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Efficacy of Ampicillin Against Methicillin-Resistant *Staphylococcus aureus* Restored Through Synergy with Branched Poly(ethylenimine)

Melissa A. Foxley¹, Anthony W. Friedline¹, Jessica M. Jensen¹, Susan L. Nimmo¹, Erin M. Scull¹, Jarrod R. King¹, Stoffel Strange¹, Min Xiao¹, Benjamin E. Smith², Kieth J. Thomas III¹, Daniel T. Glatzhofer¹, Robert H. Cichewicz¹, and Charles V. Rice^{1,*}

¹Department of Chemistry and Biochemistry, Stephenson Life Sciences Research Center, University of Oklahoma, 101 Stephenson Parkway, Norman, OK 73019 USA

²Samuel Roberts Noble Microscopy Laboratory, George Lynn Cross Hall, 770 Van Vleet Oval, University of Oklahoma, Norman, OK 73019-4110 USA

Abstract

Beta-lactam antibiotics kill *Staphylococcus aureus* bacteria by inhibiting the function of cell-wall penicillin binding proteins (PBPs) 1 and 3. However, β -lactams are ineffective against PBP2a, used by methicillin-resistant *Staphylococcus aureus* (MRSA) to perform essential cell wall crosslinking functions. PBP2a requires teichoic acid to properly locate and orient the enzyme, and thus MRSA is susceptible to antibiotics that prevent teichoic acid synthesis in the bacterial cytoplasm. As an alternative, we have used branched poly(ethylenimine), BPEI, to target teichoic acid in the bacterial cell wall. The result is restoration of MRSA susceptibility to the β -lactam antibiotic ampicillin with a MIC of 1 $\mu\text{g}/\text{mL}$, superior to that of vancomycin (MIC = 3.7 $\mu\text{g}/\text{mL}$). A checkerboard assay shows synergy of BPEI and ampicillin. Nuclear magnetic resonance (NMR) data show that BPEI alters the teichoic acid chemical environment. Laser scanning confocal microscopy (LSCM) images show BPEI residing on the bacterial cell wall where teichoic acids and PBPs are located.

Keywords

MRSA; β -lactam; penicillin binding protein; ampicillin; teichoic acid

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*Corresponding Author: (C.V.R.) rice@ou.edu. Phone: (405)325-5831. Fax (405) 325-6111. Department of Chemistry and Biochemistry, Stephenson Life Sciences Research Center, University of Oklahoma, 101 Stephenson Parkway, Norman, OK 73019 USA.

CONFLICT OF INTEREST

The authors declare no competing financial interest.

SUPPORTING INFORMATION AVAILABLE

Additional data describing the 20-hour growth assays, cytotoxicity data, and confocal microscopy images provided in a supplementary data file. Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>).

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a current and growing risk to human health. It causes serious infections that show remarkable resistance to antibiotic treatment.¹ Originally acquired exclusively in healthcare settings, MRSA is now regularly found outside the healthcare environment.² Drug resistance hinders efforts to develop safe clinical treatments for MRSA infections.³ Fortunately, progress has been made towards developing new antibiotics such as oxadiazoles,⁴ tedizolid,⁵ and teixobactin.⁶ The timing coincides with a critical period in antibiotic research and development as MRSA is developing resistance to drugs of last resort.^{1, 7, 8} Therapeutic approaches to overcome resistance factors include efflux-pump inhibitors that increase the intracellular concentration of antibiotics.⁹ Bacteria can also use β -lactamase enzymes that degrade the antibiotics¹⁰ and thus treatment requires β -lactamase inhibitors.¹¹

The cell envelope of Gram-positive bacteria is composed of a membrane, peptidoglycan, and teichoic acids (Figure 1A).⁶ Methicillin, a β -lactam antibiotic, occupies the active site of penicillin binding proteins (PBP) 1 and 3 to prevent the enzymatic cell-wall synthesis function (Figure 1B). Methicillin-resistant *S. aureus* performs cell-wall synthesis using PBP2a (Figure 1C), a transpeptidase enzyme with very low affinity for β -lactams. Hartman and Tomasz recognized and identified PBP2a in MRSA.¹² The β -lactam/transpeptidase complex is stable; however, resistance arises because the rate of complex formation is much slower than the *S. aureus* cell division time. Thus, it is nearly impossible for the complex to form *in vivo*.¹³ Fuda *et al.* also presented a structure of PBP2a with no realistic access to the active site, suggesting there had to be a conformational change that took place as a result of binding non-crosslinked peptidoglycan at a location other than the active site, setting the groundwork for future investigations of allosteric regulation. Nevertheless, the cell wall remains an especially rich antimicrobial target, containing many opportunities for disruption, such as excess peptidoglycan,¹⁴ teichoic acids^{15, 16} and novel proteins.¹⁷ While these targets have shown promise, side-effects and slow progress towards clinical usage have hindered efforts to reduce the rate of MRSA infection and mortality.¹⁸ While it is possible to stop teichoic acid expression in the cytoplasm, thereby disabling the function of PBP2a,¹⁵ the quantity of drug required for activity prevent development into a clinical MRSA treatment.¹⁸

