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C1-CBP-Vancomycin: Impact of a Vancomycin C-terminus Trimethylammonium Cation on Pharmacological Properties and Insights into its Newly Introduced Mechanism of Action

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Abstract

C1-CBP-vancomycin (**3**) was examined alongside CBP-vancomycin for susceptibility to acquired resistance upon serial exposure against two vancomycin-resistant enterococci strains where its activity proved more durable and remarkably better than many current therapies. Combined with earlier studies, this observation confirmed an added mechanism of action was introduced by incorporation of the trimethylammonium cation and that C1-CBP-vancomycin exhibits activity against vancomycin-resistant organisms through two synergistic mechanisms of action, both independent of D-Ala-D-Ala/D-Lac binding. New insights into this added mechanism of action, induced cell membrane permeabilization, can be inferred from studies that show added exogenous lipoteichoic acid reduces antimicrobial activity, rescues bacteria cell growth inhibition, and blocks induced cell permeabilization properties of C1-CBP-vancomycin, suggesting a direct binding interaction with embedded teichoic acid is responsible for the added mechanism of action and enhanced antimicrobial activity. Further studies indicate that the trimethylammonium cation does not introduce new liabilities in common pharmacological properties of the analogue and established that **3** is well tolerated in mice, displays substantial PK improvements over both vancomycin and CBP-vancomycin, and exhibits in vivo efficacy against a challenging multidrug-resistant and vancomycin-resistant *S. aureus* strain that is representative of the resistant pathogens all fear will emerge in the general population.

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Supporting Information

Full experimental details for preparation of **2** and **3**, resistance development assay, assays for lipoteichoic acid impact on antimicrobial activity, bacteria cell growth, and membrane permeability, cell wall biosynthesis inhibition assay, hemolysis assay, human primary hepatocyte cytotoxicity assay, protein binding assay, Caco-2 permeability assay, microsomal stability assay, CYP inhibition assay and table of results, tolerability (MTD) study, PK studies, and in vivo antimicrobial efficacy study, The Supporting Information is available free of charge on the ACS Publications website at DOI: XX.

The authors declare no competing financial interest.

INTRODUCTION

Since the discovery of vancomycin and the additional glycopeptide antibiotics in this family that followed,¹ they have been central to seminal developments in chemistry, biology, and medicine and are among the widely recognized naturally occurring cyclic peptides. Comprehensive reviews of Perkins,² Williams,³ Nicolaou,⁴ Courvalin,⁵ Walsh^{6,7} and Kahne,⁷ ourselves⁸ and others^{9–11} provide tabular accounts of the known glycopeptide antibiotics, a summary of their isolation and structure elucidation studies, and discussions of their biosynthesis, mechanism of action, structure–activity relationships, molecular recognition, total synthesis, mechanisms of resistance, and clinical use.

The majority of glycopeptide analogues examined to date have been derived from semisynthetic modifications of the natural products guided by their functional assessments in antimicrobial assays. This has resulted in the introduction of three semi-synthetic analogues now approved for use in the clinic: oritavancin (2014), dalbavancin (2014), and telavancin (2009) (Figure 1). Building on our total syntheses of the glycopeptide antibiotic aglycons that now include vancomycin,¹² teicoplanin,¹³ ristocetin,¹⁴ and the chloropeptins,¹⁵ we have detailed studies focused on the rational design of analogues to overcome the molecular basis of the emerging resistance to vancomycin. We first defined the origin of the destabilized binding to the induced and altered D-Ala-D-Lac (vs D-Ala-D-Ala) target in resistant bacteria¹⁶ and this led to the design and total syntheses of $[\Psi[C(=NH)NH]Tpg^4]$ vancomycin,¹⁷ $[\Psi(CH_2NH)Tpg^4]$ vancomycin¹⁷ and earlier their aglycons.¹⁸ Each exhibit dual, balanced binding to both D-Ala-D-Ala and D-Ala-D-Lac, altering a single atom¹⁹ in the binding pocket such that the new analogues remain active against vancomycin-sensitive bacteria (e.g., MRSA) and now display equally potent activity against vancomycin-resistant bacteria (e.g., VRSA, VRE). The recent total syntheses of their peripherally modified derivatives that introduce additional mechanisms of action (MOA) for antimicrobial activity have provided vancomycin analogues: (1) that contain binding pocket and one¹⁷ or two²⁰ added peripheral modifications, (2) that are endowed with two or three synergistic mechanisms of action, only one of which is dependent on D-Ala-D-Ala/D-Lac binding, (3) that display broad spectrum Gram-positive antimicrobial activity (e.g., MRSA,

VanA/VanB VRE and VRSA) at remarkable potencies (MICs = 0.01–0.005 $\mu\text{g/mL}$), and (4) that exhibit especially durable antimicrobial activity, suppressing elicited resistance.²⁰

Many may view this latter work simply as peripheral modifications introduced to increase the already superb antimicrobial potency and spectrum of activity of the pocket modified analogues. However, the modifications serve additional, even more important roles. The first modification utilized was the well-established 4-chlorobiphenylmethyl (CBP) substituent found in oritavancin and introduced onto the disaccharide by reductive amination.²¹ The second constitutes a new peripheral modification, a C-terminus trimethylammonium cation (C1) prepared by amine coupling with the vancomycin carboxylic acid.²⁰ Each modification increases antimicrobial activity by 10 to 100-fold, both display activity independent of D-Ala-D-Ala/D-Lac binding, both exert their effects through newly added MOAs independent of one another, both act on the cellular envelope impacting cell wall synthesis (CBP) or membrane integrity (C1), and both act synergistically with one another and with the MOA of vancomycin and its pocket analogues (inhibition of transpeptidase via D-Ala-D-Ala/D-Lac binding). The CBP modification results in inhibition of bacterial cell wall synthesis through direct competitive inhibition of transglycosylase (direct enzyme active site binding competitive with disaccharide substrate).^{20,22,23} We showed that the action of the trimethylammonium group is both structure²⁰ and site^{24,25} specific and does not result in cell membrane lysis but does induce membrane permeabilization further weakening the cellular envelope.²⁰

In addition to providing additional insights into the MOA underlying the activity of the trimethylammonium group and complementary to ongoing structure–activity studies further improving on this activity, the question that we also wanted to address with the studies herein was how the combined peripheral vancomycin modifications (CBP and trimethylammonium group) would impact the pharmacological properties, in vivo behavior, and antimicrobial efficacy of the compounds. Since the CBP modification is present in the clinically approved analogue oritavancin,²⁶ the key feature to be addressed was what impact the trimethylammonium cation might have when combined with the CBP modification. Although it appears to serve as a permanently charged cation and surrogate for a reversibly protonated dimethylamine like that found in dalbavancin, the analogous C-terminus dimethylamine of CBP-vancomycin does not display the activity enhancements seen with **3**.²⁰ Consequently, we examined CBP-vancomycin (**2**) and C1-CBP-vancomycin (**3**) alongside vancomycin (**1**) in order to establish the productive features or potential liabilities of the peripheral modifications, especially that of the unusual trimethylammonium cation (Figure 1). Although these studies were conducted in part to lay the foundation for anticipated pharmacological studies to be conducted on peripherally modified vancomycin analogues that contain key binding pocket modifications, they provided results that suggest even **3** or further improved analogues offer opportunities for new treatments against not only vancomycin-sensitive, but also vancomycin-resistant bacteria even without incorporation of the binding pocket modifications.

RESULTS AND DISCUSSION

Durability.

