cultures with the highest level of antibiotic that allowed growth to an OD_{600} of, at least,

0.2. For *P. aeruginosa*, the experiment was for 30 d. For MRSA LAC*, the experiment was terminated at 15 d. Isolates of MRSA LAC* were obtained from the last transfer and stored as glycerol stocks at −80 °C for use in further studies.

Whole Genome Sequencing. We used standard procedures to isolate genomic DNA from PIM1-resistant *S. aureus* mutants, and DNA was prepared for sequencing by using an Illumina Nextera DNA Library Preparation Kit. DNA was sequenced on an Illumina MiSeq instrument (paired end sequencing). Sequences were mapped onto the genome of the parent strain MRSA LAC* (35), and CLC Genomics Workbench software was used to identify single nucleotide variations, small deletions, and insertions. Large deletions were identified by manual sequence comparison. DNA sequences have been deposited in the European Nucleotide Archive (ENA) and the accession no. is PRJEB37791.

Murine Infection and Toxicity Models. Mice were housed for 1 wk in a 12-h light–dark cycle at room temperature prior to infection. Our skin infection model was as follows: Wounds (diameter about 5 mm) on the shaved dorsal skin of female C57B/6 mice, 8 to 9 wk of age were created by punch biopsy and Log phase cells of *P. aeruginosa* PAER were introduced into the wound (about 10^6 cfu in 10 μL PBS) by pipetting. The infected wounds were immediately covered with Tegaderm (3M). At 4-h postinfection antimicrobial treatment was initiated by injection through the Tegaderm after which another layer of Tegaderm was applied. After a further 24 h, we removed a 1-cm^2 tissue sample from the center of a wound, homogenized the sample, and determined cell numbers by plate counting. Our protocol was approved by the Institutional Animal Care and Use Committee of Nanyang Technological University (NTU IACUC, protocol A0362).

For the systemic infection model, we first assessed toxicity of PIM1 (single IP injection, 6 mg/kg) and PIM1D (IP injection, 15 mg/kg once daily for 7 d) in female BALB/c mice (8 to 9 wk of age) by following weight over a period of 14 d, and we monitored blood biomarkers. Weights were recorded daily (5 d for PIM1 and 14 d for PIM1D), and blood was drawn from a submandibular vein 1 d, 3 d, and 7 d after administration of the first PIM1D dose. For analysis of blood biochemistry, we used a Pointcare V3 Blood Chemistry Analyzer (MNCHIP). The protocol was approved by Animal Ethics and Welfare Committee (AEWC, protocol AEWC-2018-07) of Ningbo University.

For systemic infections, we used female BALB/c mice (8 to 9 wk of age) and challenged them with *P. aeruginosa* or MRSA. For experiments with *P. aeruginosa* (PAO1 and PAER), bacteria from Log phase cultures were washed twice with saline and suspended in 5% mucin saline solution. Bacteria suspended in mucin saline were used as inocula (10^5 cells/mL). Some 300 μL of bacterial suspension was introduced into each mouse via IP injection. At 2-h postinfection, mice (five per group) were treated with single dose of PIM1D, Imp, or saline by IP injection. Mouse survival was monitored over 7 d. Bacterial loads in peritoneal fluid, livers, kidneys, and spleens were determined with a separate set of mice that were euthanized 26-h postinfection. For the pretreatment group, organs were harvested 2-h postinfection. Bacterial numbers were determined by plate counting as described elsewhere (36). Experiments with MRSA were similar to those with *P. aeruginosa* except that we immunosuppressed the mice by IP injection of 150 and 100 mg/kg cyclophosphamide at day 4 and day 1 prior to infection. Treatments were given twice, at 2 and 26 h after infection. Bacterial loads were from organs in mice euthanized 50-h postinfection. For the no treatment group, the mice were euthanized 26- or 50-h postinfection, whichever is closer to their death time. For the pretreatment group, mice were euthanized 2 h after infection. The *P. aeruginosa* PAER infection protocol was approved by the Institutional Animal Care and Use Committee of Nanyang Technological University (NTU IACUC, protocol A0363). The *P. aeruginosa* PAO1 and MRSA protocol was approved by Animal Ethics and Welfare Committee (AEWC, protocol AEWC-2018-07) of Ningbo University.