The continued spread of resistance could be stemmed by re-sensitizing resistant strains of *S. aureus* to currently ineffective antibiotics, such as the β -lactam ampicillin. This approach can be viable by inhibiting the expression and/or functionality of proteins that contribute to resistance, such as PBP2a. PBPs are indispensable for cell growth as they create essential crosslinks between adjacent peptidoglycan segments. Targeting PBP2a with inhibitors has been shown to re-sensitize resistant strains to methicillin.¹⁹ In contrast, branched poly(ethylenimine) BPEI may indirectly disable PBP2a. Our work shows that BPEI, administered in concert with ampicillin, resensitizes MRSA to ampicillin. Laser scanning confocal microscopy (LSCM) images show that BPEI binds to the cell wall where it can interrupt the function of teichoic acids, inactivate PBP2a, and restore β -lactam antibiotic activity. Nuclear magnetic resonance (NMR) spectroscopy data demonstrate that BPEI binds to teichoic acids, likely through ionic attraction between the cationic polymer and the anionic teichoic acid. Ampicillin activity against MRSA was restored by low molecular

weight (M_W) BPEI. Checkerboard assays were used to measure the fractional inhibitory concentration (FIC) index and identify synergy between 1–8 $\mu\text{g/mL}$ ampicillin and 16 $\mu\text{g/mL}$ low- M_W BPEI; or 8 $\mu\text{g/mL}$ ampicillin and 8 $\mu\text{g/mL}$ low- M_W BPEI. However, BPEI does not increase the efficacy of vancomycin or novobiocin. Thus, BPEI's synergistic properties arise by indirectly disabling PBP2a, rendering MRSA susceptible to ampicillin that disables PBP1 and PBP3.

MATERIALS AND METHODS

Materials

The bacteria used in this work were obtained from the American Type Culture Collection (methicillin-resistant *Staphylococcus aureus* (MRSA) strain ATCC 700787, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 11775, *Bacillus subtilis* (Ehrenberg) Cohn ATCC 23059). Chemicals from Sigma-Aldrich (DMSO, ampicillin, vancomycin, and novobiocin) were used as purchased.

Preparation and Characterization of Cationic Polymers

Branched and linear polyethylenimine was obtained from Sigma-Aldrich, Inc. as high molecular weight (~25 kDa) or low molecular weight (~0.5 kDa) polymers. The molecule has polycationic character from the protonation of its amine functional groups based on its protonation constant (pK_a). Protonation constants for branched PEI molecules have been reported to be around 4.5 for primary amines, 6.7 for secondary amines, and 11.6 for tertiary amines.²⁰ Thus, at $\text{pH} = 7.2$, the BPEI has many positively-charged primary amines to interact with teichoic acid.

Growth Inhibition Assays

Compounds were tested against methicillin-resistant *Staphylococcus aureus* (MRSA) strain ATCC 700787, which also exhibits reduced susceptibility to vancomycin. A stock culture was diluted in tryptic soy broth (TSB) and delivered into pre-sterilized 96-well plates. Stock solutions of BPEI and antibiotics, prepared in DMSO, were added to each well of a 96-well plate (final DMSO concentration was 1%), followed by inoculation with MRSA in TSB. Optical density measurements were performed with a Tecan Infinite M200 plate reader and an initial OD_{600} value recorded. The plates were incubated for 20 h in a humidified incubator at 37°C. Plates were removed, orbitally shaken, and a final OD_{600} value recorded. The final OD_{600} reading was subtracted from the initial OD_{600} reading to obtain the change in OD_{600} (recorded in the figures as ΔOD_{600}). Antimicrobial activity was determined by the change in optical density. Duplicate measurements were performed and the average reported.

Using the average ΔOD_{600} values, separate MIC values for BPEI and ampicillin were determined by the lowest concentration of each that inhibited growth. From this, fractional inhibitory concentration indices (FIC) were calculated for all wells that showed inhibition.^{21, 22}

Cytotoxicity Assay

Mammalian cell cytotoxicity assays were performed on NIH/3T3 mouse fibroblast cells by adding 5,000 cells per well into 96-well plates. The cells were allowed to adhere overnight at 37°C in a humidified incubator (5% CO₂ atmosphere). Test compounds were diluted in DMSO and added to the wells so that the final concentration of DMSO per well did not exceed 1% by volume. The plates containing treated and control cells were incubated for 48 hours and cell viability was determined by MTT assay.²³ Duplicate measurements were performed and the average reported.

Synthesis of the BPEI:Dye Conjugate

Low-molecular weight BPEI (Sigma-Aldrich) was added to Alexa Fluor 488 dye provided in the Alexa Fluor 488 Protein Labeling Kit (Life Technologies) at a ratio of 200 µL BPEI (3 mg/mL stock in Milli-Q H₂O) per tube of powdered dye. After allowing the dye and BPEI to form the conjugate over 1.5 h at 25°C, the product was stored at 4°C and used without further purification.

Labeling MRSA Cells with the BPEI:Dye Conjugate

Cultures of *E. coli* ATCC 11775 and methicillin-resistant *S. aureus* ATCC 700787 at mid-log growth phase were pelleted by centrifugation at 2,000 × g for 5 minutes at room temperature, and the growth media supernatant was removed. 6 µM DAPI in phosphate-buffered saline (1X PBS, pH 7.2) was added to resuspend the cell pellet, which was allowed to incubate for 5 minutes at room temperature. The BPEI-dye conjugate was then added to a final concentration of 100 µg/mL. Two control samples were prepared with either unconjugated BPEI or Alexa Fluor 488 alone and added to MRSA cells as described above. The stained bacterial cells were immediately fixed by resuspension with 4% paraformaldehyde (PFA) followed by a 10-fold dilution in 1X PBS. Cells were added to a microscope slide immediately prior to imaging.