The discussion surrounding vancomycin analogues most often focuses on their potency or spectrum of activity, including identification of the contributing mechanisms of action and the demonstration that each independently improve antimicrobial activity potency. However, an additional and perhaps even more important feature of the expression of multiple independent mechanisms of action by a vancomycin analogue is its impact on the rate at which bacterial resistance may emerge.²⁷ In previous work, we addressed the durability of the antimicrobial activity of several vancomycin analogues that exhibit multiple synergistic mechanisms of action (MOA).²⁰ A direct correlation was observed between their ability to avoid raising resistance and the number of MOA the compound exhibits.²⁰ Herein, we examined the durability profile of C1-CBP-vancomycin (**3**) against two VanA vancomycin-resistant enterococci (VRE) strains alongside that of CBP-vancomycin (**2**) for comparison. They were examined for their susceptibility to acquired resistance upon sub-lethal ($0.5 \times \text{MIC}$) serial exposure to two VRE bacterial strains, monitoring MICs daily (Figure 2). Distinct from most related studies enlisting MRSA or other vancomycin-sensitive bacterial strains, this study was conducted with the most stringent VanA (vs VanB) vancomycin-resistant strains for which the mechanism of action associated with D-Ala-D-Ala binding is no longer effective. Consequently, it is not counted among the number of effective mechanisms of action embedded in the compound structure in the discussion below. In addition, the antibiotic susceptibility profiles of the VanA VREs used herein indicate they are resistant to most classes of antibiotics and are on the verge of being classified as multi-drug resistant VRE, indicating they have already assimilated a number of common resistance mechanisms.²⁸ Notably, because the changes for **3** were so small throughout a typical 25 day study, the examination was extended to 50 days, which is rarely reported in comparable studies.

Whereas the MIC of CBP-vancomycin (**2**) increased by as much as 32-fold after a typical 25-day serial passaging and is indicative of resistance selection, only a small 2-fold increase in the MIC was observed for C1-CBP-vancomycin (**3**). These results indicate that C1-CBP-vancomycin (**3**) has not selected for resistance after 25 days, that it clearly expresses activity through two MOAs relative to the less durable CBP-vancomycin (1 MOA, inhibition of transglycosylase), and that this second MOA protects against the selection of resistance against CBP-derived transglycosylase inhibition. The difference in durability between these two analogues was also observed when the study was extended to 50 days, where a much smaller increase in MIC was observed for C1-CBP-vancomycin compared with CBP-vancomycin (64-fold). We attribute the eventual slow loss in sensitivity with **3** to the relatively less potent activity of the second C1-derived MOA, which allows mutants insensitive to transglycosylase inhibition to slip through. This suggests that a more potent C1 variant, conveying activity on par with that of the CBP-derived transglycosylase inhibition, would preclude this loss in sensitivity. Although not yet disclosed, we have discovered such analogues and do see greater durability. Most striking, resistance to daptomycin (shown), linezolid (not shown), and tigecycline (not shown), each a frontline single target antibiotic, arises much faster (<25 days) against these two organisms and is much more pronounced

(reported in ref 20), highlighting the especially notable durability of the antimicrobial activity for **3**.

Combined, these results confirm that an added mechanism of action was introduced by incorporation of the trimethylammonium cation and that C1-CBP-vancomycin (**3**) exhibits its activity through two independent MOA against vancomycin-resistant organisms, both of which are independent of D-Ala-D-Ala/D-Lac binding. More subtle and because CBP-vancomycin (**2**) and related analogues are less potent and less durable against vancomycin-resistant bacteria (e.g., VRE and VRSA) where only one mechanism of action remains operative, their clinical use against such organisms will likely select for propagational resistance to transglycosylase inhibition. The incorporation of the second mechanism of action as is found in C1-CBP-vancomycin (**3**) improves the antimicrobial durability against such organisms and could provide a preferred clinical option against vancomycin-resistant organisms.

Additional Insights into the Added Mechanism of Action.

Consistent with its lack of activity, vancomycin fails to inhibit bacterial cell wall synthesis in the two VRE strains studied as judged by the lack of buildup of precursor UDP-MurNAc-pp (depsipentapeptide). Incorporation of the trimethylammonium cation with C1-vancomycin did not alter this behavior, indicating it also fails to inhibit cell wall synthesis in the two VRE strains (Supporting Information Figure S3). In contrast, CBP-vancomycin (**2**) effectively inhibits cell wall synthesis in the two VRE strains as judged by the buildup of precursor UDP-MurNAc-pp (depsipentapeptide) and does so not by binding the altered peptidoglycan precursor D-Ala-D-Lac, but rather by direct competitive inhibition of transglycosylase independent of D-Ala-D-Ala/D-Lac binding as reported by others.²³ Although not the focus compound of our preceding studies, we also showed that introduction of the C1 modification with C1-CBP-vancomycin (**3**) did not significantly alter this behavior, indicating that the improved antimicrobial potency and durability that accompanied the C1 introduction are derived from a newly added synergistic MOA also independent of D-Ala-D-Ala/D-Lac binding and independent of cell wall synthesis inhibition (Supporting Information Figure S3).²⁰

In these preceding studies, the antimicrobial activity as well as the durability of a series of C-terminus modified vancomycin analogues were found to correlate directly with induced bacterial cell permeability but, interestingly, not bacterial cell membrane disruption (depolarization).²⁰ The behavior of the analogues including **3** in a functional assay measuring bacterial cell permeability was the only feature that always and directly correlated with the added functional expression of antimicrobial activity and it was found to be both structurally specific (only trimethylammonium vs other trialkylammonium cations)²⁰ and site specific (C-terminus vs other sites in vancomycin).^{24,25} As a result of these studies, we have come to view the trimethylammonium group as a permanently positively charged surrogate for a protonated dimethylamine. These cumulative studies provided compelling evidence that the permeability assay is both an accurate readout of the correlated functional behaviors (induced permeability correlates with increased antimicrobial activity) and that the underlying mechanistic interpretation of consequences of the induced cell permeability is

similarly accurate (compromised membrane integrity). Moreover, it is remarkable that this effect is specific for C1 when combined with the CBP modification, suggesting the mechanism responsible for induction of membrane permeability may involve specific interactions within the bacterial cellular envelope. Among many investigated, our studies have led us to suggest a target of such analogues is membrane incorporated teichoic acid, although there may be others not yet recognized.

Teichoic acids are highly abundant alditol phosphate-containing polymers that are either linked to a glycolipid anchor embedded in the membrane (lipoteichoic acid, LTA) or covalently linked to the cell wall (wall teichoic acid, WTA) in Gram-positive bacteria.²⁹ Their polyanionic phosphate backbones bind and sequester positively charged autolysins³⁰ and they exhibit a regulatory effect on the activity of these cell wall autolytic proteins, which play crucial roles in peptidoglycan degradation, repair and cell division upon their release. Because of their indispensable biological function, teichoic acids and autolysins may serve as potential targets of antibiotics. Cationic peptides are thought to act in part by displaced release of autolysins³¹ and the novel antibiotic teixobactin was reported to bind lipid III (undecaprenyl-PP-GlcNAc), inhibit teichoic acid biosynthesis, and cause delocalization of the Atl autolysin.³² To examine if polyanionic teichoic acid serves as a possible membrane embedded binding partner for the permanently positively charged trimethylammonium vancomycin derivatives, the impact of added external lipoteichoic acid on antimicrobial activity, bacteria cell growth, and induced bacteria membrane permeabilization were examined and established.