Data Availability. Genome sequences data have been deposited in the European Nucleotide Archive, https://www.ebi.ac.uk/ena/browser/home (accession no. PRJEB37791).

1. N. Mookherjee, M. A. Anderson, H. P. Haagsman, D. J. Davidson, Antimicrobial host defence peptides: Functions and clinical potential. *Nat. Rev. Drug Discov.* **19**, 311–332 (2020).

2. M. S. Ganewatta, C. Tang, Controlling macromolecular structures towards effective antimicrobial polymers. *Polymer* **63**, A1–A29 (2015).

3. C. Ghosh, P. Sarkar, R. Issa, J. Haldar, Alternatives to conventional antibiotics in the era of antimicrobial resistance. *Trends Microbiol.* **27**, 323–338 (2019).

4. M. Mahlapuu, J. Håkansson, L. Ringstad, C. Björn, Antimicrobial peptides: An emerging category of therapeutic agents. *Front. Cell. Infect. Microbiol.* **6**, 194 (2016).

5. T. Velkov, P. E. Thompson, R. L. Nation, J. Li, Structure–activity relationships of polymyxin antibiotics. *J. Med. Chem.* **53**, 1898–1916 (2010).

6. A. O. Javan, S. Shokouhi, Z. Sahraei, A review on colistin nephrotoxicity. *Eur. J. Clin. Pharmacol.* **71**, 801–810 (2015).

7. H. Ye *et al.*, Diversified *mcr-1*-harbouring plasmid reservoirs confer resistance to colistin in human gut microbiota. *mBio* **7**, e00177(2016).

8. L. Liu *et al.*, Main-chain imidazolium oligomer material as a selective biomimetic antimicrobial agent. *Biomaterials* **33**, 8625–8631 (2012).

9. J. Guo *et al.*, Antibacterial activity of cationic polymers: Side-chain or main-chain type? *Polym. Chem.* **9**, 4611–4616 (2018).

10. Y. Yuan, S. Liang, J. Li, S. Zhang, Y. Zhang, Copolymers with both soft and rigid cationic rings as highly selective antimicrobials to combat antibiotic resistant microbes and biofilms. *J. Mater. Chem. B* **7**, 5620–5625 (2019).

11. J. Liang *et al.*, In situ synthesis of biocompatible imidazolium salt hydrogels with antimicrobial activity. *Acta Biomater.* **99**, 133–140 (2019).

12. Y. Yuan *et al.*, pH-Degradable imidazolium oligomers as antimicrobial materials with tuneable loss of activity. *Biomater. Sci.* **7**, 2317–2325 (2019).

13. S. M. Moskowitz, R. K. Ernst, S. I. Miller, PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. *J. Bacteriol.* **186**, 575–579 (2004).

14. A. P. Magiorakos *et al.*, Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* **18**, 268–281 (2012).

15. A. O. Olaitan, S. Morand, J.-M. Rolain, Mechanisms of polymyxin resistance: Acquired and intrinsic resistance in bacteria. *Front. Microbiol.* **5**, 643 (2014).

16. M. A. Farha, C. P. Verschoor, D. Bowdish, E. D. Brown, Collapsing the proton motive force to identify synergistic combinations against *Staphylococcus aureus*. *Chem. Biol.* **20**, 1168–1178 (2013).

17. S. Meylan *et al.*, Carbon sources tune antibiotic susceptibility in *Pseudomonas* aeruginosa via tricarboxylic acid cycle control. *Cell Chem. Biol.* **24**, 195–206 (2017).