Confocal Microscopy

PFA-fixed cells were mounted in 1X PBS and imaged using a Leica SP8 LSCM with a 63x/1.4NA oil objective. A 405 nm GaN diode laser line was used to image DAPI, and a 488 nm argon laser line was used to observe Alexa Fluor 488 fluorescence. Single optical sections were acquired of cells that had adhered to the coverslip, for highest axial resolution, with a pixel resolution of 80 nm × 80 nm. Instrument settings were kept fixed for all imaging to allow for direct comparison of fluorescence intensity.

Image Processing

To visualize the relative localization of fluorescence, independent of intensity, images were processed (ImageJ v1.49m) such that the total fluorescence intensity within each image was visible (Figure S8, left column). To determine the relative fluorescence intensity between images, the fluorescence intensity of both the DAPI and the Alexa Fluor 488 were normalized to the respective intensities in the MRSA sample treated with BPEI conjugated with Alexa Fluor 488 (Figure S8, right column).

WTA Purification for NMR Studies

The NMR experiments require 1–5 mg WTA isolated from 1 L of bacterial cell culture. The poly(ribitol phosphate) WTA used in this work was isolated from *B. subtilis* W23^{24, 25} (*Bacillus subtilis* (Ehrenberg) Cohn ATCC® 23059™) rather than from MRSA, allowing high volumes (500–2000 mL) of culture to be processed with minimal risk to personnel. This also allows us to take the sample into the NMR facility room which is not rated for BSL-2 work. In the rare instance of sample breakage, using WTA from *B. subtilis* W23 does not present a clean-up hazard nor require decontamination of the expensive NMR analysis probes, magnets, or spectrometers.

B. subtilis W23 cells were grown in LB broth to an OD₆₀₀ reading of approximately 0.8. After growth, the cells were harvested by centrifugation and physically disrupted using an Avestin® EmulsiFlex-C3 homogenizer. The insoluble cell wall was collected, placed in boiling 6% (w/v) SDS, washed with sterile water and EDTA then placed in a 10% trichloroacetic acid (TCA) treatment for 48 hours at 4°C. After allowing the TCA to remove the bound WTA from the cell wall, the WTA was collected in the supernatant and placed into a 500 Da molecular weight cutoff dialysis membrane. Membrane dialysis was performed at 4°C in 1.5 L of sterile water with continual water changes over a 24-hour period. The final 4 h of dialysis took place in a 1 kDa molecular weight cutoff membrane to assure sample purity. The sample was lyophilized and kept at –20°C until use.

NMR Spectroscopy

NMR samples were prepared in Eppendorf tubes by mixing teichoic acid with low-M_W BPEI in water. The pH was measured with a microscopic pH probe and adjusted to 7.2 if necessary. A 3-mm NMR tube was filled with 160 µL of a 2 mM sample of WTA/D₂O or a combination of 2 mM BPEI with 2 mM WTA in D₂O. NMR data collection use Agilent VNMR5-500 MHz equipped with a PFG probe tuned to the ³¹P resonance frequency. Data acquisition and processing were performed using VNMRJ 2.2C software on a system running Red Hat Enterprise Linux.

RESULTS AND DISCUSSION

While there are numerous MRSA strains, *Staphylococcus aureus subsp. aureus* ATCC® 700787™ was isolated in Port Chester, NY from blood culture, exhibits vancomycin intermediate resistant (VISA)²⁶ and also expresses *mecA* to produce PBP2a.²⁷ The ability of BPEI to restore ampicillin effectiveness against MRSA is shown in Table 1. The growth of MRSA *in vitro* after 20 hours of incubation was inhibited by ampicillin when the antibiotic was co-administered with low molecular-weight branched poly(ethylenimine), low-M_W BPEI. MRSA shows resistance towards ampicillin, with a minimum inhibitory concentration (MIC) of 32 µg/mL, but the presence of low-M_W BPEI (16 µg/mL) rendered MRSA susceptible to ampicillin at a 32x lower dose (MIC = 1 µg/mL). At a reduced BPEI concentration of 8 µg/mL, the ampicillin MIC was still decreased, but only to 8 µg/mL. In the absence of ampicillin, BPEI itself inhibited growth of MRSA at a concentration of 64 µg/mL. With these values, it is possible to calculate the fractional inhibitory concentration (FIC) index^{21, 22} for each combination. When the FIC is equal to or below 0.5, ampicillin

and low- M_W BPEI stop MRSA growth through synergistic effects. Synergy occurred between 8 $\mu\text{g}/\text{mL}$ ampicillin and 8 $\mu\text{g}/\text{mL}$ low- M_W BPEI (FIC = 0.375), as well as with combinations of 16 $\mu\text{g}/\text{mL}$ low- M_W BPEI with 1, 2, 4, and 8 $\mu\text{g}/\text{mL}$ ampicillin (FIC = 0.281, 0.313, 0.275, 0.500 respectively). Although MRSA does not grow in the presence of 0.5 $\mu\text{g}/\text{mL}$ ampicillin and 32 $\mu\text{g}/\text{mL}$ low- M_W BPEI, the FIC index is 0.516.