The added presence of exogenous lipoteichoic acid (100 µg/mL) caused a 10 to 20-fold increase in the minimum inhibitory concentration (MIC) of C1-CBP-vancomycin (**3**) against the same two VanA VRE strains used in the durability assessment, which is in sharp contrast with its impact on the activity of either vancomycin (no observable difference in MIC) or CBP-vancomycin (2-fold) (Table 1). Co-administration of either inorganic divalent cation (Mg^{2+}) or the C-terminus side chain $H_2N(CH_2)_3NMe_3^+$ ($C1^+$) found in **3** had no impact on this behavior and all MICs were unchanged, indicating a tighter association and suggesting a more specific diverted binding of **3** with the added lipoteichoic acid than with inorganic cations or free $C1^+$ itself. Additionally, and although not the point of the studies, the fact that the co-administration of CBP-vancomycin with free $C1^+$, which on its own has no antimicrobial activity, did not change the MIC of CBP-vancomycin itself indicates that the improved activity of C1-CBP-vancomycin requires its covalent attachment. Since the exogenously added lipoteichoic acid may partition into the bacteria cell membrane as well as the cell culture media, the diverted binding does not necessarily preclude other actions of the antibiotics. In this regard, the fact that the MICs for C1-CBP-vancomycin in the presence of lipoteichoic acid now match those of CBP-vancomycin suggest the additive is simply serving as a decoy for the action of the trimethylammonium group but is not impacting its CBP-derived residual antimicrobial activity derived from inhibition of transglycosylase and cell wall synthesis. Finally, addition of inorganic NaH_2PO_4 or a phospholipid (1-pamitoyl-2-oleylglycero-3-phosphoethanolamine, POPE) found in the bacterial cell membrane did not reduce or alter the antimicrobial activity of **3**, indicating that simple or non-selective phosphate anion binding is incapable of such diverted binding in the assay. In addition,

added Mg^{+2} , which bridges neighboring phosphate groups, also failed to alter the antimicrobial activity of **3** against the two VanA VRE strains tested and is often used to inhibit nonspecific bacterial cell membrane association and/or the uptake of cationic antibiotics.

The decrease of antimicrobial activity of C1-CBP-vancomycin (**3**) in the presence of lipoteichoic acid was further confirmed in a bacteria cell growth inhibition assay against the same two VanA VRE strains. The addition of lipoteichoic acid (100 $\mu\text{g/mL}$) was found to rescue bacteria from growth inhibition by C1-CBP-vancomycin (**3**) added at $2 \times \text{MIC}$, whereas no LTA rescued bacteria cell growth was observed for vancomycin and CBP-vancomycin (Figure 3, see also Supporting Information Figure S1). This further indicates a diverted binding interaction between C1-CBP-vancomycin (**3**) and LTA, but not vancomycin or CBP-vancomycin and demonstrates that the presence of the positively charged trimethylammonium group on the C-terminus of **3** is crucial for the binding. Notably, the addition of simple inorganic phosphate did not rescue cell growth inhibition for any of the compounds, indicating this interaction is not a simple nonspecific electrostatic interaction between ions.

Finally, we examined the impact of exogenous lipoteichoic acid on the added mechanism of action (bacteria cell membrane permeabilization) of C1-CBP-vancomycin (**3**) enlisting the same two VanA VRE strains. The effect of the compounds on cell membrane permeability was examined by measuring cytoplasmic membrane uptake of propidium iodide where this fluorescent probe only enters cells with permeabilized cell membranes and is detected by the emission of fluorescence upon intracellular nucleic acid binding. Fresh, mid log phase VanA VRE in pH 7.2 buffer at 25 °C were preincubated with propidium iodide (10 μM) for 5 min before the compounds were added, monitoring the fluorescence emission at 617 nm (excitation at 535 nm) over time, both pre (5 min) and post (15 min) compound addition (10 μM). Rapid and sustained increases in fluorescence intensity are observed immediately upon addition of compounds that induce bacterial cell membrane permeability. C1-CBP-vancomycin (**3**) induces membrane permeabilization but neither vancomycin or CBP-vancomycin (Supporting Information Figure S2)²⁰ do so and the addition of lipoteichoic acid completely suppresses this effect of **3** (Figure 4). This result is consistent with a demonstration that diverted binding of **3** by added lipoteichoic acid prevents membrane permeabilization and completely blocks the effects of the added C1^+ mechanism of action of C1-CBP-vancomycin at 17 $\mu\text{g/mL}$ (10 μM). Moreover, it is consistent with the residual antimicrobial activity of **3** observed in the presence of lipoteichoic acid ($\text{MIC} = 5\text{--}20 \mu\text{g/mL}$, Table 1) being derived exclusively from the CBP modification and its effects on cell wall synthesis via transglycosylase inhibition ($\text{MIC} = 5\text{--}10 \mu\text{g/mL}$ for CBP-vancomycin, Table 1).

These studies indicate that added exogenous lipoteichoic acid reduces the antimicrobial activity, rescues the bacteria cell growth inhibition, and blocks the induced bacterial cell membrane permeabilization properties of C1-CBP-vancomycin (**3**). Together, these studies suggest a direct and likely specific binding interaction of C1-CBP-vancomycin (**3**) with bacterial membrane embedded teichoic acid that in turn is contributing to or responsible for the functional membrane permeabilization and associated antimicrobial activity.

Impact of Trimethylammonium Salt on Analogue Key Pharmacological Parameters.

As we reported,²⁰ key compounds in a preceding study including comparisons with **2** and **3** were examined for in vitro toxicity that might result from the combined mechanisms of action, especially the introduction of the trimethylammonium group that might impact host as well as bacterial cell membrane integrity. As previously reported,²⁰ neither **2** or **3** exhibited red blood cell hemolytic activity derived from membrane lysis or disruption even at concentrations >1000-fold above their MICs (Supporting Information Figure S6). Importantly, no hemolytic activity was observed with CBP-vancomycin (**2**), the addition of the trimethylammonium cation in **3** did not alter this behavior, and both compounds were indistinguishable from vancomycin itself in this regard.

We also reported that no mammalian cell toxicity was observed for **2** and **3** in cell growth inhibition assays against three mammalian cell lines, NIH/3T3 (mouse embryonic fibroblast), HepG2 (human liver cancer cell line) and HCT116 (human colon cancer cell line) up to 100 μ M in initial studies.²⁰ We expanded on this assessment and have now also examined the cell growth inhibition activity of **3** alongside **2** against human primary hepatocytes (PHH) and found no cytotoxic activity up to 100 μ M, the highest dose tested (Supporting Information Figure S7). Consequently, the addition of the trimethylammonium group in **3** did not result in unexpected mammalian cell toxicity and **3** was not distinguishable from either **2** or vancomycin itself in this regard.

The intrinsic permeability and potential for active transport in mammalian membranes was measured in the Caco-2 bidirectional permeability assay at both pH 6.5 and 7.4 in both the apical to basolateral (A–B, passive diffusion) and the B–A (active transport) directions.³³ Neither compound (**2** or **3**) could be detected in the receiver chambers at the end of the incubation periods (40–60 min), indicating that neither display an appreciable intrinsic or active transport mediated permeability ($<0.01\text{--}0.04 \times 10^{-6}$ cm/s based on the lower limit of compound detection). This is also reported to be observed with vancomycin and teicoplanin ($P_{\text{app}} < 0.1 \times 10^{-6}$ cm/s)³⁴ and is consistent with their routes of clinical administration (iv) and low oral bioavailability ($<1\%$) as well as in vitro studies indicating that oritavancin is neither a substrate nor an inhibitor of the efflux transporter P-glycoprotein (Pgp). This is a positive attribute that contributes to the lack of mammalian cellular effects, and this assessment indicates that no unexpected permeability accompanied introduction of the trimethylammonium cation found in **3**.