18. K. R. Allison, M. P. Brynildsen, J. J. Collins, Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature* **473**, 216–220 (2011).

19. Z. Shi, M. S. Zeden, A. Gründling, Polymer resistant *Staphylococcus aureus* strains. European Nucleotide Archive. https://www.ebi.ac.uk/ena/browser/view/PRJEB37791. Deposited 14 April 2020.

20. M. Falord, U. Mäder, A. Hiron, M. Debarbouille, T. Msadek, Investigation of the *Staphylococcus aureus* GraSR regulon reveals novel links to virulence, stress response and cell wall signal transduction pathways. *PLoS One* **6**, e21323 (2011).

21. H.-S. Joo, M. Otto, Mechanisms of resistance to antimicrobial peptides in *Staphylococci*. *Biochim. Biophys. Acta* **1848**, 3055–3061 (2015).

22. S.-J. Yang *et al.*, The *Staphylococcus aureus* two-component regulatory system, GraRS, senses and confers resistance to selected cationic antimicrobial peptides. *Infect. Immun.* **80**, 74–81 (2012).

23. J. Lannergård *et al.*, Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **52**, 4017 (2008).

24. C. Von Eiff *et al.*, Phenotype microarray profiling of *Staphylococcus aureus menD* and *hemB* mutants with the small-colony-variant phenotype. *J. Bacteriol.* **188**, 687–693 (2006).

25. J.-P. Lindner, Imidazolium-based polymers via the poly-Radziszewski reaction. *Macromolecules* **49**, 2046–2053 (2016).

26. M. M. Konai, B. Bhattacharjee, S. Ghosh, J. Haldar, Recent progress in polymer research to tackle infections and antimicrobial resistance. *Biomacromolecules* **19**, 1888–1917 (2018).

27. E. Glukhov, M. Stark, L. L. Burrows, C. M. Deber, Basis for selectivity of cationic antimicrobial peptides for bacterial versus mammalian membranes. *J. Biol. Chem.* **280**, 33960–33967 (2005).

28. D. Xhindoli *et al.*, The human cathelicidin LL-37—A pore-forming antibacterial peptide and host-cell modulator. *Biochim. Biophys. Acta* **1858**, 546–566 (2016).

29. V. Pader, E. H. James, K. L. Painter, S. Wigneshweraraj, A. M. Edwards, The Agr quorum-sensing system regulates fibronectin binding but not hemolysis in the absence of a functional electron transport chain. *Infect. Immun.* **82**, 4337–4347 (2014).

30. I. Wiegand, K. Hilpert, R. E. Hancock, Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* **3**, 163–175 (2008).

31. I. O. f. Standardization, *ISO 10993-5: Biological Evaluation of Medical Devices-Part 5: Tests for in Vitro Cytotoxicity* (ISO Geneva, 2009).

32. L. Zhang, P. Dhillon, H. Yan, S. Farmer, R. E. Hancock, Interactions of bacterial cationic peptide antibiotics with outer and cytoplasmic membranes of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **44**, 3317–3321 (2000).

33. L. C. Radlinski *et al.*, Chemical induction of aminoglycoside uptake overcomes antibiotic tolerance and resistance in *Staphylococcus aureus*. *Cell Chem. Biol.* **26**, 1355–1364 (2019).

34. L. L. Ling *et al.*, A new antibiotic kills pathogens without detectable resistance. *Nature* **517**, 455–459 (2015).

35. L. Bowman, M. S. Zeden, C. F. Schuster, V. Kaever, A. Gründling, New insights into the cyclic di-adenosine monophosphate (c-di-AMP) degradation pathway and the requirement of the cyclic dinucleotide for acid stress resistance in *Staphylococcus aureus*. *J. Biol. Chem.* **291**, 26970–26986 (2016).

36. W. Chin *et al.*, A macromolecular approach to eradicate multidrug resistant bacterial infections while mitigating drug resistance onset. *Nat. Commun.* **9**, 917 (2018).

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