BPEI's potentiation of ampicillin to inhibit MRSA growth suggests that similar effects may be seen with other antibiotics. However, low- M_W BPEI did not benefit vancomycin or novobiocin against MRSA (Figures S1 and S2, respectively). These data suggest that BPEI potentiation depends on the class of antibiotic used. Novobiocin, an aminocoumarin, works by inhibiting DNA gyrase.²⁸ Vancomycin, a glycopeptide, inhibits peptidoglycan crosslinking by binding to the peptidoglycan stems.²⁹ In contrast, ampicillin occupies the active sites of transpeptidase proteins that create the crosslinks between peptide stems.

The trend of lower ampicillin MICs with higher BPEI concentrations suggests therapeutic treatment of MRSA using FDA approved β -lactam antibiotics may be viable. To be clinically viable, BPEI must be safe for human use. Low- M_W BPEI does not cause hemolysis³⁰ and is non-toxic³¹, as cytotoxicity and renal failure are more prevalent with high molecular weight polymers.^{30, 32, 33} Both linear and branched forms, LPEI and BPEI, are used as non-viral transfection agents for gene therapy.^{34–36} In this application, LPEI has undergone clinical trial testing and is awaiting final FDA approved for gene delivery to patients.^{37, 38} These studies also report that the cytotoxicity of PEI increases as its molecular weight increases^{35, 39} and that most branched PEIs are nontoxic below 25 kDa.⁴⁰ To test the cytotoxicity of BPEI used in these experiments (MW \sim 0.5 kDa), mouse fibroblasts (NIH/3T3 cells) were incubated over three days in the presence of varying concentrations of BPEI (supplemental data, Figure S3). Viability was determined using the MTT assay, following the protocol of Hansen *et al.*²³ At 2.65 $\mu\text{g}/\text{mL}$, only 1.5% of the fibroblasts became nonviable over the three-day incubation period compared against the control sample. This indicates negligible cytotoxicity from low- M_W BPEI. From the checkerboard assay (Table 1), synergy occurs with 16 $\mu\text{g}/\text{mL}$ low- M_W BPEI which causes \sim 10% reduction in fibroblast viability (Figure S3). At 26.5 $\mu\text{g}/\text{mL}$ low- M_W BPEI, only a 16.3% reduction in viability compared to the control sample was observed. Thus, BPEI concentrations that induce synergy are also associated with low cytotoxicity; providing guarded optimism in conducting *in vivo* testing. Low- M_W BPEI is non-toxic³¹ whereas high- M_W BPEI penetrates mammalian cells leading to cytotoxicity and renal failure.^{30,32, 33} Low- M_W BPEI, up to 1000 $\mu\text{g}/\text{mL}$, does not affect the membrane of human cells as judged by the lack of hemolysis in red blood cells and the lack of lactate hydrogenase leakage from HEP-2 cells.³⁰ The BPEI concentration for this toxicity testing is \sim 25 times larger than the highest amount, 64 $\mu\text{g}/\text{mL}$, used for antibiotic potentiation.

Antibiotic potentiation by BPEI does not extend to Gram-negative *Escherichia coli* (ATCC 11775). Supplementary data show results of growing *E. coli* in the presence of ampicillin alone and with a fixed BPEI concentration (2.65 $\mu\text{g}/\text{mL}$, Figure S4), while Figure S8 shows the result of testing BPEI with a fixed ampicillin concentration (0.37 $\mu\text{g}/\text{mL}$). In the first data set, the observed ampicillin MIC was identical at 3.7 $\mu\text{g}/\text{mL}$ with or without BPEI addition. In the second data set, BPEI did not show inhibitory effects on growth of *E. coli* up

to 53 $\mu\text{g}/\text{mL}$, either by itself or with 0.37 $\mu\text{g}/\text{mL}$ ampicillin. This is in contrast to the MRSA data in which the ampicillin MIC decreased by a factor of 32 when the BPEI concentration was 16 $\mu\text{g}/\text{mL}$. Additionally, our data differs from previously reported results showing PEI-induced antibiotic synergy against Gram-negative bacteria, including *E. coli* and *P. aeruginosa*.^{41, 42} This could be due to differences in branching or molecular weight between the polymers tested. Our tests use a low- M_{W} BPEI whereas the prior reports use a high- M_{W} LPEI.

The data in Table 1 suggests that BPEI may interact with PBP2a. This would prevent the enzyme from functioning properly while allowing the β -lactam to disable PBP1 and PBP3. If true, BPEI's interaction with MRSA should be confined to the cell wall. By conjugating BPEI to a fluorescent marker, Alexa-Fluor 488, we were able to visualize BPEI localization in bacterial cultures using LSCM. Individual transverse optical sections clearly show BPEI interaction with the MRSA cell wall region (Figure 2A). Using DAPI, a DNA-binding fluorescent dye, as a marker for the cytoplasm within the cells (Figure 2B) the merged image (Figure 2C) confirms that BPEI was not detected within the cytoplasm, verifying that BPEI does not traverse the lipid bilayer membrane. Similar optical sections of *E. coli* cells treated with BPEI-conjugated Alexa-Fluor 488 revealed minimal fluorescence intensity within the cell envelope, indicating a weaker interaction between the *E. coli* cell envelope and BPEI (Figures 2D–F). This may explain the absence of antibiotic potentiation against *E. coli* in our present study. Additional LSCM images of the samples are provided as supplemental data (Figure S8).