In humans, 75–80% of vancomycin is excreted unchanged after the first 24 h following administration. Consequently, there is seemingly no apparent metabolism of the drug.³⁵ Additionally, the concentration of vancomycin in liver tissue and bile 24 h after administration has also been reported to be below detection limits as well.³⁵ Both **2** and **3** were examined side-by-side for metabolic stability using both mouse liver microsomes (mlm) and human liver microsomes (hlm). Both showed little or no metabolic instability ($t_{1/2} > 60$ min, Figure 5). Most notable of these assessments was the observation that $104 \pm 4\%$ of **3**, bearing both the CBP and trimethylammonium cation modifications, remains following 60 min incubation with hlm. Importantly, this indicates that no new or even apparent human metabolic liability accompanies introduction of the trimethylammonium

cation and that it may even further improve the already excellent hlm stability exhibited by CBP-vancomycin (70% of **2** remains at 60 min, est. $t_{1/2}$ = 120 min) (Figure 5 and Supporting Information Figures S8), at least in the in vitro assay. It has been similarly reported that oritavancin, of which **2** is a close structural analogue, also displays no hlm in vitro metabolism in these assays.³⁶

The compounds **2** and **3** were also compared for their inhibition or induction of metabolizing CYP enzymes found in the liver that may impact drug–drug interactions, using assays that measure the inhibition of specific substrate metabolism for the individual CYP isozymes (CYP1A, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A) in human liver microsomes. Both CBP-vancomycin (**2**) and C1-CBP-vancomycin (**3**) displayed modest nonspecific CYP inhibition with IC₅₀ values at or above 10 μ M (10–30 μ M) for all isozymes (Supporting Information Table S1). This behavior is indicative of a nonspecific inhibition of enzyme activity, which may occur through a physical effect of the compounds on the microsomes or CYP enzymes themselves. Since inhibition is only observed at concentrations where the solubilities of both **2** and **3** are marginal and each inhibition curve displayed large Hill coefficients (>2–3 vs ideally 1), indicating the noncompetitive and nonspecific inhibition,³⁷ it is likely associated with the compound solubility properties in the assay. Nonetheless, it represents a pharmacological property worthy of more detailed study. Whereas vancomycin is not a source for CYP drug interactions either by induction or inhibited metabolism,³⁵ weak nonspecific in vitro CYP inhibition has been reported for other clinically approved semi-synthetic glycopeptide antibiotics, including the oritavancin that contains the CBP modification.^{36a} Importantly, addition of the trimethylammonium cation as found in **3** did not alter the CYP inhibition behavior relative to **2** and appears to somewhat mitigate the effects, typically displaying less potent inhibition.

Finally, in comparison to vancomycin (ca. 50%)³⁵ and oritavancin (85%),^{36b} both **2** and **3** were established to be highly protein bound in both mouse and human plasma behaving much like the structurally related oritavancin (Supporting Information). Notably, the addition of the trimethylammonium cation in **3** did not significantly alter the behavior of **2**, where both were found to be highly protein bound in an initial assessment.

Combined, these studies indicate that the introduction of the C1 trimethylammonium cation does not result in mammalian cell toxicity or hemolytic activity and does not introduce new liabilities in common pharmacological properties of the vancomycin analogue, including intrinsic or active transport, stability to hlm metabolism, inhibition/induction of CYP enzymes, or protein binding.

In vivo Assessments.

A key question that we wished to address was whether the peripheral modifications (CBP and trimethylammonium group) would adversely impact in vivo properties and antimicrobial efficacy. Since the CBP modification is present in the clinically approved analogue oritavancin,²⁶ the key feature we wanted to address was what impact the trimethylammonium group might have when combined with the CBP modification. Consequently, we elected to examine CBP-vancomycin (**2**) and C1-CBP-vancomycin (**3**) alongside vancomycin in tolerability, PK, and a challenging in vivo efficacy assessment in

order to establish the productive features or liabilities of the peripheral modifications, especially that of the trimethylammonium cation modification.

Maximum tolerated doses (MTD) and dosing vehicles for iv administration in mice ($n = 3$) were established for the three compounds to be tested side-by-side and found to be 300 mg/kg for vancomycin, 75 mg/kg for CBP-vancomycin (**2**), and 50 mg/kg for C1-CBP-vancomycin (**3**). Not surprisingly given the safety and tolerability of the clinical vancomycin analogue oritavancin that bears a peripheral 4-chlorobiphenylmethyl substituent, the CBP-vancomycin (**2**) MTD was found to be only 4-fold lower than vancomycin. The further addition of the trimethylammonium salt in **3** did not significantly alter this tolerability. Notably, CBP-vancomycin (100-fold) and C1-CBP-vancomycin (1000-fold) are progressively more potent than vancomycin, and would be administered at accordingly much lower doses, making the small MTD distinctions even more impressive. These studies established that there is no significant additional acute toxicity associated with the added trimethylammonium cation modification, a key question these initial studies were designed to answer.

The PK properties in mice (iv, $n = 3$ /time point @ MTD and 10 mg/kg) were established for the three compounds and conducted side-by-side (Table 2). These studies revealed that **3** ($t_{1/2} = 6-7$ h), bearing the CBP group and trimethylammonium cation, improved the short terminal half-life (0.5–1.3 h, $t_{1/2}$), lower exposure (AUC), consistent volume of distribution (V_d), and rapid clearance (CL) of vancomycin and mitigated the poor dose proportionality³⁸ and extended terminal half-life ($t_{1/2}$) of CBP-vancomycin (**2**) that makes clinical administration of the structurally related drug oritavancin challenging. Both vancomycin and **3** displayed well behaved dose proportional PK across all parameters, whereas CBP-vancomycin (**2**) did not, exhibiting a relatively lower plasma exposure at the higher dose (Table 2). Perhaps surprising to some, the trimethylammonium cation in **3** behaved exceptionally well in vivo, with **3** displaying a terminal half-life of 6–7 h and illustrating that it is not subject to rapid metabolism. These preliminary studies established that **3**, bearing both the peripheral CBP group and trimethylammonium cation, displays substantial PK improvements over both vancomycin and CBP-vancomycin (**2**).

An initial efficacy study in neutropenic mice ($n = 5$ /dosed group) was conducted using a well-established thigh infection model with an especially challenging vancomycin-resistant MRSA strain (VanA VRSA, VRS2³⁹). This *S. aureus* strain is both a MRSA strain, being resistant to common classes of antibiotics, as well as vancomycin resistant (VRSA) and is representative of the resistant pathogens that all fear will emerge in the general population. The right thigh of each mouse was injected intramuscularly (im) with the pathogen. After 2 h, the compounds and control vehicle were administered by subcutaneous (sc) injection. After 26 h, the bacterial CFU/g of harvested thighs were established and compared with baseline bacterial count at 2 h and vehicle control at 26 h. The results were remarkable especially with this challenging pathogen. As expected, vancomycin was inactive in this model at the compared doses shown. In contrast, both **2** and **3** were efficacious, both outperformed the clinical drug linezolid (administered po twice at 2 and 12 h), and both demonstrated dose dependent efficacious bactericidal activity (CFU/g <2 h baseline), resulting in a nearly 3-log₁₀ reduction in bacterial count even without optimized dosing

(Figure 6). Importantly, the study revealed that **3**, bearing both the CBP group and trimethylammonium cation, matches or is even more efficacious than CBP-vancomycin (**2**) (compare 12.5 mg/kg dose). When the dose at which the effect is bacteriostatic (harvested thigh CFU/g = 2 h baseline) is taken as a measure of the relative potency/efficacy, the activity of **3** was established to be greater than that of **2**: **3** > **2** > linezolid > vancomycin. This study also examined the sc administration of **2** and **3** twice (at 2 and 12 h), but only at the 12.5 and 25 mg/kg doses for **3** (Supporting Information Figure S9). Here the distinctions between **2** and **3** were clearer (**3** > **2**), but the overall efficacy was only marginally improved over the already impressive efficacy observed with the otherwise identical single dose treatments.