The microscopy data, showing BPEI located in the cell wall region and not the cytoplasm, suggests that the observed antibiotic potentiation against MRSA is caused by an interaction of BPEI with some component of the bacterial cell wall. One major component of the Gram-positive cell wall is teichoic acid, a phosphodiester polymer whose anionic phosphate groups have been shown to interact strongly with metal cations.^{43–45} BPEI, with its polycationic properties, has the potential for very strong electrostatic interactions with the polyanionic WTA molecules. This interaction would involve the primary amines of BPEI and the phosphate groups of WTA (Figure 3). This interaction can be observed using nuclear magnetic resonance (NMR) studies of mixed BPEI-teichoic acid solutions when compared to NMR spectra of teichoic acid alone.

The 1-D ^{31}P spectra (Figures 4A and B) show significant changes after mixing WTA with low- M_{W} BPEI. WTA is a phosphodiester polymer with heterogeneous arrangement of N-acetylglucosamine (NAG), D-alanine, and hydroxyl groups. This creates variations in conformation of the poly(ribitol) backbone and differences in the phosphate conformations that generate distinct ^{31}P NMR peaks. In the presence of low- M_{W} BPEI, the ^{31}P NMR peak at 1.3 ppm is very intense, demonstrating that a large fraction of the phosphates have similar conformations. However, signals near 4 ppm are produced by phosphates in a deshielded environment. The downfield shift arises from a loss of electron density around the phosphorous nucleus, an effect that could be caused by a hydrogen bond between the phosphate oxygen and a BPEI amine group. The addition of BPEI also increases the intensity of cross peaks in the $^1\text{H}\{^{31}\text{P}\}$ HMBC (heteronuclear multiple bond coupling) NMR data (Figures 4C and D). This experiment relies on strong through-bond coupling

between the ^1H and ^{31}P nuclei. For flexible molecules, internal motion and dynamics causes relaxation of NMR signals^{46, 47} and thus the $^1\text{H}\{^{31}\text{P}\}$ HMBC signals are difficult to observe. When molecular motion is restricted, the signals are stronger.

Phosphate:amine binding from the WTA:BPEI interactions likely occurs through electrostatic attraction between the numerous cationic primary amines of BPEI and anionic phosphate groups of WTA (Figure 3). If this assumption is correct linear PEI, with only the 2 primary amines at its terminal ends, should not affect ampicillin's MIC values. The 0.6 kDa form of LPEI (similar in mass to the 0.5 kDa BPEI) does not inhibit MRSA growth (supplemental data figure S7) until its concentration is very high (54 $\mu\text{g}/\text{mL}$). Low molecular weight quaternary ammonium compounds have recently been shown to overcome resistance if the number of cationic sites is increased.⁴⁸ Therefore, the optimal cationic amine polymer should have a high number of primary amines while minimizing cytotoxicity with a low molecular weight.

Because low- M_w BPEI binds to WTA, the cationic polymer has the ability to change WTA properties by altering molecular structure and/or creating steric bulk from the branched polymer (Figure 1D). This would change, or prevent, the interaction of WTA with PBP2a and thus disable the enzyme. The same effect can be created through WTA-deficient strains of MRSA, which are re-sensitized to amoxicillin, ampicillin, methicillin, nafcillin, and ceftizoxime.¹⁵ An inhibitor of WTA synthesis, tunicamycin, re-sensitizes MRSA to β -lactams such as methicillin, oxacillin, cefotaxime, and several others.⁴⁹ Inhibition of another regulatory gene, *tarG*, also re-sensitizes MRSA strains to traditional β -lactams⁵⁰⁻⁵² like imipenem.¹⁶ Thus WTA, while not essential to viability,⁵⁰⁻⁵² is involved in β -lactam resistance.⁵³ WTA helps to optimally localize PBP2a, and WTA-deficient mutants show a decreased functionality of the protein.¹⁵ It additionally localizes PBP4, which is essential for the highly cross-linked peptidoglycan exhibited by MRSA and for the expression of β -lactam resistance in community-acquired strains.⁵⁴ Thus, restoration of β -lactam activity in therapeutic clinical usage could be achieved with antibiotics or other compounds that target WTA synthesis or interrupt the ability of WTA to localize PBP2a in the proper configuration required for peptidoglycan crosslinking. If this perspective is true, there should be little or no benefit when BPEI and ampicillin are used to treat non-resistant *S. aureus* strains that do not express PBP2a. Supplemental data (Figure S6) shows that the ampicillin MIC against methicillin-susceptible *S. aureus* ATCC 25923 was 50 ng/mL. When combined with 2.65 $\mu\text{g}/\text{mL}$ of BPEI, the ampicillin MIC of *S. aureus* ATCC 25923 was not reduced.

Rather than developing new inhibitors which require exhaustive clinical testing, we have discovered that some FDA-approved β -lactam antibiotics, such as ampicillin, can regain their efficacy against MRSA. The benefits to human health could be dramatic if the ampicillin + BPEI combination, used as a routine antibiotic therapy, can eliminate *S. aureus* infections while simultaneously preventing the growth of ampicillin-resistant bacteria. Further experiments will be necessary to determine the extent of the BPEI-teichoic acid interaction and whether this interaction changes the structure of PBP2a. Importantly, the data for *S. aureus* ATCC 25923 provide a potential route to treat, and prevent, antibiotic resistant infections. By using a combination of BPEI and ampicillin to treat a non-resistant *S. aureus* infection, the emergence of β -lactam resistant strains *in vivo* could be slowed. This

benefit would not be possible with ampicillin alone. Combination treatments of an antibiotic with a compound that blocks the resistance pathway have been shown to be a viable therapeutic strategy. For example, β -lactam antibiotics can be deactivated by bacteria that possess β -lactamases. The increasing incidence of β -lactamases has been problematic since the 1960s.⁵⁵ Researchers discovered that amoxicillin could be co-administered with the β -lactamase inhibitor, clavulanic acid. In such combination therapies, commercialized as Augmentin®, the antibiotic is not deactivated, but rather can successfully inhibit cell wall synthesis and kill the bacterium.^{55, 56} The amoxicillin and clavulanic acid treatment provides a compelling precedent demonstrating that combination therapies can be a successful antibacterial approach. While this approach effectively treats methicillin-susceptible *S. aureus* (MSSA) infections, MRSA bacteria will endure.⁵⁷ Our approach has the potential of simultaneously eliminating MSSA and MRSA to limit tissue damage from toxins, decreasing morbidity and mortality. Patients will not have to endure multiple treatments with an array of antibiotics to clear the infection, thereby improving quality of life.⁵⁸ Fewer medical complications and courses of treatment will result in better patient outcomes at a lower cost to patients and providers.⁵⁸

Colonies of MRSA bacteria invade host tissue to release toxins that cause tissue injury, leading to significant patient morbidity. The patient suffers while numerous first- and second-line antibiotics are prescribed to no avail. This increases the threat of MRSA to public health.⁵⁹ Timely MRSA diagnosis⁵⁸ and delivering drugs of last resort are essential to prevent mortality.⁶⁰ In 2011, MRSA infected 80,500 people; nearly 1 in 7 cases resulted in death (11,300; 14%)⁶¹. Several antibiotics of last resort (vancomycin, linezolid, daptomycin) are effective at killing MRSA, and no MRSA strain is resistant to more than one of them.^{62, 63} Although there has never been a *S. aureus* isolate resistant to all approved antibiotics, patients still die from MRSA infections. MRSA infections are deadly because drugs of last resort are given after morbidity from staphylococcal toxins, too late to prevent mortality.^{58, 62} Vancomycin, a primary treatment option after MRSA diagnosis, presents additional barriers of high cost and toxicity.^{64–68} New antibiotics, such as oxadiazoles,⁴ tedizolid,⁵ and teixobactin,⁶⁹ are awaiting FDA approval to meet the critical need for new treatments because *S. aureus* strains resistant to vancomycin and β -lactams have emerged.^{1, 7, 8} Although we envision improving human health with β -lactam plus polymer combinations, this represents a new pathway to develop other antibiotic treatments. Disabling PBP2a with cationic polymers enables advancement of antibiotic drug discovery by providing ways for other researchers to reinvigorate antibiotic development efforts that have stalled in the face of PBP2a.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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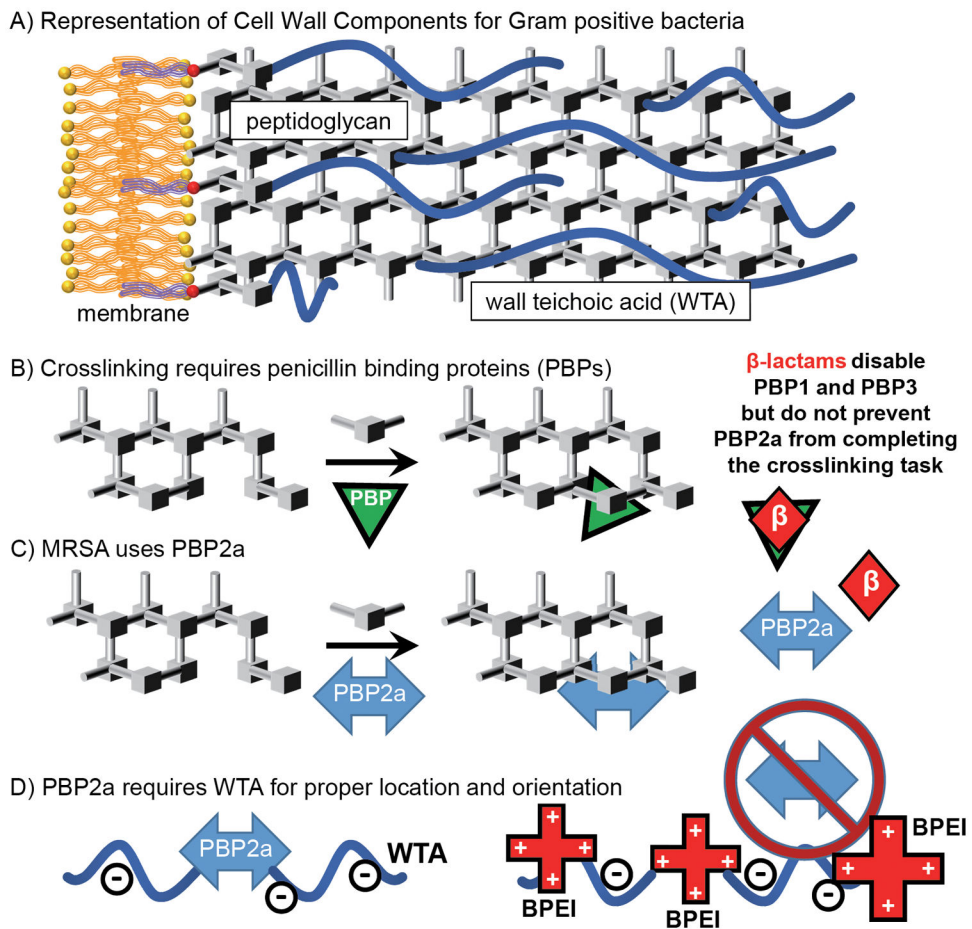


Figure 1. Schematic⁶ of Gram-positive cell wall components (A) and crosslinking of peptidoglycan using PBPs 1 and 3, which can be inhibited by β -lactams (B), and PBP2a, which cannot (C). PBP2a requires WTA to be properly localized; BPEI can bind to WTA to prevent PBP2a from functioning properly (D).