These in vivo efficacy results are even more impressive because they were achieved without key pocket modifications to the vancomycin structure needed to overcome the molecular basis of resistance to vancomycin. These vancomycin derivatives are active against the VRSA because of the added mechanisms of action provided by the peripheral modifications, reflecting their direct inhibition of transglycosylase (CBP) and induced bacterial membrane permeability (C1). Moreover, and even more importantly, whereas **2** (with 1 MOA) raises resistance in VanA VRE, **3** does so much more slowly (with 2 MOAs) as shown in Figure 2. As such, **3** would be expected to perform as a more durable antibiotic, being less prone to selecting for subsequent resistance.

Together, these studies show that incorporation of the trimethylammonium cation modification into CBP-vancomycin is well tolerated and improves both the PK properties and maintains or even improves antimicrobial efficacy in mice. Although these studies were designed to probe potential liabilities of the peripheral modifications, especially that of the unconventional trimethylammonium cation and its combination with the well-established CBP modification, they have provided a unique derivative of vancomycin worthy of further investigation and potential development. Just as significant and as a result of these studies, expectations are that added binding pocket modifications will further improve the in vivo potency as much as 100-fold over **3**, reinstate the durable D-Ala-D-Ala/D-Lac binding MOA, and may provide well tolerated, efficacious, and especially durable antibiotics with three independent MOAs. From the studies detailed herein, there does not appear to be obvious liabilities to the introduction of the unconventional C-terminus trimethylammonium cation, it may impart useful compound physical properties that positively impact both in vitro and in vivo behavior, and it conveys an independent MOA (induced bacterial membrane permeability) that improves potency and durability.

CONCLUSIONS

In earlier studies,²⁰ we showed that introduction of the C-terminus trimethylammonium cation into C1-CBP-vancomycin (**3**) led to a synergistic 10-fold increase in antimicrobial activity due to incorporation of an added MOA, additionally inducing bacterial cell membrane permeabilization. For vancomycin-sensitive bacteria, it is a compound that acts by three synergistic independent MOAs, derived from: (1) inhibition of transpeptidase and cell wall synthesis by binding and sequestering the enzyme substrate D-Ala-D-Ala in peptidoglycan precursors, (2) active site binding and direct competitive inhibition of

transglycosylase and cell wall synthesis independent of D-Ala-D-Ala binding by peripheral CBP introduction onto the disaccharide, and (3) induced bacterial membrane permeabilization (reduced membrane integrity) by C-terminus trimethylammonium cation (C1) introduction. Although vancomycin-resistant organisms overcome cell wall synthesis inhibition derived from peptidoglycan D-Ala-D-Ala binding by remodeling late-stage precursors with D-Ala-D-Lac, they remain susceptible to C1-CBP-vancomycin through the two independent MOAs introduced through the synergistic CBP and C1 peripheral modifications. Herein and complementary to these earlier studies,²⁰ C1-CBP-vancomycin (**3**) was found to display not only more potent antimicrobial activity than CBP-vancomycin against VRE, but also more durable activity, further suppressing elicited or selected resistance. It does so by expressing an added synergistic mechanism of action independent of bacterial cell wall synthesis inhibition or binding to D-Ala-D-Ala/D-Lac that entails inducing cell membrane permeability, but not cell lysis or disruption. Expression of this functional activity is structurally (trimethylammonium vs other trialkylammonium cations) and site (C-terminus vs other sites) specific for an added C-terminus trimethylammonium cation. It was shown that added exogenous lipoteichoic acid reduces the antimicrobial activity, rescues the bacteria cell growth inhibition, and blocks the induced bacterial cell membrane permeabilization properties of C1-CBP-vancomycin (**3**), suggesting a direct and likely specific binding interaction of C1-CBP-vancomycin (**3**) mediated by the trimethylammonium cation with bacterial membrane embedded teichoic acid that in turn is contributing to or responsible for the added functional membrane permeabilization and associated synergistic antimicrobial activity.

C1-CBP-vancomycin was found to exhibit no mammalian cell toxicity and does not display mammalian cell membrane hemolytic activity. The introduction of the trimethylammonium cation in **3** had no apparent impact on intrinsic passive or active transport permeability in mammalian cells and did not introduce aberrant metabolism. It displayed excellent hlm stability and the trimethylammonium cation introduction did not result in selective or added substantial inhibition or induction of CYP enzymes. The studies established that **3** is well tolerated in vivo in mice and that there is no significant additional acute toxicity associated with the added trimethylammonium cation modification. C1-CBP-vancomycin (**3**) was found to display substantial PK improvements over both vancomycin (**1**) and CBP-vancomycin (**2**) in mice, including displaying a terminal half-life of 6–7 h that further illustrates it is not subject to rapid metabolism, and it exhibited superb in vivo antimicrobial efficacy in mice against a challenging vancomycin-resistant *S. aureus* (VanA VRSA, VRS2) strain. This *S. aureus* strain is a MRSA strain resistant both to all common classes of antibiotics as well as vancomycin (VRSA) and is representative of the resistant pathogens all fear will eventually emerge in the general population. Together, these latter studies show that incorporation of the trimethylammonium cation modification found in C1-CBP-vancomycin (**3**) is well tolerated, improves PK properties, and maintains or even improves antimicrobial efficacy in mice.

Although these studies were designed to probe potential productive features or intrinsic liabilities of the peripheral modifications, especially that of the unconventional trimethylammonium cation and its combination with the well-established CBP modification,

they have provided a prototypical representative of a new class of vancomycin analogues,^{40,41} including those additionally detailed by Cooper, Wender and Haidar, that express an added independent synergistic mechanism of action and a unique derivative of vancomycin worthy of further investigation and potential development.

EXPERIMENTAL

Preparation of CBP-Vancomycin (2) and C1-CBP-Vancomycin (3).

A solution of vancomycin (2.50 g, 1.68 mmol, 1.0 equiv) in DMF (200 mL) was treated with 4-(4-chlorophenyl)-benzaldehyde (550 mg, 2.54 mmol, 1.5 equiv) and *i*-Pr₂NEt (2.18 g, 2.93 mL, 16.8 mmol, 10 equiv) at 25 °C. The reaction mixture was warmed at 70 °C for 2 h before NaCNBH₃ (1.0 M in THF, 34 mL, 34 mmol, 20 equiv) was added. The mixture was then warmed at 70 °C for 5 h before it was cooled to 25 °C. The solvent was removed, and the residue was triturated by addition of H₂O (200 mL). The precipitated solid was collected by filtration and was washed with CH₂Cl₂ (100 mL) and MeOH (100 mL), providing 1.64 g of crude CBP-vancomycin (**2**) as a white solid. Part of this sample (1.18 g) was used directly in the next step without further purification. The filtrate was concentrated, combined with 0.46 g of the remaining crude CBP-vancomycin (**2**) collected by filtration, and the mixture was purified by semi-preparative reverse-phase HPLC (Vydac 218TP1022-C18, 10 µm, 22 × 250 mm, 20–80% MeCN/H₂O–0.07% TFA gradient over 40 min, 10 mL/min, *t_R* = 14.5 min) to afford CBP-vancomycin (**2**) (636 mg) as a white solid identical to the material previously reported.²⁰