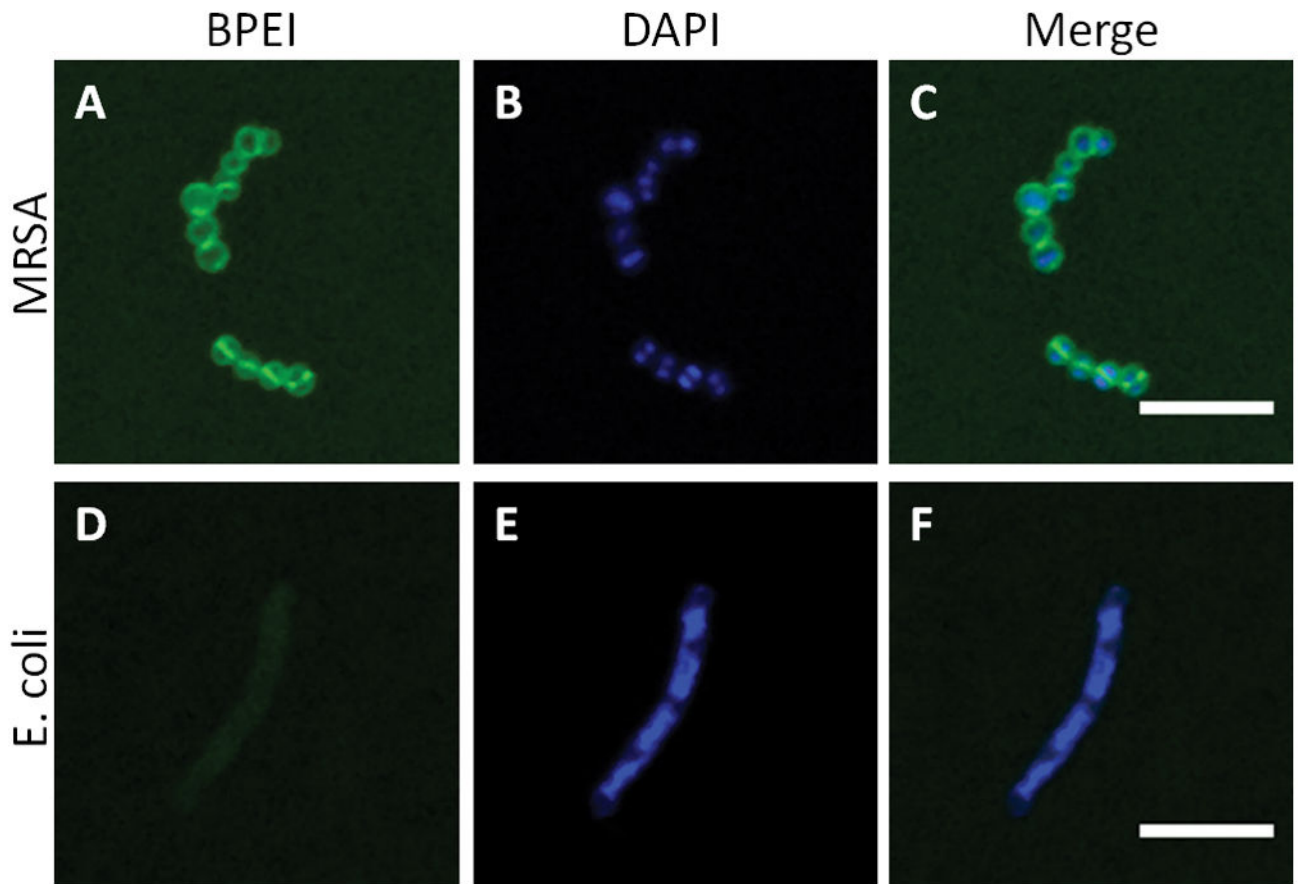


Figure 2.

Optical sections of BPEI binding to MRSA and *E. coli*. Paraformaldehyde-fixed MRSA, stained with BPEI-Alexa Fluor 488 (A) and DAPI (B), is imaged by LSCM. The merged image (C) shows BPEI binding to the cell surface but not within the cytoplasm. In contrast, PFA-fixed *E. coli* stained with BPEI-Alexa Fluor 488 (D) and DAPI (E), and the merged image (F), shows a relatively low affinity between BPEI and *E. coli*. Scale bar = 5 μ m.