A solution of crude **2** (1.18 g, 0.70 mmol, 1.0 equiv) in DMF/DMSO (1/1, 70 mL) was treated with **C1** (Me₃N⁺(CH₂)₃NH₃⁺·2CF₃COO[−], 1 M in DMF/DMSO = 1/1, 1.4 mL, 1.4 mmol, 2 equiv), *N*-methylmorpholine (distilled, 1 M in DMF/DMSO = 1/1, 4.2 mL, 4.2 mmol, 6 equiv), and HBTU (0.5 M in DMF/DMSO = 1/1, 2.8 mL, 1.4 mmol, 2 equiv) at 5 °C. The reaction mixture was stirred at 5 °C for 4 h and quenched with the addition of 50% MeOH in H₂O (20 mL) at 5 °C. The mixture was concentrated and purified by semi-preparative reverse-phase HPLC (Vydac 218TP1022-C18, 10 µm, 22 × 250 mm, 20–80% MeCN/H₂O–0.07% TFA gradient over 40 min, 10 mL/min, *t_R* = 12.5 min) to afford **3** (571 mg) as a white solid identical to the material previously reported.²⁰

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Levine D Vancomycin: A History. *Clin. Infect. Dis* 2006, 42, 5–12.
2. Perkins HR Vancomycin and Related Antibiotics. *Pharmacol. Ther* 1982, 16, 181–197. [PubMed: 6752974]
3. (a) Williams DH The Glycopeptide Story – How to Kill the Deadly ‘Superbugs.’ *Nat. Prod. Rep* 1996, 13, 469–477. [PubMed: 8972102] (b) Williams DH; Bardsley B The Vancomycin Group of Antibiotics and the Fight against Resistant Bacteria. *Angew. Chem., Int. Ed* 1999, 38, 1172–1193.
4. Nicolaou KC; Boddy CNC; Bräse S; Winssinger N Chemistry, Biology, and Medicine of the Glycopeptide Antibiotics. *Angew. Chem., Int. Ed* 1999, 38, 2096–2152.
5. (a) Courvalin P Resistance of Enterococci to Glycopeptides. *Antimicrob. Agents Chemother* 1990, 34, 2291–2296. [PubMed: 2088183] (b) Courvalin P Vancomycin Resistance in Gram-positive Cocci. *Clin. Infect. Dis* 2006, 42, 25–34.
6. (a) Walsh CT Vancomycin Resistance: Decoding the Molecular Logic. *Science* 1993, 261, 308–309. [PubMed: 8392747] (b) Hubbard BK; Walsh CT Vancomycin Assembly: Nature’s Way. *Angew. Chem., Int. Ed* 2003, 42, 730–735.
7. Kahne D; Leimkuhler C; Lu W; Walsh C Glycopeptide and Lipoglycopeptide Antibiotics. *Chem. Rev* 2005, 105, 425–448. [PubMed: 15700951]
8. (a) James RC; Pierce JG; Okano A; Xie J; Boger DL Redesign of Glycopeptide Antibiotics: Back to the Future. *ACS Chem. Biol* 2012, 7, 797–804. [PubMed: 22330049] (b) Okano A; Isley NA; Boger DL Total Synthesis of Vancomycin Related Glycopeptide Antibiotics and Key Analogues. *Chem. Rev* 2017, 117, 11952–11993. [PubMed: 28437097]
9. Binda E; Marinelli F; Marcone GL Old and New Glycopeptide Antibiotics: Action and Resistance. *Antibiotics* 2014, 3, 572–594. [PubMed: 27025757]
10. Butler MS; Hansford KA; Blaskovich MAT; Halai R; Cooper MA Glycopeptide Antibiotics: Back to the Future. *J. Antibiot* 2014, 67, 631–644. [PubMed: 25118105]
11. (a) Boneca IG; Chiosis G Vancomycin Resistance: Occurrence, Mechanisms and Strategies to Combat it. *Expert Opin. Ther. Targets* 2003, 7, 311–328. [PubMed: 12783569] (b) Blaskovich MAT; Hansford KA; Butler MS; Jia Z; Mark MS; Cooper MA Developments in Glycopeptide Antibiotics. *ACS Infect. Dis* 2018, 4, 715–735. [PubMed: 29363950] (c) Dhanda G; Sarkar P; Samaddar S; Haidar J Battle against Vancomycin-Resistant Bacteria: Recent Developments in Chemical Strategies. *J. Med. Chem* 2019, 62, 3184–3205. [PubMed: 30404451]
12. Boger DL; Miyazaki S; Kim SH; Wu JH; Castle SL; Loiseleur O; Jin Q Total Synthesis of the Vancomycin Aglycon. *J. Am. Chem. Soc* 1999, 121, 10004–10011.
13. Boger DL; Kim SH; Mori Y; Weng J-H; Rogel O; Castle SL; McAtee JJ First and Second Generation Total Synthesis of the Teicoplanin Aglycon. *J. Am. Chem. Soc* 2001, 123, 1862–1871. [PubMed: 11456806]
14. Crowley BM; Mori YC; McComas CC; Tang D; Boger DL Total Synthesis of the Ristocetin Aglycon. *J. Am. Chem. Soc* 2004, 126, 4310–4317. [PubMed: 15053621]
15. Shimamura H.; Breazzano SP; Garfunkle J; Kimball FS; Trzupek JD; Boger DL Total Synthesis of Complestatin: Development of a Pd(0)-mediated Indole Annulation for Macrocyclization. *J. Am. Chem. Soc* 2010, 132, 7776–7783. [PubMed: 20469945]
16. McComas CC; Crowley BM; Boger DL Partitioning the Loss in Vancomycin Binding Affinity for D-Ala-D-Ala into Lost H-bond and Repulsive Lone Pair Contributions. *J. Am. Chem. Soc* 2003, 125, 9314–9315. [PubMed: 12889959]
17. Okano A; Nakayama A; Wu K; Lindsey EA; Schammel AW; Feng Y; Collins KC; Boger DL Total Synthesis and Initial Examination of [Ψ [C(=S)NH]Tpg⁴]vancomycin, [Ψ [C(=NH)NH]Tpg⁴]-vancomycin, [Ψ [CH₂NH]Tpg⁴]vancomycin and their (4-chlorobiphenyl)methyl Derivatives: Synergistic Binding Pocket and Peripheral Modifications for the Glycopeptide Antibiotics. *J. Am. Chem. Soc* 2015, 137, 3693–3704. [PubMed: 25750995]
18. (a) Crowley BM; Boger DL Total Synthesis and Evaluation of [Ψ (CH₂NH)Tpg⁴]vancomycin aglycon: Reengineering Vancomycin for Dual D-Ala-D-Ala and D-Ala-D-Lac Binding. *J. Am. Chem. Soc* 2006, 128, 2885–2892. [PubMed: 16506767] (b) Xie J; Okano A; Pierce JG; James RC; Stamm S; Crane CM; Boger DL Total Synthesis of [Ψ [C(=S)NH]Tpg⁴]vancomycin Aglycon,

- [Ψ[C(=NH)NH]Tpg⁴]-vancomycin Aglycon, and Related Key Compounds: Reengineering Vancomycin for Dual D-Ala-D-Ala and D-Ala-D-Lac Binding. *J. Am. Chem. Soc* 2012, 134, 1284–1297. [PubMed: 22188323]
19. Boger DL The Difference a Single Atom Can Make: Synthesis and Design at the Chemistry–Biology Interface. *J. Org. Chem* 2017, 82, 11961–11980. [PubMed: 28945374]
 20. Okano A; Isley NA; Boger DL Peripheral Modifications of [Ψ[CH₂NH]Tpg⁴]vancomycin with Added Synergistic Mechanisms of Action Provide Durable and Potent Antibiotics. *Proc. Natl. Acad. Sci. USA* 2017, 114, E5052–E5061. [PubMed: 28559345]
 21. (a) Nicas TI; Mullen DL; Flokowitsch JE; Preston DA; Snyder NJ; Zweifel MJ; Wilkie SC; Rodriguez MJ; Thompson RC; Cooper RD Semisynthetic Glycopeptide Antibiotics Derived from LY264826 Active against Vancomycin-resistant Enterococci. *Antimicrob. Agents Chemother* 1996, 40, 2194–2199. [PubMed: 8878606] (b) Cooper RDG; Snyder NJ; Zweifel MJ; Staszak MA; Wilkie SC; Nicas TI; Mullen DL; Butler TF; Rodriguez MJ; Huff BE; Thompson RC Reductive Alkylation of Glycopeptide Antibiotics: Synthesis and Antibacterial Activity. *J. Antibiot* 1996, 49, 575–581. [PubMed: 8698642] (c) Nagarajan R; Schabel AA; Occolowitz JL; Counter FT; Ott JL; Felty–Duckworth AM Synthesis and Antibacterial Evaluation of N-alkyl Vancomycins. *J. Antibiot* 1989, 42, 63–72. [PubMed: 2921228]
 22. (a) Allen NE; Hobbs JN Jr.; Nicas TI Inhibition of Peptidoglycan Biosynthesis in Vancomycin-susceptible and -resistant Bacteria by a Semisynthetic Glycopeptide Antibiotic. *Antimicrob. Agents Chemother* 1996, 40, 2356–2362. [PubMed: 8891144] (b) Goldman RC; Baizman ER; Longley CB; Branstrom AA Chlorobiphenyl-desleucyl-vancomycin Inhibits the Transglycosylation Process Required for Peptidoglycan Synthesis in Bacteria in the Absence of Dipeptide Binding. *FEMS Microbiol. Lett* 2000, 183, 209–214. [PubMed: 10675585] (c) Allen NE; Nicas TI Mechanism of Action of Oritavancin and Related Glycopeptide Antibiotics, *FEMS Microbiol. Rev* 2003, 26, 511–532. [PubMed: 12586393]
 23. (a) Ge M; Chen Z; Onishi HR; Kohler J; Silver LL; Kerns R; Fukuzawa S; Thompson C; Kahne D Vancomycin Derivatives that Inhibit Peptidoglycan Biosynthesis without Binding D-Ala-D-Ala. *Science* 1999, 284, 507–511. [PubMed: 10205063] (b) Chen L; Walker D; Sun B; Hu Y; Walker S; Kahne D Vancomycin Analogues Active Against VanA-resistant Strains Inhibit Bacterial Transglycosylase without Binding Substrate. *Proc. Natl. Acad. Sci. USA* 2003, 100, 5658–5663. [PubMed: 12714684]
 24. Wu Z-C; Isley NA N-Terminus Alkylation of Vancomycin: Ligand Binding Affinity, Antimicrobial Activity, and Site-specific Nature of Quaternary Trimethylammonium Salt Modification. *ACS Infect. Dis* 2018, 4, 1468–1474. [PubMed: 30067012]
 25. Wu Z-C Exploration of the Site-specific Nature and Generalizability of a Trimethylammonium Salt Modification on Vancomycin: A-ring Derivatives. *Tetrahedron* 2019, 75, 3160–3165. [PubMed: 31327878]
 26. Markham A Oritavancin: First Global Approval. *Drugs* 2014, 74, 1823–1828. [PubMed: 25239268]
 27. Silver LL Multi-targeting by Monotherapeutic Antibacterials. *Nat. Rev* 2007, 6, 41–55.
 28. For VanA *E. faecalis* (VanA VRE, BM-4166): resistant to erythromycin, gentamicin, chloramphenicol, and ciprofloxacin as well as vancomycin and teicoplanin; sensitive to daptomycin. For VanA *E. faecium* (VanA VRE, ATCC BAA-2317): resistant to ampicillin, benzylpenicillin, ciprofloxacin, erythromycin, levofloxacin, nitrofurantoin, and tetracycline as well as vancomycin and teicoplanin, insensitive to linezolid; sensitive to tigecycline and dalbapristine.
 29. (a) Brown S; Santa Maria JP Jr.; Walker S Wall Teichoic Acids of Gram-positive Bacteria. *Ann. Rev. Microbiol* 2013, 67, 313–336. [PubMed: 24024634] (b) Percy MG; Gründling A Lipoteichoic Acid Synthesis and Function in Gram-positive Bacteria. *Ann. Rev. Microbiol* 2014, 68, 81–100. [PubMed: 24819367]
 30. Hölte J-V; Tomasz A Lipoteichoic Acid: a Specific Inhibitor of Autolysin Activity in *Pneumococcus*. *Proc. Natl. Acad. Sci. USA* 1975, 72, 1690–1694. [PubMed: 239401]
 31. (a) Bierbaum G; Sahl H-G Induction of Autolysis of Staphylococci by the Basic Peptide Antibiotics Pep 5 and Nisin and their Influence on the Activity of Autolytic Enzymes. *Arch. Microbiol* 1985, 141, 249–254. [PubMed: 4004448] (b) Bierbaum G; Sahl HG Autolytic System

- of *Staphylococcus Simulans* 22: Influence of Cationic Peptides on Activity of N-acetylmuramoyl-L-alanine Amidase. *J. Bacteriol* 1987, 169, 5452–5458. [PubMed: 2890620]
32. (a) Ling LL; Schneider T; Peoples AJ; Spoering AL; Engels I; Conlon BP; Mueller A; Schäberle TF; Hughes DE; Epstein S; Jones M; Lazarides L; Steadman VA; Cohen DR; Felix CR; Fetterman KA; Millett WP; Nitti AG; Zullo AM; Chen C; Lewis K A New Antibiotic Kills Pathogens without Detectable Resistance. *Nature* 2015, 517, 455–459. [PubMed: 25561178] (b) Homma T; Nuxoll A; Gandt AB; Ebner P; Engels I; Schneider T; Götz F; Lewis K; Conlon BP Dual Targeting of cell wall Precursors by Teixobactin Leads to Cell Lysis. *Antimicrob. Agents Chemother* 2016, 60, 6510–6517. [PubMed: 27550357]
 33. (a) Borchardt RT Assessment of Transport Barriers using Cell and Tissue Culture Systems. *Drug Dev. Ind. Pharm* 1990, 16, 2595–2612. (b) Hidalgo IJ; Raub TJ; Borchardt RT Characterization of the Human Colon Carcinoma Cell Line (Caco-2) as a Model System for Intestinal Epithelial Permeability. *Gastroenterology* 1989, 96, 736–749. [PubMed: 2914637]
 34. Biganzoli E; Cavenaghi LA; Rossi R; Brunati MC; Nolli ML Use of a Caco-2 Cell Culture Model for the Characterization of Intestinal Absorption of Antibiotics. *II Farmaco* 1999, 54, 594–599.
 35. Matzke GR; Zhanel GG; Guay DR Clinical Pharmacokinetics of Vancomycin. *Clin. Pharmacokinet* 1986, 11, 257–282. [PubMed: 3530582] (b) Piscitelli SC Drug Interactions in Infectious Diseases Chapter 10, Glycopeptides, Lipopeptides, and Lipoglycopeptides. New York: Humana Press, 2011.
 36. (a) Tatarkiewicz J; Staniszevska A; Bujalska-Zadro ny M New Agents Approved for Treatment of Acute Staphylococcal Skin Infections. *Arch. Med. Sci.: AMS* 2016, 12, 1327–1336. [PubMed: 27904526] (b) Guskey MT; Tsuji BT A Comparative Review of the Lipoglycopeptides: Oritavancin, Dalbavancin, and Telavancin. *Pharmacotherapy* 2010, 30, 80–94. [PubMed: 20030476]
 37. Prinz H Hill Coefficients, Dose-response Curves and Allosteric Mechanisms. *J. Chem. Biol* 2010, 3, 37–44. [PubMed: 19779939]
 38. Fetterly GJ; Ong CM; Bhavnani SM; Loutit JS; Porter SB; Morello LG; Ambrose PG; Nicolau DP Pharmacokinetics of Oritavancin in Plasma and Skin Blister Fluid Following Administration of a 200-milligram Dose for 3 Days or a Single 800-milligram Dose. *Antimicrob. Agents Chemother* 2005, 49, 148–152. [PubMed: 15616289]
 39. VRS2 is a VanA VRSA that is resistant to not only vancomycin, but also other classes of antibiotics (MRSA), including methicillin, oxacillin, gentamicin, erythromycin, clindamycin, levofloxacin, trimethoprim-sulfamethoxazole, ceftriaxone, amoxicillin-clavulanic acid, piperacillin-tazobactam, cefepime, and imipenem. It remains sensitive to linezolid (MIC = 2 µg/mL), daptomycin (MIC = 0.25 µg/mL), mupirocin (MIC = 2 µg/mL), and tigecycline (MIC = 0.25 µg/mL). Measured MICs for vancomycin, CBP-vancomycin (**2**), and C1-CBP-vancomycin (**3**) were >16, 4, and 4 µg/mL, respectively, against this VRSA strain.
 40. (a) Yarlagadda V; Akkapeddi P; Manjunath GB; Haldar J Membrane Active Vancomycin analogues: A strategy to Combat Bacterial Resistance. *J. Med. Chem* 2014, 57, 4558–4568. [PubMed: 24846441] (b) Blaskovich MAT; Hansford KA; Gong Y; Butler MS; Muldoon C; Huang JX; Ramu S; Silva AB; Cheng M; Kavanagh AM; Ziora Z; Premraj R; Lindahl F; Bradford TA; Lee JC; Karoli T; Pelingon R; Edwards DJ; Amado M; Elliott AG; Phetsang W; Daud NH; Deecke JE; Sidjabat HE; Ramaologa S; Zuegg J; Betley JR; Beevers APG; Smith RAG; Roberts JA; Paterson DL; Cooper MA Protein-inspired Antibiotics Active Against Vancomycin- and Daptomycin-resistant Bacteria. *Nat. Commun* 2018, 9, 22. [PubMed: 29295973] (c) Antonoplis A; Zang X; Huttner MA; Chong KKL; Lee YB; Co JY; Amieva MR; Kline KA; Wender PA; Cegelski L A Dual-function Antibiotic-transporter Conjugate Exhibits Superior Activity in Sterilizing MRSA Biofilms and Killing Persister Cells. *J. Am. Chem. Soc* 2018, 140, 16140–16151. [PubMed: 30388366] (d) Yarlagadda V; Sarkar P; Samaddar S; Haldar J A Vancomycin Derivative with a Pyrophosphate-Binding Group: A Strategy to Combat Vancomycin-resistant Bacteria. *Angew. Chem., Int. Ed* 2016, 55, 7836–7840.
 41. Wright PM; Seiple IB; Myers AG The Evolving Role of Chemical Synthesis in Antibacterial Drug Discovery. *Angew. Chem., Int. Ed* 2014, 53, 8840–8869.