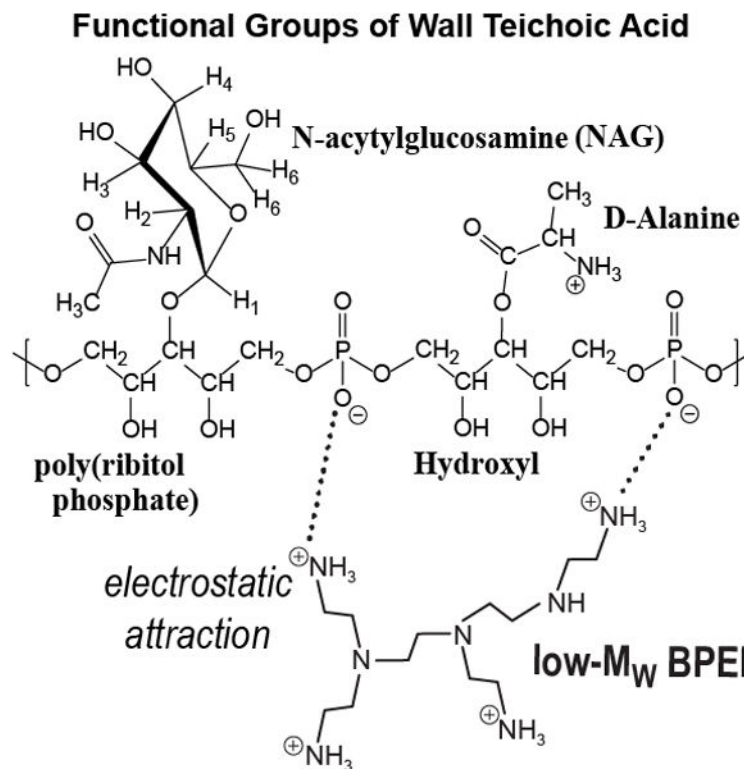


Figure 3. Chemical structure of the interaction between BPEI and the functional groups of wall teichoic acid. The cationic polymer is attracted to the anionic WTA. The result is a heterogeneous macromolecular complex that likely has increased steric bulk compared to WTA alone. These factors may prevent WTA from arranging PBP2a in its proper orientation required for crosslinking of peptidoglycan.

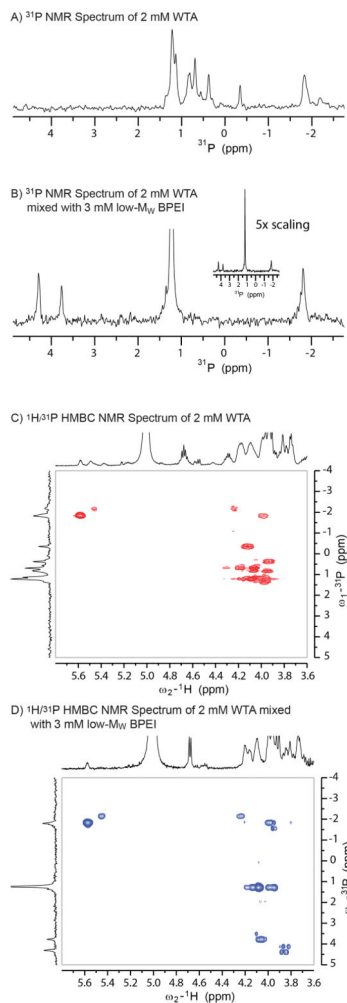


Figure 4.

^{31}P NMR spectra of WTA before and after the addition of low- M_w BPEI (A and B) show significant changes in phosphate chemical shift caused by changes in the chemical environment. These changes are also manifested in the heteronuclear multiple-bond correlation (HMBC) spectra (C and D). The P-31 signals near 4 ppm are correlated with the proton signals of NAG sugar groups. However, clear identification of specific interactions is prevented by the heterogeneous nature of WTA functional groups, BPEI branching, and WTA:BPEI binding interactions.

Table 1

MRSA Growth Inhibition Assay in the Presence of Ampicillin and low-M_w BPEI

BPEI Conc. (µg/mL)	Ampicillin Concentration (µg/mL)										
	0	0.125	0.250	0.500	1	2	4	8	16	32	64
0	0.4887	0.4361	0.4282	0.4342	0.4289	0.4294	0.4375	0.4344	0.3287	0.0003	0.0014
1	0.5088	0.4124	0.4289	0.4310	0.4324	0.4283	0.4097	0.3943	0.0661	-0.0024	-0.0037
2	0.4937	0.4388	0.4374	0.4510	0.4372	0.4540	0.3814	0.2333	0.0062	-0.0020	-0.0021
4	0.5054	0.4217	0.4693	0.4289	0.4373	0.4115	0.2587	0.1954	-0.0006	-0.0033	-0.0018
8	0.4835	0.4423	0.4449	0.4610	0.2826	0.2411	0.1070	-0.0018	-0.0011	-0.0025	-0.0002
16	0.4066	0.3178	0.2238	0.2931	0.0416	-0.0053	-0.0017	-0.0012	0.0003	-0.0006	-0.0007
32	0.1860	0.0997	0.1531	-0.0007	0.0798	0.0006	-0.0022	0.0023	0.0009	0.0001	0.0002
64	0.0001	0.0012	0.0005	-0.0003	-0.0001	0.0003	0.0006	-0.0018	0.0175	0.0003	0.0011

MRSA cells (ATCC 700787) were used to inoculate growth media containing ampicillin and low-M_w BPEI at 37 °C for 20 hours. Table entries are the optical density (OD) of the growth media measured at a wavelength of 600 nm. Each entry is the average of duplicate measurements. The bold line separates combinations that allowed MRSA growth (OD₆₀₀ values above 0.05) from those combinations that prevented MRSA growth. The ampicillin MIC is 32 µg/mL demonstrating antibiotic resistance without the presence of BPEI. Values highlighted in bold text were determined to arise from synergy between ampicillin and BPEI.

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