Figure 1.

Structures of vancomycin (1), CBP-vancomycin (2), and C1-CBP-vancomycin (3) and the clinically approved semisynthetic glycopeptide antibiotics.

Figure 2. Resistance acquisition on serial passaging of two strains of VanA VRE in the presence of 0.5 $\times$ MIC levels of compound: CBP-vancomycin (2), C1-CBP-vancomycin (3), and daptomycin. One of two replicate experiments shown.

Figure 3.

Impact of vancomycin derivatives on bacteria growth (VanA VRE, BM 4166 and ATCC BAA-2317) in the absence and presence of added lipoteichoic acid (LTA, 100 $\mu\text{g/mL}$) or inorganic phosphate (NaH_2PO_4 , 100 $\mu\text{g/mL}$).

Permeability Assay, VRE, VanA *E. faecalis* BM 4166

Permeability Assay, VRE, VanA *E. faecium* ATCC BAA-2317

Figure 4. Impact of added lipoteichoic acid (LTA, 100 $\mu\text{g/mL}$) on bacteria cell permeabilization by C1-CBP-vancomycin (10 μM , 17 $\mu\text{g/mL}$). No permeabilization is observed with vancomycin or CBP-vancomycin (Supporting Information Figure S2) at the same concentration (10 μM).

Figure 5.
Human liver microsome (hlm) stability expressed as % compound remaining after incubation with hlm after the noted time. The results shown are an average of duplicate assessments.

Figure 6.

Efficacy of vancomycin, CBP-vancomycin (**2**), C1-CBP-vancomycin (**3**), and linezolid against the multidrug resistant and vancomycin-resistant *S. aureus* (VRSA) strain VRS-2 in the mouse thigh infection model ($n = 5/\text{dose}$). Compounds **2**, **3**, vancomycin and control vehicle were administered sc once at 2 h at the doses indicated. The reference standard linezolid was administered orally (po) twice at 2 and 12 h at the doses indicated. Left: dose-dependent reduction in bacterial load. Right: dose-dependent bactericidal effect (relative to 2 h baseline): **3** **2** > linezolid > vancomycin.

Table 1.Impact of added lipoteichoic acid (LTA) on antimicrobial activity of vancomycin derivatives, MIC ($\mu\text{g/mL}$)^a

Compound	VanA VRE		VanA VRE	
	<i>E. faecalis</i> (BM 4166)		<i>E. faecium</i> (ATCC BAA-2317)	
	-LTA	+LTA	-LTA	+LTA
vancomycin (1)	250	250	250	250
CBP-vancomycin (2)	5	10	2.5	5
(+100 $\mu\text{g/mL}$ C1 ⁺)	5	10	2.5	5
(+100 $\mu\text{g/mL}$ Mg ²⁺)	5	10	2.5	5
C1-CBP-vancomycin (3)	1	20	0.3	5
(+100 $\mu\text{g/mL}$ C1 ⁺)	1	20	0.3	5
(+100 $\mu\text{g/mL}$ Mg ²⁺)	1	20	0.3	5
H ₂ N(CH ₂) ₃ NMe ₃ ⁺ (C1 ⁺)	>800	>800	>800	>800
C1-CBP-vancomycin (3)	1		0.3	
(+100 $\mu\text{g/mL}$ NaH ₂ PO ₄)				
C1-CBP-vancomycin (3)	1		0.3	
(+100 $\mu\text{g/mL}$ POPE)				

^aMIC = Minimum inhibitory concentration. Assays run in the absence or presence of additives (lipoteichoic acid (LTA), NaH₂PO₄, or POPE, 100 $\mu\text{g/mL}$) as well as the absence or presence of H₂N(CH₂)₃NMe₃⁺ (C1⁺, 100 $\mu\text{g/mL}$) or Mg²⁺ (MgCl₂, 100 $\mu\text{g/mL}$).

Table 2.Comparison PK properties of **2** and **3** versus vancomycin.^a

Parameter	Vancomycin		CBP-vancomycin (2)		C1-CBP-vancomycin (3)	
	300 mg/kg	10 mg/kg	75 mg/kg	10 mg/kg	50 mg/kg	10 mg/kg
C _{max} (μg/mL)	1665	62.8	125	65.0	58.9	14.1
t _{max} (h)	0.083	0.083	0.25	0.083	0.25	0.50
AUC (μg-h/mL)	935	21.8	575	135	430	81.1
V _d (L/kg)	0.62	0.34	1.24	1.28	1.04	1.25
CL (L/h/kg)	0.32	0.46	0.13	0.074	0.12	0.12
t _{1/2} (h)	1.35	0.52	6.6	12.0	6.2	7.0

^a Compounds administered iv @ MTD and 10 mg/kg in mice (n = 3/time point, measured at 0.083, 0.25, 0.5, 1, 2, 4, 8, and 24 h).